Wydział Farmaceutyczny

Katedra Analityki Medycznej

Zakład Chemii Klinicznej i Hematologii Laboratoryjnej

ROZPRAWA DOKTORSKA

Katarzyna Izabela Wadowska

Diagnostyczne i prognostyczne wykorzystanie oznaczeń potencjalnych biomarkerów raka płuca u pacjentów narażonych środowiskowo na ksenobiotyki dymu papierosowego

Diagnostic and prognostic application of potential biomarkers of lung cancer in patients with environmental exposure to tobacco smoke xenobiotics

Promotor:

dr hab. Mariola Śliwińska-Mossoń

Wrocław, 2023

Spis treści

1. CYKL PUBLIKACJI STANOWIĄCYCH PODSTAWĘ ROZPRAWY DOKTORSKIEJ 4

2. WYBRANE ASPEKTY TEORETYCZNE 5

2.1. EPIDEMIOLOGIA RAKA PŁUCA 5
2.2. KLASYFIKACJA RAKA PŁUCA 6
2.3. CZYNNIKI ETIOLOGICZNE RAKA PŁUCA 9
2.4. BIOMARKERY W DIAGNOSTYCE NOWOTWOROWEJ 10

3. CEL PRACY 13

3.1. ARTYKUŁ PRZEGLĄDOWY 14
3.2. I ARTYKUŁ ORYGINALNY 14
3.3. II ARTYKUŁ ORYGINALNY 16
3.4. III ARTYKUŁ ORYGINALNY 17

4. MATERIAŁY I METODY ZASTOSOWANE W BADANIACH 18

4.1. MATERIAŁ BADANY 18
4.1.1. I i II artykuł oryginalny 18
4.1.2. III artykuł oryginalny 19
4.2. METODYKA BADAŃ 20
4.2.1. Artykuł przeglądowy 20
4.2.2. I artykuł oryginalny 20
4.2.3. II artykuł oryginalny 22
4.2.4. III artykuł oryginalny 22
4.3. ANALIZA STATYSTYCZNA 24
4.3.1. I artykuł oryginalny 25
4.3.2. II artykuł oryginalny 26
4.3.3. III artykuł oryginalny 26

5. NAJWAŻNIEJSZE WYNIKI BADAŃ 28

5.1. ARTYKUŁ PRZEGLĄDOWY 28
5.2. I ARTYKUŁ ORYGINALNY 29
5.3. II ARTYKUŁ ORYGINALNY 31
5.4. III ARTYKUŁ ORYGINALNY 34

6. WNIOSKI 36

6.1. ARTYKUŁ PRZEGLĄDOWY 36

6.2. I ARTYKUŁ ORYGINALNY 36
6.3. II ARTYKUŁ ORYGINALNY 37
6.4. III ARTYKUŁ ORYGINALNY 38
6.5. WNIOSKI KOŃCOWE 38

7. STRESZCZENIE 40

8. SUMMARY 44

9. PIŚMIENNICTWO 47

10. ŹRÓDŁA FINANSOWANIA 52

11. WYKAZ SKRÓTÓW 53

12. SPIS RYSUNKÓW I TABEL 55

12.1. SPIS RYSUNKÓW 55
12.2. SPIS TABEL 55

13. SPIS ZAŁĄCZNIKÓW 56

13.1. ZAŁĄCZNIK 1 – ARTYKUŁ PRZEGLĄDOWY 57
13.2. ZAŁĄCZNIK 2 – I ARTYKUŁ ORYGINALNY 58
13.3. ZAŁĄCZNIK 3 – II ARTYKUŁ ORYGINALNY 59
13.4. ZAŁĄCZNIK 4 – III ARTYKUŁ ORYGINALNY 60
13.5. ZAŁĄCZNIK 5 – CAŁKOWITY DOROBEK NAUKOWY 61
13.6. ZAŁĄCZNIK 6 – OŚWIADCZENIA WSPÓŁAUTORÓW PUBLIKACJI WŁĄCZONYCH DO CYKLU 62

1. Cykl publikacji stanowiących podstawę rozprawy doktorskiej

I. Katarzyna Wadowska, Iwona Bil-Lula, Łukasz Trembecki, Mariola Śliwińska-Mossoń: Genetic markers in lung cancer diagnosis: a review. *International Journal of Molecular Sciences*, **2020**, vol. 21, nr 13, art. 4569 [24 s.], DOI:10.3390/ijms21134569
IF_{2020}=5,924; MEiN=140 pkt

II. Katarzyna Wadowska, Piotr Błasiak, Adam Rzechonek, Iwona Bil-Lula, Mariola Śliwińska-Mossoń: New insights on old biomarkers involved in tumour microenvironment changes and their diagnostic relevance in non-small cel lung carcinoma. *Biomolecules*, **2021**, vol. 11, nr 8, art. 1208 [24 s.], DOI:10.3390/biom11081208
IF_{2021}=6,064; MEiN=100 pkt

III. Katarzyna Wadowska, Piotr Błasiak, Adam Rzechonek, Iwona Bil-Lula, Mariola Śliwińska-Mossoń: Hepcidin as a diagnostic biomarker in anaemic lung cancer patients. *Cancers*, **2023**, vol. 15, nr 1, art. 224 [24 s.], DOI:10.3390/cancers15010224
IF_{2022}=5,2; MEiN=200 pkt

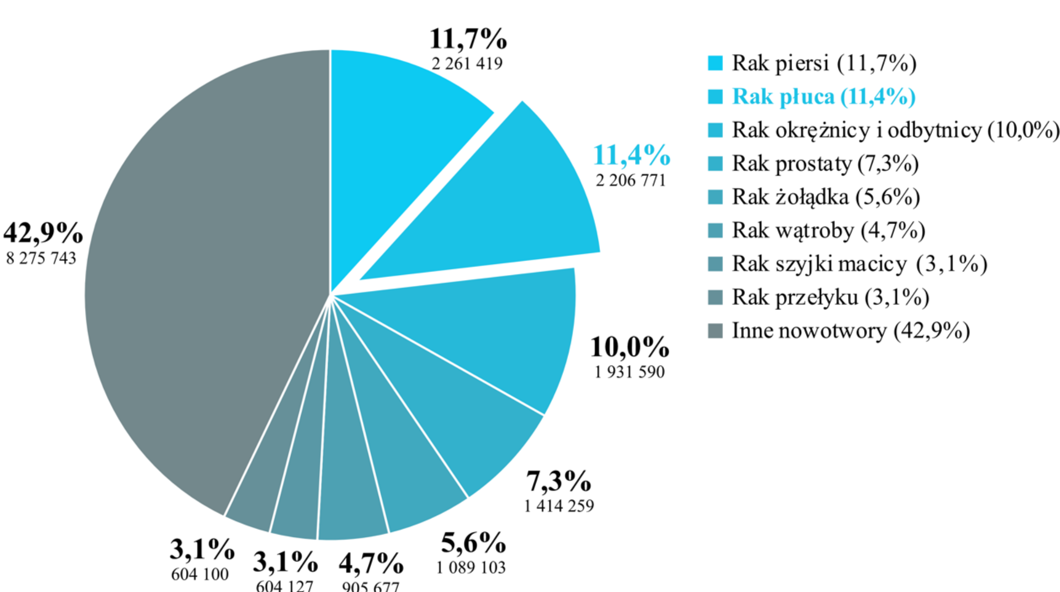
IV. Katarzyna Wadowska, Piotr Błasiak, Adam Rzeczonek, Mariola Śliwińska-Mossoń: Analysis of MMP-2 -735C/T (rs2285053) and MMP-9 -1562C/T (rs3918242) polymorphisms in the risk assessment of developing lung cancer. *International Journal of Molecular Sciences*, **2023**, vol. 24, nr 13, art. 10576 [20 s.], DOI:10.3390/ijms241310576
IF_{2022}=5,6; MEiN=140 pkt

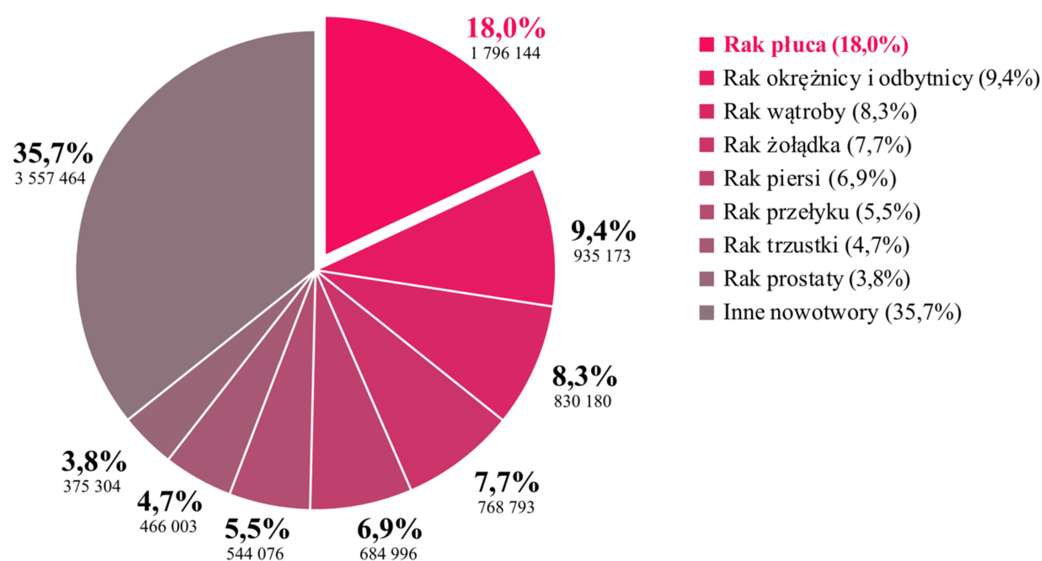
Sumaryczny współczynnik IF za cykl publikacji: 22,788
Sumaryczna wartość punktacji MEiN za cykl publikacji: 580
Wykaz całkowitego dorobku naukowego został potwierdzony przez Bibliotekę Uniwersytetu Medycznego i stanowi załącznik numer 5.

2. Wybrane aspekty teoretyczne

2.1. Epidemiologia raka płuca

Na początku XX wieku rak płuca był uznawany za rzadki nowotwór. Przed 1900 rokiem w literaturze medycznej opisano zaledwie 400 przypadków raka płuca. [1] Sytuacja diametralnie się zmieniła, gdyż w 2020 roku rak płuca był najczęstszą przyczyną nowotworowej śmierci na świecie, odpowiedzialną za około 20% zgonów z powodu nowotworu. [2,3]





Rysunek 1. Statystyki (a) zachorowalności i (b) śmiertelności z powodu raka na świecie. [2,3]

W 2020 roku z powodu raka płuca na świecie zmarło 1 796 144 osób, co stanowiło niewiele mniej niż łączna liczba zgonów z powodu raka okrężnicy i odbytnicy (935 173), raka piersi (684 996) i raka prostaty (375 304), czyli najczęściej występujących nowotworów obok raka płuca (Rysunek 1, sekcja b). [2,3] Co więcej, szacuje się, że liczba zgonów z powodu raka płuca wzrośnie do trzech milionów zgonów na świecie rocznie w 2035 roku. [4]

Rak płuca jest również pierwszą przyczyną nowotworowych zgonów w Polsce. Według raportu „Powiew świeżego powietrza. Analiza porównawcza polityki w zakresie raka płuca w Europie" *The Economist Intelligence Unit* (2020 rok), rocznie w powodu raka płuca umiera nawet 24 tys. Polaków i rejestruje się około 23 tys. nowych zachorowań. [5] Większa liczba zgonów z powodu raka płuca w stosunku do liczby zachorowań świadczy o brakach w rejestrowaniu przypadków zachorowań na ten nowotwór w Polsce. [6]

Wysoka śmiertelność z powodu raka płuca jest efektem wysokiej zachorowalności na ten nowotwór (Rysunek 1, sekcja a), ale przede wszystkim wynika ona z tego, że zdecydowana większość pacjentów (75%) jest diagnozowana w zaawansowanych stadiach choroby, dla których skuteczność leczenia jest obniżona. [7] Rokowania w raku płuca są bardzo niekorzystne i poprawiły się tylko nieznacznie w ciągu ostatnich 50 lat. W latach 1974-1976 5-letnie przeżycie pacjentów z rakiem płuca wynosiło 12,4%, w latach 1996-2002 15,0% i wzrosło do zaledwie średnio 22,0% dla wszystkich pacjentów z rakiem płuca w 2020 roku (w 2020 roku 5-letnie przeżycie dla mężczyzn wynosiło 18%, natomiast dla kobiet 25%). [8] Wskaźniki 5-letniego przeżycia także różnią się znacząco w zależności od podtypu raka płuca oraz stadium zaawansowania choroby. [9]

2.2. Klasyfikacja raka płuca

Raki płuca tradycyjnie dzieli się na niedrobnokomórkowe raki płuca (z ang. *non-small cell lung carcinoma*, NSCLC), które stanowią około 85% wszystkich rozpoznań oraz drobnokomórkowe raki płuca (z ang. *small cell lung carcinoma*, SCLC) stanowiące obecnie około 13% wszystkich pierwotnych nowotworów płuca. Raka płuca cechuje znaczna heterogeniczność typów histologicznych na poziomie genetycznym, epigenetycznym oraz niegenetycznym (między innymi immunologicznym), która pełni kluczową rolę w kontekście odpowiedzi i oporności na terapię oraz determinuje przebieg postępowania diagnostycznego. [10–13] Obecna klasyfikacja nabłonkowych

nowotworów płuca według Światowej Organizacji Zdrowia (z ang. *World Health Organisation*, WHO) wprowadziła między innymi nowy podział raków gruczołowych i płaskonabłonkowych oraz wskazała na konieczność wykorzystania badań immunohistochemicznych w diagnostyce patomorfologicznej raka płuca, a także biologii molekularnej i badań genetycznych w celu indywidualizacji leczenia. [6,14,15] Tabela 1 przedstawia podział typów histologicznych niedrobnokomórkowego raka płuca w oparciu o klasyfikację WHO z 2015 roku oraz wytycznych praktyki klinicznej w onkologii organizacji *National Comprehensive Cancer Network* (NCCN) z 2023 roku. [6,16]

Tabela 1. Klasyfikacja patomorfologiczna niedrobnokomórkowego raka płuca.

Typ	Podtyp
Rak płaskonabłonkowy	Rogowaciejący (*keratinising squamous cell carcinoma*) Nierogowaciejący (*non-keratinising squamous cell carcinoma*) Zmiana przedinwazyjna (*squamous cell carcinoma in situ*)
Rak gruczołowy	Groniasty (*acinar adenocarcinoma*) Brodawkowy (*papillary adenocarcinoma*) Drobnobrodawkowy (*micropapillary adenocarcinoma*) Tapetujący (*lepidic adenocarcinoma*) Lity (*solid adenocarcinoma*) Naciekający z wytwarzaniem śluzu (*invasive mucinous adenocarcinoma*)* Koloidalny (*colloid adenocarcinoma*)* Z komórek typu płodowego (*fetal adenocarcinoma*)* Z komórek typu jelitowego (*enteric-type adenocarcinoma*)* Zmiana przedinwazyjna (*adenocarcinoma in situ*)
Rak gruczołowo-płaskonabłonkowy	
Rak wielkokomórkowy	
Raki mięsakowate	Rak mięsakowaty pleomorficzny (*pleomorphic sarcomatoid carcinoma*) Mięsakorak (*carcinosarcoma*) Blastoma płuc (*pulmonary blastoma*)
Raki niesklasyfikowane (*not otherwise specified*, NOS)	

*warianty inwazyjnego raka gruczołowego płuca według NCCN.

Klasyfikacja patomorfologiczna raka płuca według WHO z 2015 roku obejmuje dodatkowo nowotwory neuroendokrynne [6,16], do których sklasyfikowano raka drobnokomórkowego, raka wielkokomórkowego neuroendokrynnego (*large cell carcinoma*), rakowiaka typowego i atypowego (*typical and atypical carcinoid*) oraz

zmianę przedinwazyjną, to jest rozlaną hiperplazję idiopatyczną płuc neuroendokrynnokomórkową (*diffuse idiopatic pulmonary neuroendocrine hyperplasia*).

Dokładne określenie zaawansowania raka płuca jest podstawą kwalifikacji chorego do leczenia i obejmuje ocenę guza pierwotnego (cecha T, z ang. *tumour*), zajęcie regionalnych węzłów chłonnych (cecha N, z ang. *lymph nodes*) oraz wystąpienie przerzutów odległych (cecha M, z ang. *metastasis*). W Tabeli 2 zebrano możliwości terapeutyczne w zależności od stopnia zaawansowania raka płuca w oparciu o 8. edycję klasyfikacji TNM z 2017 roku. [1,6,11,17]

Tabela 2. Postępowanie terapeutyczne w niedrobnokomórkowym raku płuca na podstawie stopnia zaawansowania z 8. Edycji TNM.

<table>
<tr><th colspan="4">8. Edycja Stopnia Zaawansowania TNM</th><th>Możliwości terapeutyczne</th></tr>
<tr><td>Stopień IA$_1$</td><td>T1a</td><td>N0</td><td>M0</td><td rowspan="3">Wyłącznie zabieg operacyjny</td></tr>
<tr><td>Stopień IA$_2$</td><td>T1b</td><td>N0</td><td>M0</td></tr>
<tr><td>Stopień IA$_3$</td><td>T1c</td><td>N0</td><td>M0</td></tr>
<tr><td>Stopień IB</td><td>T2a</td><td>N0</td><td>M0</td><td rowspan="4">< 4 cm ∅ wyłącznie zabieg operacyjny
> 4 cm ∅ zabieg operacyjny z następującą chemioterapią adjuwantową
Nie ma wskazań do radioterapii uzupełniającej w przypadku stopnia zaawansowania I lub II z ujemnymi marginesami. Pacjentom ze stopniem zaawansowania I i II, którzy odmawiają lub nie kwalifikują się do zabiegu chirurgicznego, powinna zostać zaproponowana radioterapia jako postępowanie terapeutyczne</td></tr>
<tr><td>Stopień IIA</td><td>T2b</td><td>N0</td><td>M0</td></tr>
<tr><td rowspan="2">Stopień IIB</td><td>T1a-2b</td><td>N1</td><td>M0</td></tr>
<tr><td>T3</td><td>N0</td><td>M0</td></tr>
<tr><td rowspan="3">Stopień IIIA</td><td>T1-2b</td><td>N2</td><td>M0</td><td rowspan="6">W przypadku N0 lub N1 – zabieg operacyjny z następującą chemioterapią adjuwantową.
W przypadku N2 lub N3 leczenie bez zabiegu operacyjnego, z zastosowaniem chemioradioterapii. Optymalna strategia leczenia nie została jeszcze ostatecznie ustalona. Mimo wielu możliwych strategii leczenia żadna nie daje wysokiego prawdopodobieństwa wyleczenia. III stopień zaawansowania jest bardzo heterogenny i nie istnieje pojedyncza terapia, która byłaby uniwersalna dla wszystkich pacjentów</td></tr>
<tr><td>T3</td><td>N1</td><td>M0</td></tr>
<tr><td>T4</td><td>N0/N1</td><td>M0</td></tr>
<tr><td rowspan="3">Stopień IIIB</td><td>T1-2b</td><td>N3</td><td>M0</td></tr>
<tr><td>T3/T4</td><td>N0/N1</td><td>M0</td></tr>
<tr><td>T3/T4</td><td>N3</td><td>M0</td></tr>
<tr><td>Stopień IVA</td><td>dowolny T</td><td>dowolny N</td><td>M1a/M1b</td><td rowspan="2">Przeciwbólowe leczenie paliatywne oraz odpowiednie użycie radioterapii i terapii systemowej</td></tr>
<tr><td>Stopień IVB</td><td>dowolny T</td><td>dowolny N</td><td>M1c</td></tr>
</table>

TNM - Klasyfikacja TNM nowotworów złośliwych (z ang. *tumour – lymph nodes – metastasis*, to jest ocena stanu guza pierwotnego – regionalnych węzłów chłonnych – przerzutów odległych); T1 - guz o średnicy ≤3 cm, otoczony miąższem płucnym lub opłucną trzewną, bez naciekania oskrzeli głównych; T1a - guz o wymiarze ≤1 cm; T1b - guz o wymiarze >1 cm, ale ≤2 cm; T1c - guz o wymiarze >2 cm, ale ≤3 cm; T2 - guz o wymiarze >3 cm, ale ≤5 cm albo guz, który nacieka główne oskrzele, ale nie dochodzi do rozwidlenia tchawicy i/lub nacieka opłucną trzewną, i/lub powoduje niedodmę lub zapalenie płuc sięgające okolicy wnęki, obejmujące zarówno część, jak i całe płuco; T2a - guz o wymiarze >3 cm, ale ≤4 cm; T2b - guz o wymiarze >4 cm, ale ≤5 cm; T3 - guz o wymiarze >5 cm, ale ≤7 cm albo guz każdej

wielkości, który nacieka ścianę klatki piersiowej (w tym guz górnego otworu klatki piersiowej) i/lub nerw przeponowy, i/lub osierdzie ścienne, albo guz każdej wielkości ze współwystępowaniem zmian satelitarnych w innym płacie tego samego płuca; T4 - guz o wymiarze >7 cm lub guz każdej wielkości, który nacieka przeponę i/lub śródpiersie, i/lub serce, i/lub duże naczynia, i/lub tchawicę, i/lub nerw krtaniowy wsteczny, i/lub przełyk, i/lub rozwidlenie tchawicy, i/lub kręgi, albo guz każdej wielkości ze współwystępowaniem zmian satelitarnych w innym płacie tego samego płuca; N0 - nie stwierdza się przerzutów w regionalnych węzłach chłonnych; N1 - przerzuty w węzłach chłonnych okołooskrzelowych i/lub wnękowych po stronie guza pierwotnego oraz wewnątrzpłucnych lub ich bezpośrednie naciekanie; N2 - przerzuty w węzłach chłonnych śródpiersia po stronie guza pierwotnego i/lub rozwidlenia tchawicy; N3 - przerzuty w węzłach chłonnych śródpiersia lub wnęki po stronie przeciwnej, pod mięśniem pochyłym i/lub nadobojczykowych po stronie guza pierwotnego lub po stronie przeciwnej; M0 - nie stwierdza się przerzutów odległych; M1 - obecność przerzutów odległych; M1a - odrębne ognisko lub ogniska raka w drugim płucu, ogniska raka w opłucnej/osierdziu lub wysięk nowotworowy w jamie opłucnej/osierdzia; M1b - pojedyncze przerzuty odległe (poza klatką piersiową); M1c - mnogie przerzuty odległe (poza klatką piersiową) w jednym lub wielu narządach.

Wyróżnia się klasyfikację TNM kliniczną (cTNM, z ang. *clinical TNM*) oraz patomorfologiczną (pTNM, z ang. *pathological TNM*), przy czym ocena kliniczna określa zasięg anatomiczny nowotworu na podstawie badań obrazowych, natomiast ocena patomorfologiczna określa ostateczny stopień zaawansowania raka płuca w materiale operacyjnym, jest także dokładniejsza i lepiej opisuje rokowania chorych. Dodatkowo w ocenie patomorfologicznej materiału operacyjnego ustala się stopień złośliwości nowotworu na podstawie cechy G (z ang. *grade*) to jest stopnia zróżnicowania, gdzie G1 oznacza zróżnicowanie wysokie, G2 zróżnicowanie umiarkowane, G3 zróżnicowanie niskie, a G4 raka niezróżnicowanego. Stopień złośliwości nowotworu nie determinuje doboru metody leczenia, uzupełnia natomiast klasyfikację histologiczną niedrobnokomórkowego raka płuca. [6,11]

2.3. Czynniki etiologiczne raka płuca

Czynniki ryzyka zachorowania na raka płuca są związane przede wszystkim ze stylem życia pacjentów, a także ich środowiskowymi i zawodowymi ekspozycjami. Za jeden z najważniejszych czynników etiologicznych raka płuca uznaje się palenie tytoniu, a dokładniej ekspozycję na rakotwórcze składniki dymu tytoniowego w wyniku czynnego czy też biernego palenia, które są odpowiedzialne za około 90% zachorowań wśród mężczyzn i 80% wśród kobiet. Ocenia się, że względne ryzyko zachorowania na raka płuca jest o 10 do 30 razy wyższe u palaczy wyrobów tytoniowych w porównaniu z osobami niepalącymi i zależy ono w głównej mierze od wypalonych paczkolat, to jest liczby paczek papierosów wypalanych dziennie pomnożonych przez lata palenia. [12,18]

Do czynników ryzyka zachorowania na raka płuca zalicza się również narażenie na fizyczne i chemiczne czynniki środowiskowe i zawodowe, między innymi azbest, radon, związki arsenu, chromu i niklu czy węglowodory aromatyczne. Dodatkowo

narażenie na związki organiczne, takie jak chlorek winylu, formaldehyd, benzo[a]piren, metale, jak beryl, kadm, chrom, nikiel, pyły metali ciężkich czy krzemionkę, często działa synergistycznie z paleniem tytoniu. [6,18,19]

Pozostałymi przyczynami zwiększającymi podatność na raka płuca są dziedziczone uwarunkowania genetyczne, obejmujące (1) geny o wysokiej penetracji i niskiej częstotliwości – mutacje komórek linii zarodkowej takie jak ATM, BRCA1, DIS3, ERCC2, FANCG, MRE11A, PALB2, PIK3C2G czy XRCC2 w raku gruczołowym płuca oraz BRCA2, BRIP1, DIS3, FANCA, FANCC, MAP3K15, czy PARP3 w raku płaskonabłonkowym płuca; (2) geny o niskiej penetracji i wysokiej częstotliwości – polimorfizmy pojedynczego nukleotydu genów uczestniczących w unieczynnieniu szkodliwych składników dymu tytoniowego (enzymy metabolizmu I i II fazy) czy zaburzenia genów odpowiedzialnych za naprawę uszkodzeń DNA; (3) nabyte polimorfizmy epigenetyczne takie jak metylacja DNA. [6,8,20,21]

2.4. Biomarkery w diagnostyce nowotworowej

Biomarker to gen, białko lub inna biologiczna cząsteczka znajdująca się we krwi, moczu, innych płynach ustrojowych czy tkankach, która może dostarczyć informacji na temat procesu fizjologicznego lub patologicznego toczącego się w organizmie pacjenta. [22,23] Idealny biomarker powinien charakteryzować się wysoką czułością i swoistością diagnostyczną, czyli odpowiednio wysokim prawdopodobieństwem uzyskania dodatniego wyniku badania u osoby chorującej na daną chorobę oraz uzyskania ujemnego wyniku badania u osoby niechorującej na tę chorobę, a także dużym polem powierzchni pod krzywą (AUC, z ang. *area under the curve*) krzywej ROC (z ang. *receiver-operator characteristics*), czyli graficznej reprezentacji czułości i swoistości diagnostycznej. Ponadto idealny biomarker powinien cechować się wysoką wartością predykcyjną wyniku dodatniego (PPV, z ang. *positive predictive value*), czyli wysokim prawdopodobieństwem istnienia danej choroby w przypadku uzyskania dodatniego wyniku badania. Czułość i swoistość diagnostyczna, współrzędne krzywej ROC oraz AUC, a także wartość predykcyjna wyniku dodatniego należą do statystycznych wskaźników wartości diagnostycznej badań laboratoryjnych i dostarczają praktycznej wiedzy niezbędnej przy interpretacji wyników badań. [23,24]

Nowotwór każdego pacjenta charakteryzuje się specyficznym wzorem biomarkerów, które nazywa się markerami nowotworowymi. Marker nowotworowy to tradycyjnie również białko lub inna biologiczna cząsteczka, która jest obecna

w komórkach nowotworowych bądź produkowana przez komórki nowotworowe lub inne komórki organizmu w odpowiedzi na toczący się w organizmie proces nowotworzenia. Markery nowotworowe są źródłem informacji na temat nowotworu, to jest typu histologicznego, stopnia złośliwości nowotworu, tego czy i na jaką terapię odpowie dany nowotwór, pomagając tym samym w doborze odpowiedniej terapii oraz monitorowaniu choroby w trakcie i po zastosowaniu leczenia wraz z oceną skuteczności terapii, wykrywaniem choroby resztkowej, przerzutów odległych lub wznowy nowotworu. [10,22,25,26]

Istnieje szereg przebadanych markerów nowotworowych, z których kilka znajduje zastosowanie w diagnostyce raka płuca – są to antygen rakowo-płodowy (CEA, z ang. *carcinoembryonic antigen*), fragmenty cytokeratyny 19 (CYFRA 21-1), enolaza swoista dla neuronów (NSE, z ang. *neuron-specific enolase*), antygen raka płaskonabłonkowego (SCC-Ag, z ang. *squamous cell carcinoma antigen*) oraz prekursor peptydu uwalniającego gastrynę (ProGRP, z ang. *pro-gastrin-releasing peptide*). [10,11,27] W Tabeli 3 zestawiono możliwości diagnostyczne trzech najczęściej wykorzystywanych markerów nowotworowych w diagnostyce raka płuca.

Tabela 3. Możliwości diagnostyczne markerów nowotworowych stosowanych w diagnostyce laboratoryjnej raka płuca.

Marker	Choroby nienowotworowe	Choroby nowotworowe	Wartość w raku płuca
CEA (CAM5) [10,26,28]	kobiety w ciąży, palacze tytoniu, choroba wrzodowa, nieswoiste zapalenie jelit, zapalenie trzustki, marskość wątroby, polipy okrężnicy, niedoczynność tarczycy	rak jelita grubego i okrężnicy, rak piersi, rak płuca, rak żołądka, rak trzustki, rak rdzeniasty tarczycy, raki głowy i szyi, rak wątroby, chłoniak, czerniak	najwyższa czułość diagnostyczna wśród pacjentów z gruczolakorakiem (44-63%); obserwuje się spadek stężenia CEA o >40% wyjściowego stężenia u 77% chorych z całkowitą lub częściową remisją procesu nowotworowego; wyjściowe podwyższone stężenie CEA wiąże się z gorszym rokowaniem u chorych poddanych zabiegowi operacyjnemu
CYFRA 21-1 [10,26]	nienowotworowe schorzenia oraz stany zapalne płuc, gruźlica, marskość wątroby, choroba Crohna	niedrobnokomórkowy rak płuca, gruczolakorak szyjki macicy, płaskonabłonkowy rak przełyku	najwyższa czułość diagnostyczna wśród pacjentów z rakiem płaskonabłonkowym (52-70%); tendencja do wzrostu stężenia CYFRA 21-1 w zależności od stadium zaawansowania choroby – stadium I i II podwyższone wartości u 38-41% chorych, stadium IIIA u 43-59%, stadium IIIB u 68-79%, a w stadium IV u 69-88% chorych; wyjściowe stężenie CYFRA 21-1 >3,6 ng/mL jest uznawane za niekorzystny czynnik rokowniczy

Marker	Choroby nienowotworowe	Choroby nowotworowe	Wartość w raku płuca
NSE [10,26,29]	zapalenie opon mózgowo-rdzeniowych, zawał mózgu, krwiak wewnątrzmózgowy, epilepsja, zespół Guillaina i Barrégo, choroba Creutzfeldta i Jakoba, schizofrenia	drobnokomórkowy rak płuca, neuroblastoma, glejaki, czerniak, nasieniak, rak nerkowokomórkowy	u chorych z postacią raka drobnokomórkowego ograniczoną do jednej połowy klatki piersiowej wskaźnik podwyższonych wyników NSE wynosi 39-69%, a u chorych z postacią uogólnioną 67-87%; duża przydatność w monitorowaniu chemioterapii; wysokie wyjściowe stężenie NSE jest uznawane za niekorzystny czynnik prognostyczny u pacjentów z drobnokomórkowym rakiem płuca

CEA (z ang. *carcinoembryonic antigen*, antygen rakowo-płodowy), CYFRA 21-1 (fragmenty cytokeratyny 19), NSE (z ang. *neuron-specific enolase*, enolaza swoista dla neuronów).

3. Cel pracy

Rak płuca jest problematyczny ze względu na wysoką zapadalność oraz umieralność na ten nowotwór, jak również ze względu na podstępny charakter choroby – rak płuca należy do nowotworów, które we wczesnych stadiach zaawansowania przebiegają bezobjawowo, a kiedy objawy już wystąpią, mogą być lekceważone przez chorych. [11] Problematyczna jest również diagnostyka raka płuca ze względu na lokalizację guza oraz różnorodność typów histologicznych. Stosowane obecnie narzędzia diagnostyczne, to jest konwencjonalna rentgenografia (RTG) klatki piersiowej czy badanie cytologiczne plwociny nie są wystarczająco czułymi badaniami przesiewowymi w diagnostyce raka płuca i przede wszystkim nie zmniejszają umieralności z powodu raka płuca. [30] Dostępne w praktyce klinicznej markery nowotworowe, takie jak CEA, CYFRA 21-1 czy NSE również nie umożliwiają rozpoznania raka płuca we wczesnym stadium zaawansowania choroby i według najnowszych wytycznych postępowania diagnostyczno-terapeutycznego, nie zaleca się oceny stężeń surowiczych tych markerów w ramach wstępnej diagnostyki, monitorowania przebiegu leczenia i dalszej obserwacji pacjentów z rakiem płuca. [6] W związku z powyższym konieczne jest poszukiwanie narzędzi diagnostycznych, które zapewniłyby możliwość stawiania rozpoznania we wczesnych stadiach zaawansowania raka płuca oraz możliwość prowadzenia badań przesiewowych w grupie zwiększonego ryzyka zachorowania na ten nowotwór.

Teorie dotyczące diagnostyki chorób nowotworowych przyjmują założenie o wzajemnych oddziaływaniach organizmu chorego na nowotwór i nowotworu na organizm chorego. [26] W swoich rozważaniach oparłam się na koncepcji mikrośrodowiska guza, czyli środowiska składającego się z komórek nowotworowych, macierzy zewnątrzkomórkowej oraz mikrośrodowiska stanu zapalnego z komórkami immunologicznymi oraz cytokinami prozapalnymi, oddziałującymi na procesy nowotworzenia, progresji nowotworowej i przerzutowania. Ponadto zmiany zachodzące w mikrośrodowisku guza mogą oddziaływać na ogólnoustrojową odpowiedź chorego, włączając w to rozwój przewlekłego stanu zapalnego czy związane z nim zaburzenia metaboliczne. [26,31–33]

Celem niniejszej rozprawy doktorskiej było poszerzenie aktualnej wiedzy w zakresie mechanizmów leżących u podstaw patogenezy raka płuca w oparciu o teorię mikrośrodowiska guza. Przedmiotem przeprowadzonych badań było wyselekcjonowanie

biomarkerów mirkośrodowiska guza płuca wraz z analizą ich wartości diagnostycznej, a co za tym idzie, możliwości wykorzystania tych biomarkerów w szeroko pojmowanej diagnostyce laboratoryjnej raka płuca i oceną użyteczności klinicznej. Użyteczność kliniczna badanych biomarkerów była analizowana w kontekście możliwości poprawy przeżywalności pacjentów z rakiem płuca, na którą powinny się składać (1) rozpoznanie raka płuca we wczesnym stadium zaawansowania choroby, (2) dobór właściwego postępowania terapeutycznego, to jest w kontekście typu histologicznego i stadium zaawansowania raka płuca wraz z wykorzystaniem terapii celowanych, (3) ocena sprawności organizmu (występowanie zespołu wyniszczenia nowotworowego lub niedokrwistości towarzyszącej nowotworom), wpływającej na procesy leczenia i potencjalne sukcesy terapeutyczne. [6]

3.1. Artykuł przeglądowy

Markery genetyczne w diagnostyce raka płuca

Czynniki genetyczne są jednym z kluczowych elementów patogenezy raka płuca. Zrozumienie profilu molekularnego guza wydaje się istotne w kontekście postępowania diagnostycznego czy doboru terapii celowanej. [8,15,21,34] Można się spodziewać, że na przestrzeni najbliższych lat WHO opublikuje klasyfikację raka płuca w oparciu o profil molekularny nowotworów i będzie stopniowo odchodzić od klasycznej klasyfikacji opartej na typie histologicznym. [6]

Artykuł stanowi zbiór i omówienie wiedzy z zakresu mechanizmów kancerogenezy oraz progresji nowotworowej opartej na zmianach genetycznych i ścieżkach molekularnych do czasu pierwszej połowy 2020 roku. Celem artykułu przeglądowego było podsumowanie aktualnej wiedzy na temat dostępnych biomarkerów genetycznych w diagnostyce przesiewowej raka płuca, metod ich oznaczania oraz metod leczenia raka płuca opartych na markerach genetycznych z wykorzystaniem terapii celowanych.

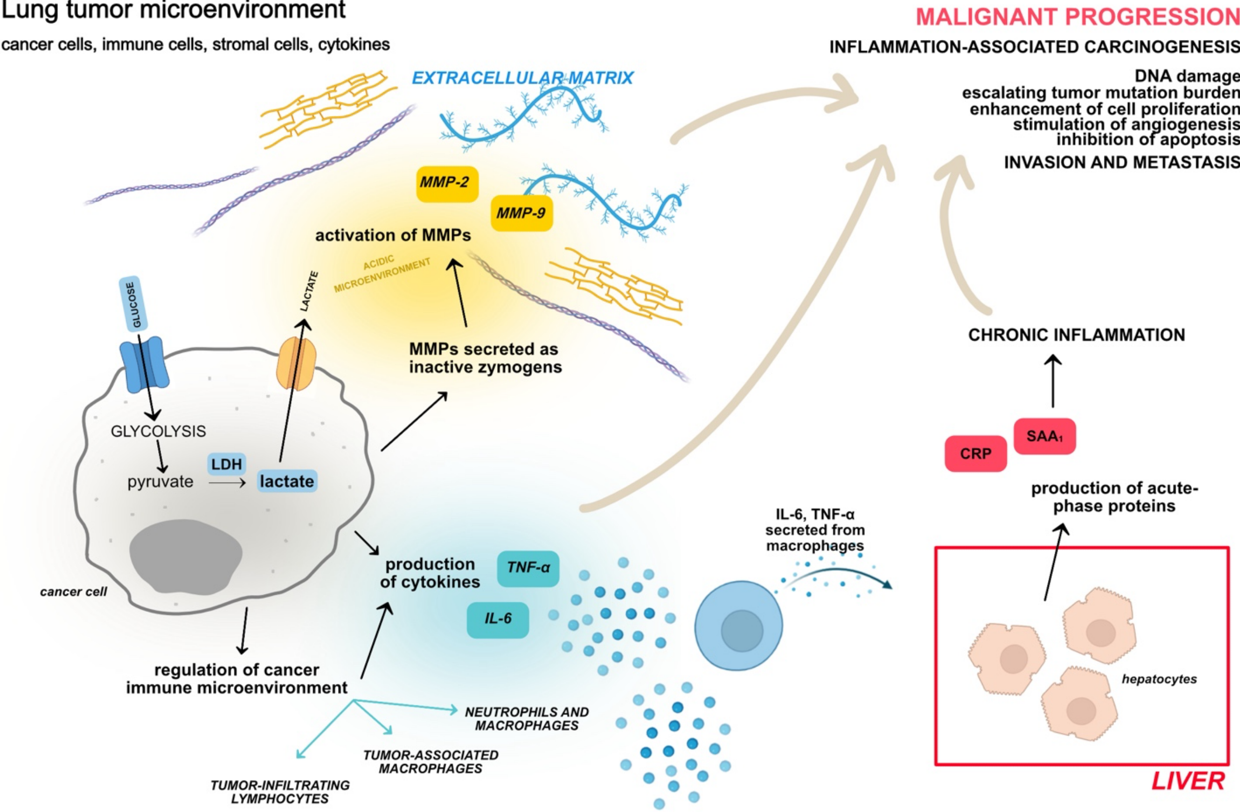
3.2. I artykuł oryginalny

Nowe spojrzenie na stare biomarkery zaangażowane w zmiany w mikrośrodowisku guza i ich znaczenie diagnostyczne w niedrobnokomórkowym raku płuca

Niniejsza praca została oparta na nowoczesnym podejściu w diagnostyce biochemicznej nowotworów złośliwych, to jest na uzupełnieniu badania stężeń markerów nowotworowych o badanie stężeń cytokin prozapalnych, receptorów cytokin prozapalnych, czynników wzrostu czy enzymów proteolitycznych. [26] W pracy

opracowano trzy panele biomarkerów: (1) nowotworowy z wybranymi markerami – CEA, CYFRA 21-1 oraz NSE; (2) stanu zapalnego utworzony z cytokin prozapalnych IL-6 (interleukina 6) i TNF-α (czynnik martwicy nowotworu-α, z ang. *tumour necrosis factor-α*), białek ostrej fazy CRP (białko C-reaktywne, z ang. *C-reactive protein*), i SAA_1 (surowiczy amyloid A_1, z ang. *serum amyloid A_1*), a także metaloproteinaz macierzy zewnątrzkomórkowej -2 i -9 (MMP-2, MMP-9, z ang. *matrix metalloproteinases*); oraz (3) metabolizmu glukozy utworzony z glukozy, mleczanu i dehydrogenazy mleczanowej. Parametry wchodzące w skład każdego panelu są albo dobrze znanymi, „starymi" biomarkerami, stosowanymi w codziennej praktyce klinicznej i rutynowej diagnostyce laboratoryjnej, albo biomarkerami kompleksowo przebadanymi przez naukowców, z licznymi dowodami naukowymi ich roli w patogenezie chorób nowotworowych. Ponadto parametry zostały dobrane tak, aby były ze sobą powiązane i tworzyły ciąg przyczynowo skutkowy w patogenezie raka płuca, co zostało schematycznie przedstawione na Rysunku 2 (Rysunek 1 w I artykule oryginalnym).

Nadrzędnym celem niniejszej pracy było sprawdzenie czy połączenie parametrów biochemicznych wchodzących w skład panelu stanu zapalnego i panelu metabolizmu glukozy z markerami nowotworowymi istotnie wpłynie na podniesienie statystycznych wskaźników wartości diagnostycznej markerów nowotworowych, tym samym dostarczając nowego, rzetelnego i taniego narzędzia w laboratoryjnej diagnostyce raka płuca. Biomarkery były analizowane najpierw pojedynczo, a następnie w panelach składających się z kombinacji od dwóch do pięciu biomarkerów, w celu oceny ich wartości diagnostycznych w różnicowaniu pacjentów z gruczolakorakiem od pacjentów z rakiem płaskonabłonkowym płuca, jak również pacjentów ze stadium IIB od IIA i IIIA od IIB. Nacisk został położony na dwa najczęściej występujące podtypy raka niedrobnokomórkowego płuca oraz na stadia zaawansowania nowotworu IIA, IIB oraz IIIA, które determinują dobór leczenia i przebieg postępowania terapeutycznego.



Rysunek 2. Uproszczona grafika przedstawiająca funkcję wybranych biomarkerów (glukoza, dehydrogenaza mleczanowa, mleczan, MMP-2, MMP-9, IL-6, TNF-α, CRP i SAA_1) oraz zależności pomiędzy nimi, jakie zachodzą w mikrośrodowisku guza płuca.

3.3. II artykuł oryginalny

Hepcydyna jako biomarker diagnostyczny w niedokrwistości u pacjentów z rakiem płuca

Niedokrwistość jest jednym z najczęstszych powikłań u pacjentów onkologicznych, które występuje u około 40% chorych z guzami litymi. Pacjenci z rakiem płuca charakteryzują się najwyższą częstością występowania niedokrwistości, która rozwija się u około 50-70% chorych i niekorzystnie wpływa na ich jakość życia, leczenie oraz przeżycie. [35–38]

W patogenezie niedokrwistości uczestniczą cytokiny prozapalne oraz hormon stanu zapalnego – hepcydyna, która jest odpowiedzialna za regulację dostępności żelaza dla rozwijających się krwinek czerwonych. [39] Celem pracy była analiza przydatności diagnostycznej hormonu hepcydyny oraz cytokin prozapalnych IL-6 i TNF-α i białek ostrej fazy CRP i SAA_1 u operacyjnych pacjentów z rakiem płuca w kontekście oceny podstaw patogenezy niedokrwistości i stopnia jej ciężkości, jak również możliwości

przewidywania występowania powikłania pooperacyjnego w postaci rozwinięcia się niedokrwistości pooperacyjnej.

3.4. III artykuł oryginalny

Analiza polimorfizmów MMP-2 -735C/T (rs2285053) i MMP-9 -1562C/T (rs3918242) w ocenie ryzyka zachorowania na raka płuca

Do populacji zwiększonego ryzyka zachorowania na raka płuca kwalifikuje się (1) osoby z wysoką ekspozycją środowiskową lub zawodową na czynniki rakotwórcze zawarte na przykład w dymie tytoniowym, (2) nosicieli mutacji o wysokiej penetracji i niskiej częstości w genach linii zarodkowej, (3) nosicieli mutacji o niskiej penetracji i wysokiej częstości (na przykład polimorfizmy pojedynczego nukleotydu w genach odpowiedzialnych za metabolizm kancerogenów lub naprawę DNA) narażonych na niskie i większe dawki czynników rakotwórczych. [40] Analiza markerów polimorficznych pozwala na rozpoznanie chorób o podłożu genetycznym, o nieznanych produktach konkretnych genów lub molekularnym charakterze zmian prowadzących do rozwoju choroby. [41]

W niniejszej pracy przebadano warianty o niskiej penetracji, to jest polimorfizmy w regionach promotorów genów MMP-2 i MMP-9 odpowiednio w pozycjach -735C/T (rs2285053) i -1562C/T (rs3918242) w kontekście ich potencjalnego wpływu na ryzyko zachorowania na raka płuca zarówno u pacjentów niepalących, jak i palących wyroby tytoniowe.

Celem pracy była odpowiedź na następujące pytania:

(1) Czy na podstawie analizy polimorfizmów MMP-2-735C/T oraz MMP-9-1562C/T można zidentyfikować populację o zwiększonym ryzyku zachorowania na raka płuca?
(2) Czy polimorfizmy MMP-2-735C/T i MMP-9-1562C/T wpływają na surowicze stężenia odpowiednio MMP-2 i MMP-9?
(3) Czy podtypy histologiczne raka płuca różnią się częstością występowania poszczególnych genotypów MMP-2-735C/T i MMP-9-1562C/T?

4. Materiały i metody zastosowane w badaniach

4.1. Materiał badany

4.1.1. I i II artykuł oryginalny

Nowe spojrzenie na stare biomarkery zaangażowane w zmiany w mikrośrodowisku guza i ich znaczenie diagnostyczne w niedrobnokomórkowym raku płuca *oraz* ***Hepcydyna jako biomarker diagnostyczny w niedokrwistości u pacjentów z rakiem płuca***

Grupę badaną stanowiło 112 pacjentów z nowotworami płuca zakwalifikowanych do leczenia operacyjnego. Wszyscy pacjenci zostali zrekrutowani do projektu badawczego przez lekarzy torakochirurgów z Katedry i Kliniki Chirurgii Klatki Piersiowej, Dolnośląskiego Centrum Onkologii, Pulmonologii i Hematologii we Wrocławiu oraz podpisali świadome zgody na udział w badaniu. Protokół projektu badawczego był zgodny z zasadami Deklaracji helsińskiej (2000) opracowanymi przez Światowe Towarzystwo Medyczne. Projekt obejmujący zbiórkę materiału biologicznego oraz prowadzenie na tym materiale badań naukowych, został pozytywnie zaopiniowany przez Komisję Bioetyczną przy Uniwersytecie Medycznym we Wrocławiu (NR KB: 106/2020 wraz z przedłużeniem NR KB: 214/2022) pod warunkiem zachowania anonimowości uzyskanych danych.

Przed leczeniem operacyjnym, od wszystkich pacjentów zostały pobrane próbki krwi żylnej do probówek zawierających (1) antykoagulant, kwas wersenowy (z ang. *ethylenediaminetetraacetic acid*, EDTA) oraz (2) aktywator krzepnięcia. Próbki krwi zaraz po przetransportowaniu do Katedry Analityki Medycznej były odwirowane w warunkach 2000× g przez 8–10 minut w temperaturze pokojowej, w celu uzyskania osocza i surowicy pacjenta. Następnie osocze i surowica były porcjowane, żeby unikać wielokrotnych cykli rozmrażania–zamrażania próbek w czasie prowadzenia badań. Materiał biologiczny był przechowywany w temperaturze -80°C do czasu jego użycia.

Wszyscy pacjenci byli leczeni operacyjnie z wykorzystaniem wideotorakoskopii (z ang. *video-assisted thoracoscopic surgery*, VATS) lub torakotomii i przeszli zabieg usunięcia płata płuca (lobektomia, u 61 ze 112 pacjentów, 54.46%), resekcji klinowej guza (28 ze 112 pacjentów, 25.00%), biopsji guza (9 ze 112 pacjentów, 8.04%), usunięcia segmentu płuca (segmentektomia, u 7 ze 112 pacjentów, 6.25%), usunięcia dwóch płatów

płuca (bilobektomia, u 5 ze 112 pacjentów, 4.46%) lub usunięcia całego płuca (pulmonektomia, u 2 ze 112 pacjentów, 1.79%).

Ocena histopatologiczna materiału tkankowego pobranego śródoperacyjnie potwierdziła rozpoznanie nowotworu płuca, które zostało ustalone zgodnie z wytycznymi praktyki klinicznej w onkologii organizacji NCCN i z określeniem stopnia nowotworu według ósmej edycji klasyfikacji TNM, opracowanej przez Amerykański Wspólny Komitet ds. Raka (z ang. *American Joint Committee on Cancer's*, AJCC). Grupę badaną stanowiło 98 pacjentów z niedrobnokomórkowym rakiem płuca (98 ze 112 pacjentów, 87.50%), z czego u 50 zdiagnozowano gruczolakoraka (50 ze 112, 44.64%), u 35 raka płaskonabłonkowego (35 ze 112, 31.25%), a u 13 pacjentów inne podtypy raka niedrobnokomórkowego płuca (13 ze 112, 11.61%, w tym 5 z rakiem wielkokomórkówym neuroendokrynnym, czterech z typowym rakowiakiem, dwóch z rakiem pleomorficznym i dwóch z rakiem nieokreślonym inaczej). U pozostałych 14 pacjentów (14 ze 112, 12.50%) zdiagnozowano inne nowotwory niż niedrobnokomórkowy rak płuca zlokalizowane w płucach, w tym u jednego pacjenta postawiono rozpoznanie drobnokomórkowego raka płuca, u dwóch międzybłoniaka, u czterech łagodnego guzka w płucach, a u siedmiu pacjentów przerzuty innych nowotworów (między innymi raka piersi, raka jelita grubego, raka żołądka lub przełyku) do płuc.

Wszystkie dostępne dane kliniczne, patologiczne i laboratoryjne pacjentów zostały zebrane z systemu informatycznego, z którego korzystał szpital (Asseco Medical Management Solutions, AMMS).

4.1.2. III artykuł oryginalny

Analiza polimorfizmów MMP-2 -735C/T (rs2285053) i MMP-9 -1562C/T (rs3918242) w ocenie ryzyka zachorowania na raka płuca

Praca obejmuje badanie retrospektywne z grupą badaną i grupą kontrolną. Grupa badana została opisana powyżej w podrozdziale 6.1.1. Grupę kontrolną stanowiło 47 niepalących i 51 palących osób. Materiał biologiczny (surowica, krew pełna do izolacji DNA) oraz podstawowe informacje o osobach z grupy kontrolnej zostały przekazane do Katedry Analityki Medycznej, Zakładu Chemii Klinicznej i Hematologii Laboratoryjnej z Biobanku Sieci Badawczej Łukasiewicz – PORT Polskiego Ośrodka Rozwoju Technologii. Wszystkie dostarczone próbki materiału biologicznego były przechowywane w temperaturze -80°C do czasu ich użycia. Komisja Bioetyczna przy

Uniwersytecie Medycznym we Wrocławiu wydała pozytywną opinię (NR KB: 507/2021) na prowadzenie badań naukowych na uzyskanym materiale biologicznym od zdrowych osób pod warunkiem zachowania anonimowości uzyskanych danych.

4.2. Metodyka badań

4.2.1. Artykuł przeglądowy

Do przygotowania artykułu przeglądowego została przeprowadzona kwerenda bibliograficzna przy użyciu bazy danych MEDLINE, wyszukiwarki PubMed w internetowych bazach danych oraz przeglądarki Google Scholar do wyszukiwania danych naukowych. Podczas wyszukiwania aktualnej literatury tematu korzystano ze słów kluczowych i ich kombinacji, między innymi „(lung cancer or lung tumour or lung neoplasm) AND genetic biomarker", „(lung cancer or lung tumour or lung neoplasm) AND (microRNA or miRNA)", lung carcinogenesis AND genetic changes".

4.2.2. I artykuł oryginalny

Nowe spojrzenie na stare biomarkery zaangażowane w zmiany w mikrośrodowisku guza i ich znaczenie diagnostyczne w niedrobnokomórkowym raku płuca

Do przygotowania pierwszego artykułu oryginalnego zostały wykonane oznaczenia 12 biomarkerów w surowicy i/lub osoczu pacjentów z rakiem płuca. Stężenia markerów nowotworowych CEA, CYFRA 21-1 i NSE, metaloproteinaz macierzy zewnątrzkomórkowej MMP-2 i MMP-9, cytokin prozapalnych IL-6 i TNF-α oraz białka ostrej fazy SAA_1 zostały oznaczone z zastosowaniem komercyjnie dostępnych testów immunoenzymatycznych (z ang. *enzyme immunoassay*, EIA), głównie testów fazy stałej ELISA (z ang. *enzyme-linked immunosorbent assay*), zgodnie z instrukcjami i zaleceniami dostarczonymi przez producentów (Tabela 4). Do wykonania oznaczeń powyższych parametrów został wykorzystany wielofunkcyjny czytnik płytek SPARK TK BIOTECH (Tecan, Männedorf, Switzerland).

Tabela 4. Testy wykorzystane do wykonania oznaczeń stężeń biomarkerów.

Lp.	Oznaczony biomarker	Nazwa testu	Producent	Numer katalogowy	Kraj pochodzenia
1	CEA	CEA ELISA	DRG Instrument GmbH	EIA 1868	Marburg, Germany
2	CYFRA21-1	CYFRA21-1 EIA	Fujirebio Diagnostics AB	211-10	Göteborg, Sweden

3	NSE	Human Enolase 2/Neuron-specific Enolase Quantikine ELISA Kit	R&D Systems, Inc.	DENL20	Minnesota, MN, USA
4	MMP-2	Total MMP-2 Quantikine ELISA Kit	R&D Systems, Inc.	MMP200	Minnesota, MN, USA
5	MMP-9	Human MMP-9 Quantikine ELISA Kit	R&D Systems, Inc.	DMP900	Minnesota, MN, USA
6	IL-6	Human IL-6 DuoSet ELISA	R&D Systems, Inc.	DY206	Minnesota, MN, USA
7	TNF-α	Human TNF-alpha DuoSet ELISA	R&D Systems, Inc.	DY210	Minnesota, MN, USA
8	SAA_1	Human Serum Amyloid A1 DuoSet ELISA	R&D Systems, Inc.	DY3019-05	Minnesota, MN, USA
9	CRP	C-REACTIVE PROTEIN	BioSystems S.A.	31321	Barcelona, Spain
10	Glukoza	GLUCOSE-HK	BioSystems S.A.	11538	Barcelona, Spain
11	Mleczan	LACTATE	BioSystems S.A.	11736	Barcelona, Spain
12	Dehydrogenaza mleczanowa	LACTATE DEHYDROGENASE, LDH	BioSystems S.A.	11580	Barcelona, Spain

Białko ostrej fazy CRP oraz parametry metabolizmu glukozy (glukoza, mleczan, dehydrogenaza mleczanowa) zostały oznaczone za pomocą testów komercyjnych firmy BioSystems S.A. przy użyciu automatycznego analizatora biochemicznego a15 (BioSystems, Barcelona, Spain).

Różnice w stężeniach biomarkerów wśród pacjentów z rakiem płuca były badane w zależności od (1) podtypu raka płuca (pacjenci z gruczolakorakiem vs. z rakiem płaskonabłonkowym vs. z innymi nowotworami raka niedrobnokomórkowego płuca vs. z innymi nowotworami niż niedrobnokomórkowy płuca) oraz (2) stadium zaawansowania choroby (pacjenci ze stadium IA vs. IB vs. IIA vs. IIB vs. IIIA vs. IIIB vs. IVA).

4.2.3. II artykuł oryginalny

Hepcydyna jako biomarker diagnostyczny w niedokrwistości u pacjentów z rakiem płuca

W drugim artykule oryginalnym zostały wykorzystane wyniki oznaczeń stężeń cytokin prozapalnych IL-6 i TNF-α oraz białek ostrej fazy CRP i SAA_1, wykonanych metodami opisanymi powyżej w podrozdziale 6.2.1. Ponadto wykonano oznaczenie stężenia hormonu peptydowego hepcydyny z zastosowaniem komercyjnego testu Human Hepcidin DuoSet ELISA (Catalog Number: DY8307-05, R&D Systems, Inc., Minnesota, MN, USA) zgodnie z instrukcją i zaleceniami producenta oraz użyciem wielofunkcyjnego czytnika płytek SPARK TK BIOTECH (Tecan, Männedorf, Switzerland).

W pracy zostały wykorzystane również wyniki oznaczeń morfologii krwi obwodowej, wykonanych w szpitalnym diagnostycznym laboratorium medycznym z użyciem automatycznych analizatorów hematologicznych XN-550 oraz XN-1000 (Sysmex Corporation, Kobe, Japan).

Różnice w stężeniach biomarkerów wśród pacjentów z rakiem płuca były badane w zależności od płci pacjenta w połączeniu z liczbą wykonanych oznaczeń morfologii krwi obwodowej w trakcie hospitalizacji. Na podstawie wyników oznaczeń morfologii krwi obwodowej pacjenci zostali podzieleni na grupy: (1) z niedokrwistością od momentu przyjęcia do szpitala, (2) z niedokrwistością rozwiniętą w trakcie hospitalizacji oraz (3) bez niedokrwistości w trakcie hospitalizacji.

4.2.4. III artykuł oryginalny

Analiza polimorfizmów MMP-2 -735C/T (rs2285053) i MMP-9 -1562C/T (rs3918242) w ocenie ryzyka zachorowania na raka płuca

Z próbek krwi pełnej pobranej na EDTA od pacjentów z rakiem płuca i od zdrowych osób z grupy kontrolnej zostało wyizolowane DNA przy użyciu gotowego zestawu komercyjnego Syngen Blood/Cell DNA Mini Kit (300) (Cat. No: SY221012, Syngen Biotech, Wroclaw, Poland), opartego na metodzie kolumnowej. Opracowany materiał został poddany ocenie pod kątem czystości wyizolowanego DNA oraz wydajności izolacji przy użyciu spektrofotometru NanoDrop™ Lite (Thermo Fisher Scientific, Waltham, MA, USA). Tak przygotowany materiał był gotowy do dalszych badań i przechowywany w temperaturze -80°C do czasu jego użycia.

Wyizolowane DNA zostało poddane analizie polimorfizmów z użyciem metody polimorfizmu długości fragmentów restrykcyjnych w połączeniu z techniką łańcuchowej reakcji polimerazy (z ang. *polymerase-chain reaction–restriction fragment length polymorphism*, PCR-RFLP). Metoda ta składa się z czterech etapów: (1) amplifikacji regionów promotora zawierających polimorfizmy z zastosowaniem PCR przeprowadzonym w warunkach szczegółowo opisanych w Tabeli 5 (Tabela 9 artykułu) i użyciem systemu detekcji PCR w czasie rzeczywistym – CFX96™ Real-Time System z C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA); (2) cięcia specyficznymi endonukleazami restrykcyjnymi zamplifikowanych sekwencji DNA w warunkach opisanych w Tabeli 5 (Tabela 9 artykułu) i użyciem aparatu CFX96™ Real-Time System z C1000 Touch Thermal Cycler; (3) rozdziału elektroforetycznego produktów trawienia enzymem restrykcyjnym w warunkach opisanych w Tabeli 5 (Tabela 9 artykułu) i użyciem aparatu do elektroforezy poziomej i zasilacza do elektroforezy firmy Bio-Rad (Hercules, CA, USA); (4) analizy żelu w świetle UV z zastosowaniem systemu obrazowania żeli ChemiDoc™ XRS+ z oprogramowaniem Image Lab™ (Bio-Rad, Hercules, CA, USA).

Tabela 5. Protokół opracowanej i zastosowanej metody PCR/RFLP do analizy polimorfizmów MMP-2-735C/T (rs2285053) i MMP-9-1562C/T (rs3918242).

	***MMP-2*-735 C/T**	***MMP-9*-1562 C/T**
I. Amplifikacja regionów promotora z zastosowaniem PCR		
PCR Mix: (ilości podane dla jednej reakcji)	Forward primer: 0.6 μL Reverse primer: 0.6 μL Polimeraza Gold Taq (5 U/μL): 0.2 μL 10x bufor Gold: 2 μL 25 mM $MgCl_2$: 1.6 μL 10 mM dNTP Mix: 0.4 μL DNA: 2 μL Woda do PCR: 12.6 μL	Forward primer: 0.6 μL Reverse primer: 0.6 μL Polimeraza Gold Taq (5 U/μL): 0.2 μL 10x bufor Gold: 2 μL 25 mM $MgCl_2$: 1.6 μL 10 mM dNTP Mix: 0.4 μL DNA: 2 μL Woda do PCR: 12.6 μL
Warunki PCR:	Aktywacja: 15 min w 95°C 35 cykli: • Denaturacja: 30 sek w 95°C; • Annealing: 30 sek w 60°C; • Wydłużanie: 30 sek w 72°C. Końcowe wydłużanie: 15 min w 72°C Wstrzymanie: ∞ w 4 °C	Aktywacja: 15 min w 95°C 35 cykli: • Denaturacja: 30 sek w 95°C; • Annealing: 30 sek w 60°C; • Wydłużanie: 30 sek w 72°C. Końcowe wydłużanie: 15 min w 72°C Wstrzymanie: ∞ w 4 °C
II. Trawienie powielonej sekwencji DNA przy użyciu enzymów restrykcyjnych		
Mieszanina reakcyjna:	Produkt po PCR: 10 μL 10(X) bufor Anza™: 2 μL Enzym HinfI: 1 μL Woda do PCR: 7 μL	Produkt po PCR: 10 μL 10(X) bufor B: 2 μL Enzym PaeI: 1 μL Woda do PCR: 18 μL

	MMP-2-735 C/T	*MMP-9*-1562 C/T
Warunki reakcji:	Inkubacja: 16 godz w 37°C Inaktywacja: 20 min w 65°C	Inkubacja: 16 godz w 37°C Inaktywacja: 20 min w 65°C
III. Elektroforeza produktów trawienia fragmentów DNA		
Żel agarozowy:	Agaroza: 1.5 g Bufor TBE 1(x): 100 mL Barwnik Gold DNA: 5 µL	Agaroza: 1.5 g Bufor TBE 1(x): 100 mL Barwnik Gold DNA: 5 µL
Warunki reakcji:	50V przez 5 min 120V przez 120 min	50V przez 5 min 120V przez 120 min
Produkty ostateczne:	CC: 300 bp TT: 254 bp, 46 bp CT: 300 bp, 254 bp, 46 bp	CC: 435 bp TT: 247 bp, 188 bp CT: 435 bp, 247 bp, 188 bp

Oznaczenia: PCR-RFLP – metoda polimorfizmu długości fragmentów restrykcyjnych w połączeniu z techniką łańcuchowej reakcji polimerazy (z ang. *polymerase-chain reaction–restriction fragment length polymorphism*); Mix – mieszanina reakcyjna; dNTP – deoksynukleotydy; 10(X) – 10-krotnie stężony; V – jednostka wolt; CC – genotyp homozygotyczny CC; TT – genotyp homozygotyczny TT; CT – genotyp heterozygotyczny CT.

Ponadto w pracy zostały wykorzystane wyniki oznaczeń stężeń MMP-2 i MMP-9 u pacjentów z rakiem płuca wykonanych metodami opisanymi powyżej w podrozdziale 6.2.1. Przy użyciu tych samych testów komercyjnych zostały wykonane oznaczenia stężeń MMP-2 i MMP-9 u osób z grupy kontrolnej.

W pracy były badane różnice w ryzyku zachorowania na raka płuca poprzez porównywanie zmiennych zależnych jakościowych i ilościowych pomiędzy pacjentami z rakiem płuca z grupy badanej a zdrowymi z grupy kontrolnej. Następnie były porównywane stężenia biomarkerów MMP-2 i MMP-9 pomiędzy pacjentami z grupy badanej i grupy kontrolnej z uwzględnieniem odpowiednio genotypu MMP-2 -735C/T i MMP-9 -1562C/T (otrzymano pacjentów z grupy badanej z genotypem CC vs. z genotypem CT vs. z genotypem TT vs. zdrowych z grupy kontrolnej z genotypem CC vs. z genotypem CT vs. z genotypem TT dla obu polimorfizmów).

4.3. Analiza statystyczna

Do analizy statystycznej została stworzona baza danych, na którą składały się informacje kliniczne i patologiczne pacjentów z rakiem płuca ($n = 37$ zmiennych), wyniki badań laboratoryjnych wykonanych w szpitalu ($n = 55$ zmiennych) oraz wyniki badań oznaczonych w Zakładzie Chemii Klinicznej i Hematologii Laboratoryjnej ($n = 26$). Dane do analiz statystycznych były gromadzone i przetwarzane w programie Microsoft® Excel dla komputerów Mac w wersji 16.72 (23040900). Analiza statystyczna była prowadzona w pakiecie oprogramowania Statistica (TIBCO Software Inc., Palo Alto, CA, USA) w wersji 13.1 z użyciem dodatkowego zestawu medycznego (Plus Package, wersja

5.0.96). We wszystkich analizach wartości p <0.05 były uznawane za istotne statystycznie.

4.3.1. I artykuł oryginalny

Nowe spojrzenie na stare biomarkery zaangażowane w zmiany w mikrośrodowisku guza i ich znaczenie diagnostyczne w niedrobnokomórkowym raku płuca

Przed rozpoczęciem analizy statystycznej wartości stężeń oznaczonych biomarkerów były poddane transformacji logarytmicznej w celu uzyskania rozkładów normalnych w każdej z porównywanych grup. Test Shapiro-Wilka był użyty w celu testowania założenia normalności rozkładów.

W przypadku spełnienia założenia normalności rozkładów średnie między grupami były porównywane przy użyciu testu jednoczynnikowej analizy wariancji (z ang. *one-way analysis of variance*, *one-way* ANOVA) z lub bez poprawki Welcha, w zależności od złamania lub spełnienia założenia o równości wariancji grupowych. Założenie o jednorodności wariancji było weryfikowane za pomocą testu Browna-Forsythe'a. Następnie do sprawdzenia, które z analizowanych średnich różniły się istotnie między sobą były przeprowadzone testy post-hoc z wykorzystaniem testu rozsądnej istotnej różnicy (RIR) Tukeya. W przypadku, kiedy założenie normalności rozkładów nie było spełnione, mediany między grupami były porównywane przy użyciu nieparametrycznej alternatywy, to jest jednoczynnikowej analizy wariancji rang Kruskala-Wallisa (ANOVA Kruskala-Wallisa) i testu post-hoc wielokrotnych porównań między średnimi rang dla wszystkich prób (test Dunna). Do oceny zależności między stężeniami badanych biomarkerów została przeprowadzona analiza korelacji z obliczeniem wartości współczynników korelacji liniowej Pearsona.

Praca została uzupełniona o ocenę wartości klinicznej badanych zmiennych w różnicowaniu gruczolakoraka od raka płaskonabłonkowego płuca oraz stadium IIB od IIA i IIIA od IIB przy użyciu modeli regresji logistycznej z analizą krzywych ROC. Analiza wartości klinicznej została przeprowadzona dla pojedynczych biomarkerów oraz dla paneli składających się z 2 do 5 biomarkerów. Panele biomarkerów o najwyższej wartości diagnostycznej były wyznaczane za pomocą wyliczonego kryterium informacyjnego Akaikego (z ang. *Akaike information criterion*, AIC) w modelu regresji logistycznej.

4.3.2. II artykuł oryginalny

Hepcydyna jako biomarker diagnostyczny w niedokrwistości u pacjentów z rakiem płuca

Różnice w częstotliwości występowania niedokrwistości na kolejnych etapach hospitalizacji były sprawdzone przy użyciu testu niezależności χ^2 (chi-kwadrat) Pearsona.

Test Shapiro-Wilka był użyty w celu testowania założenia normalności rozkładów stężeń biomarkerów wśród badanych grup. W przypadku spełnienia założenia normalności rozkładów, parametryczny test t-Studenta oraz jednoczynnikowa ANOVA były stosowane w celu porównania stężeń biomarkerów pomiędzy odpowiednio dwoma lub trzema (i więcej) badanymi grupami. Do analizy parametrów morfologii krwi obwodowej pomiędzy kolejnymi oznaczeniami laboratoryjnymi została użyta ANOVA dla powtarzanych pomiarów. ANOVA została uzupełniona analizą post-hoc przy użyciu testu Studenta-Newmana-Keulsa oraz jego modyfikacji – testu Duncana porównań wielokrotnych. W przypadku, kiedy założenie normalności rozkładów nie było spełnione, mediany między grupami były porównywane przy użyciu nieparametrycznej alternatywy, to jest jednoczynnikowej analizy wariancji rang Kruskala-Wallisa (ANOVA Kruskala-Wallisa) i testu post-hoc wielokrotnych porównań między średnimi rang dla wszystkich prób (test Dunna).

Analiza statystyczna została uzupełniona o analizę korelacji z obliczeniem wartości współczynników korelacji liniowej Pearsona pomiędzy poszczególnymi biomarkerami i parametrami morfologii krwi obwodowej. Ponadto, w pracy zostały wykorzystane modele regresji logistycznej z analizą krzywych ROC i wyliczeniem AIC do oceny diagnostycznej efektywności badanych biomarkerów w różnicowaniu pacjentów z rakiem płuca bez niedokrwistości vs. pacjentów z niedokrwistością od czasu przyjęcia do szpitala vs. pacjentów z niedokrwistością rozwiniętą w trakcie hospitalizacji z uwzględnieniem ich płci.

4.3.3. III artykuł oryginalny

Analiza polimorfizmów MMP-2 -735C/T (rs2285053) i MMP-9 -1562C/T (rs3918242) w ocenie ryzyka zachorowania na raka płuca

Zmienne zależne jakościowe wyrażone na skali nominalnej były analizowane pomiędzy grupą badaną a grupą kontrolną oraz pomiędzy pacjentami z poszczególnymi podtypami raka płuca przy użyciu testu niezależności χ^2 (chi-kwadrat) Pearsona.

Zmienne zależne ilościowe były testowane pod kątem założenia normalności rozkładów z wykorzystaniem testu Shapiro-Wilka. Dla każdej analizowanej zmiennej zostało wyliczone ryzyko zachorowania na raka płuca na podstawie modelu regresji logistycznej (z podaniem ilorazu szans oraz 95% przedziału ufności) zmiennej dychotomicznej – pacjent z rakiem płuca vs. pacjent zdrowy.

W przypadku spełnienia założenia normalności rozkładów, parametryczny test t-Studenta oraz jednoczynnikowa ANOVA były stosowane w celu porównania stężeń biomarkerów pomiędzy odpowiednio dwoma lub trzema (i więcej) badanymi grupami. ANOVA została uzupełniona analizą post-hoc przy użyciu testu RIR Tukeya dla nierównych liczności (test Spjotvolla i Stoline'a). W przypadku, kiedy założenie normalności rozkładów nie było spełnione, mediany między grupami były porównywane przy użyciu nieparametrycznej alternatywy, to jest jednoczynnikowej analizy wariancji rang Kruskala-Wallisa (ANOVA Kruskala-Wallisa) i testu post-hoc wielokrotnych porównań między średnimi rang dla wszystkich prób (test Dunna).

5. Najważniejsze wyniki badań

5.1. Artykuł przeglądowy

Markery genetyczne w diagnostyce raka płuca

Rak płuca charakteryzuje się wysokim obciążeniem mutacjami nowotworowymi (z ang. *tumour mutational burden*, TMB) w porównaniu z innymi nowotworami złośliwymi. Gruczolakoraka i raka płaskonabłonkowego płuca cechują najwyższe wskaźniki mutacji modyfikujących białka, z wartościami odpowiednio 3,5 i 3,9 na megabazę, podczas gdy średni współczynnik mutacji we wszystkich typach nowotworów wynosi 1,8 na megabazę. [13,42]

Dostępność sekwencjonowania nowej generacji (z ang. *new generation sequencing*, NGS) pozwoliła na pełną charakterystykę profilu mutacji raka płuca w projekcie Międzynarodowego Konsorcjum Genomu Raka (z ang. *International Cancer Genome Consortium*, ICGC) i Atlasu Genomu Raka (z ang. *The Cancer Genome Atlas*, TCGA), które są zaangażowane w rozwój medycyny spersonalizowanej i dostępności terapii celowanych, przede wszystkim dla pacjentów z gruczolakorakiem i rakiem płaskonabłonkowym płuca. NCCN rekomenduje wykonywanie panelu badań genetycznych u pacjentów z NSCLC – obecnie – stanu genów EGFR (z ang. *epidermal growth factor mutations*), ALK (z ang. *anapestic lymphoma kinase rearrangements*) i ROS1 (z ang. *c-ros oncogene 1 rearrangements*) w celu wykrycia mutacji i rearanżacji określających rodzaj leczenia systemowego. [34,43–48]

Według przeprowadzonej kwerendy bibliograficznej ogromny potencjał w diagnostyce raka płuca ma badanie płynnej biopsji, która pozwala na monitorowanie choroby niemalże w czasie rzeczywistym, z wielu lokalizacji guza oraz jest źródłem krążących komórek nowotworowych, krążącego DNA komórkowego (w tym DNA pochodzącym z komórek nowotworowych), egzosomów, wolnego mikroRNA, krążących histonów i nukleosomów. Podczas gdy badanie tomografii komputerowej jest w stanie wykryć nowotwór płuca o wielkości mniejszej od 7-10 mm, zbudowany z około miliarda komórek, badanie płynnej biopsji jest w stanie wykryć wystarczającą ilość DNA nowotworowego uwalnianego z nowotworu zbudowanego z około 50 milionów komórek, czyli z około 20 razy mniejszego guza. [49–53]

W badaniu płynnej biopsji analizie mogą być także poddane mikroRNA, które nie są tkankowo specyficzne, ale przez nabywanie unikatowego profilu genetycznego

komórek nowotworowych w czasie procesu nowotworzenia, krążące mikroRNA różnią się w zależności od mikrośrodowiska nowotworu czy stadium zaawansowania raka. Osoczowe zmiany ekspresji mikroRNA na etapie badań klinicznych były w stanie przewidzieć rozwój raka płuca o 24 miesiące wcześniej niż klasycznie stosowane metody w diagnostyce przesiewowej raka płuca, czyli cytologia plwociny, RTG klatki piersiowej czy badanie niskodawkowej tomografii komputerowej płuc. [54,55]

5.2. I artykuł oryginalny

Nowe spojrzenie na stare biomarkery zaangażowane w zmiany w mikrośrodowisku guza i ich znaczenie diagnostyczne w niedrobnokomórkowym raku płuca

W panelu markerów nowotworowych jedyna zaobserwowana istotna statystycznie różnica występowała w stężeniu markera CYFRA 21-1 pomiędzy pacjentami z rakiem płaskonabłonkowym płuca i pacjentami z innymi podtypami raka niedrobnokomórkowego płuca (me = 5.49 ng/mL vs. me = 2.47 ng/mL, p = 0.0426). Najwyższe stężenia CEA były obserwowane w gruczolakoraku płuca oraz w stadium IIIA raka płuca. W przypadku stężenia markera CYFRA 21-1 najwyższe wartości występowały wśród pacjentów z rakiem płaskonabłonkowym płuca oraz w stadium IVA, natomiast stężenie NSE było najwyższe u pacjentów z innymi nowotworami niż niedrobnokomórkowy płuca i w stadium IVA.

W panelu parametrów stanu zapalnego nie było różnic w stężeniach biomarkerów pomiędzy podtypami raka płuca. Występowały natomiast różnice istotne statystycznie w stężeniu IL-6 pomiędzy stadiami zaawansowania raka płuca, z najwyższym stężeniem w stadiach IIB, IIIA i IIIB (odpowiednio me = 44.14 pg/mL, me = 47.36 pg/mL i me = 37.00 pg/mL) i najniższym w stadium IIA (me = 8.52 pg/mL).

W panelu parametrów metabolizmu glukozy obserwowane były różnice istotne statystycznie w stężeniach glukozy zarówno pomiędzy podtypami raka płuca, jak i pomiędzy stadiami zaawansowania choroby. Stężenie glukozy było najwyższe u pacjentów z innymi nowotworami niż niedrobnokomórkowy rak płuca (me = 130.50 mg/dL) i najniższe u pacjentów z gruczolakorakiem płuca (me = 100 mg/dL), a także najwyższe w stadium IIB (me = 132.00 mg/dL) i najniższe w stadium IIIB (me = 94 mg/dL) zaawansowania raka płuca. Występowały również różnice istotne statystycznie w aktywności dehydrogenazy mleczanowej z najwyższą aktywnością w stadiach IIB i IVA (odpowiednio me = 277.85 U/L, me = 317.07 U/L) oraz najniższą w stadiach IB i IIIA (odpowiednio me = 188.43 U/L i me = 159.60 U/L).

Ponadto pomiędzy analizowanymi biomarkerami istniały statystycznie istotne korelacje. Pomiędzy stężeniami parametrów CYFRA 21-1 i IL-6 istniała dodatnia korelacja wśród pacjentów z gruczolakorakiem i rakiem płaskonabłonkowym (odpowiednio współczynniki korelacji r wynosiły 0.43 i 0.44 z $p = 0.014$ i $p = 0.012$). Dodatkowo stężenie CYFRA 21-1 było dodatnio skorelowane u pacjentów z rakiem płaskonabłonkowym płuca ze stężeniem CEA ($r = 0.36$, $p = 0.048$) i CRP ($r = 0.41$, $p = 0.023$). Wśród pacjentów z gruczolakorakiem i rakiem płaskonabłonkowym występowała także istotna statystycznie dodatnia korelacja między IL-6 i MMP-9 (odpowiednio r = 0.53 i r = 0.49 z p = 0.002 i p = 0.006). Dodatnia korelacja występowała także pomiędzy białkami ostrej fazy SAA_1 i CRP we wszystkich podtypach raka płuca z wyjątkiem pacjentów z gruczolakorakiem ($r = 0.44$, $p = 0.014$ w raku płaskonabłonkowym, $r = 0.72$, $p = 0.028$ w innych podtypach raka niedrobnokomórkowego płuca, $r = 0.80$, $p = 0.003$ w innych nowotworach płuca niż rak niedrobnokomórkowy) oraz pomiędzy SAA_1 i mleczanem wśród pacjentów z gruczolakorakiem ($r = 0.44$, $p = 0.011$) i rakiem płaskonabłonkowym ($r = 0.36$, $p = 0.045$).

Ocena użyteczności klinicznej badanych biomarkerów została przeprowadzona przy użyciu regresji logistycznej wraz z analizą krzywych ROC. W analizie diagnostycznej pojedynczych markerów, glukoza była najlepszym biomarkerem do różnicowania pacjentów z rakiem płaskonabłonkowym od tych z gruczolakorakiem płuca ($p = 0.0048$, czułość 74.3%, swoistość 70.0%, $AUC = 0.674$). IL-6, glukoza i mleczan charakteryzowały się najlepszymi statystykami wartości diagnostycznej przy różnicowaniu pacjentów ze stadium zaawansowania raka płuca IIB od IIA (odpowiednio $p < 0.0001$, czułość 57.1%, swoistość 100.0%, $AUC = 0.849$; $p = 0.0192$, czułość 52.4%, swoistość 100.0%, $AUC = 0.754$; $p = 0.0223$, czułość 100.0%, swoistość 52.4%, $AUC = 0.726$). Natomiast najlepszym biomarkerem w różnicowaniu pacjentów ze stadium zaawansowania raka płuca IIIA od IIB była dehydrogenaza mleczanowa ($p = 0.0036$, czułość 81.0%, swoistość 83.3%, $AUC = 0.762$).

W celu poprawienia wartości diagnostycznej analizowanych biomarkerów, zostały one zebrane w panele. Kombinacje markerów (1) CYFRA 21-1, NSE i SAA_1 ($p = 0.0088$, czułość 75.8%, swoistość 67.7%, $AUC = 0.7565$), (2) CYFRA 21-1, NSE, SAA_1 i GLU ($p = 0.0046$, czułość 69.7%, swoistość 67.7%, $AUC = 0.7693$), (3) CEA, CYFRA 21-1, SAA_1, GLU ($p = 0.0061$, czułość 69.7%, swoistość 58.1%, $AUC = 0.7517$), (4) CEA, CYFRA 21-1, NSE, SAA_1 ($p = 0.0069$, czułość 75.8%, swoistość 71.0%, AUC

= 0.7605), (5) CEA, CYFRA 21-1, SAA_1 ($p = 0.0090$, czułość 66.7%, swoistość 58.1%, $AUC = 0.7243$) były najlepsze w różnicowaniu pacjentów z gruczolakorakiem od pacjentów z rakiem płaskonabłonkowym płuca. Kombinacje (1) IL-6, GLU i LDH (p < 0.0001, czułość 100.0%, swoistość 100.0%, AUC = 0.8333), (2) CEA, IL-6, SAA_1, MMP-9 i LAC ($p = 0.0001$, czułość 100.0%, swoistość 100.0%, $AUC = 1.0000$), (3) GLU i LAC ($p = 0.0018$, czułość 95.2%, swoistość 66.7%, $AUC = 0.9365$), (4) IL-6 i GLU ($p = 0.0033$, czułość 89.5%, swoistość 66.7%, $AUC = 0.8947$), (5) CEA, SAA_1 i LAC ($p = 0.0442$, czułość 94.4%, swoistość 50.0%, $AUC = 0.8425$) najlepiej różnicowały pacjentów ze stadium zaawansowania IIB od IIA. Natomiast kombinacje (1) TNF-α, LAC i LDH ($p = 0.0032$, czułość 54.6%, swoistość 88.2%, $AUC = 0.8609$), (2) CEA, IL-6, LDH ($p = 0.0063$, czułość 72.7%, swoistość 94.4%, $AUC = 0.8686$), (3) CEA i LDH ($p = 0.0067$, czułość 54.6%, swoistość 88.9%, $AUC = 0.7929$), (4) CYFRA 21-1, NSE, LAC, LDH ($p = 0.0057$, czułość 54.5%, swoistość 88.2%, $AUC = 0.8449$), (5) CEA, CYFRA 21-1, NSE, SAA_1 ($p = 0.0380$, czułość 63.6%, swoistość 82.4%, $AUC = 0.8288$) najlepiej różnicowały pacjentów ze stadium zaawansowania raka płuca IIIA od IIB, znaczenie zwiększając czułość i swoistość diagnostyczną wybranych biomarkerów.

5.3. II artykuł oryginalny

Hepcydyna jako biomarker diagnostyczny w niedokrwistości u pacjentów z rakiem płuca

W pracy były obserwowane różnice istotne statystycznie w częstotliwości występowania niedokrwistości pomiędzy kobietami i mężczyznami na etapie przyjęcia do szpitala (odpowiednio 1 z 41, 2.4% kobiet oraz 32 z 71, 45.1% mężczyzn miało niedokrwistość przy przyjęciu do szpitala, $p < 0.0001$) oraz w dniu operacji po zabiegu (odpowiednio 5 z 37, 13.5% kobiet i 38 z 61, 62.3% mężczyzn miało niedokrwistość pooperacyjną, $p < 0.0001$). Ponadto występowały różnice istotne statystycznie w częstotliwości występowania niedokrwistości pomiędzy kolejnymi etapami hospitalizacji zarówno wśród kobiet, spośród których 12 z 40 (30.0%) rozwinęło niedokrwistość w trakcie hospitalizacji, jak i wśród mężczyzn, spośród których 16 z 39 (41.0%) rozwinęło niedokrwistość w trakcie hospitalizacji.

Wartości parametrów czerwonokrwinkowych, czyli średnia objętość erytrocytu (z ang. *mean corpuscular volume*, MCV), średnia masa hemoglobiny w erytrocycie (z ang. *mean corpuscular haemoglobin*, MCH) oraz średnie stężenie hemoglobiny w erytrocycie (z ang. *mean corpuscular haemoglobin concentration*, MCHC)

w oznaczeniach morfologii krwi obwodowej wykonywanych na każdym etapie hospitalizacji, zarówno u kobiet, jak i u mężczyzn mieściły się w zakresie wartości referencyjnych, sugerując występowanie niedokrwistości normocytowej normochromicznej.

Kobiety i mężczyźni byli analizowani osobno z podziałem w zależności od liczby wykonanych oznaczeń morfologii krwi obwodowej w czasie hospitalizacji (jedno badanie, dwa badania lub trzy i więcej badań) oraz występowaniem niedokrwistości. Badanie morfologii krwi obwodowej było wykonane tylko raz u 4 kobiet i 7 mężczyzn, dwa razy u 23 kobiet i 38 mężczyzn oraz trzy i więcej razy u 14 kobiet i 26 mężczyzn. Kobiety, które miały wykonane 3 i/lub więcej oznaczeń morfologii krwi obwodowej, spędzały średnio 3 do 4 dni więcej w szpitalu niż pacjentki z jednym lub dwoma oznaczeniami morfologii, ponadto w największym odsetku występowała u nich niedokrwistość (odpowiednio 71.43% vs. 2.44% vs. 13.51%). Podobna zależność występowała wśród mężczyzn, którzy mieli wykonane 3 i/lub więcej oznaczeń morfologii krwi obwodowej. Spędzali oni średnio 2 do 3 dni więcej w szpitalu niż pacjenci z jednym lub dwoma oznaczeniami morfologii i również w największym odsetku występowała u nich niedokrwistość (odpowiednio 88.46% vs. 45.71% vs. 62.30%).

Kobiety, u których rozwinęła się niedokrwistość w trakcie pobytu w szpitalu, miały niższe surowicze stężenia hepcydyny oraz wyższe stężenia IL-6 niż pacjentki bez niedokrwistości. Zależność ta była obserwowana zarówno w grupie kobiet z dwoma jak i z trzema i/lub więcej oznaczeniami morfologii krwi obwodowej. W grupie z trzema i/lub więcej badaniami morfologii krwi obwodowej u pacjentek z niedokrwistością pooperacyjną stężenie hepcydyny było istotnie statystycznie niższe ($\bar{x}$ = 99.34 ng/mL), a IL-6 wyższe ($\bar{x}$ = 53.72 pg/mL) niż u pacjentek bez niedokrwistości (odpowiednio $\bar{x}$ = 331.41 ng/mL, p = 0.000694 oraz $\bar{x}$ = 24.64 pg/mL, p = 0.030727). Co istotne, średnie stężenia hemoglobiny przy przyjęciu do szpitala nie różniły się pomiędzy pacjentkami, które rozwinęły niedokrwistość i pacjentkami bez niedokrwistości w czasie hospitalizacji (odpowiednio $\bar{x}$ = 12.64 g/dL i $\bar{x}$ = 12.63 g/dL).

Podobna zależność była obserwowana u mężczyzn w grupie z dwoma oznaczeniami morfologii krwi obwodowej. U mężczyzn z niedokrwistością pooperacyjną stężenie hepcydyny było istotnie statystycznie niższe ($\bar{x}$ = 90.84 ng/mL) niż u mężczyzn bez niedokrwistości w czasie hospitalizacji ($\bar{x}$ = 161.50 ng/mL, p = 0.007905).

Zaobserwowano również, że mężczyźni z niedokrwistością przy przyjęciu do szpitala byli średnio starsi o 5 lat od mężczyzn i kobiet bez niedokrwistości przy przyjęciu do szpitala. W dodatku mężczyźni ci mieli wyższe stężenia CRP od mężczyzn i kobiet bez niedokrwistości (odpowiednio me=15.06 mg/L, me=7.56 mg/L, me=2.27 mg/L).

Pomiędzy analizowanymi biomarkerami a parametrami morfologii krwi obwodowej obserwowano istotne statystycznie korelacje. Zarówno wśród mężczyzn jak i wśród kobiet z trzema i/lub więcej oznaczeniami morfologii krwi obwodowej występowała istotna statystycznie dodatnia korelacja pomiędzy stężeniem hepcydyny a parametrami morfologii krwi obwodowej (hemoglobina, hematokryt, liczba erytrocytów) z oznaczeń wykonanych kilka dni po zabiegu operacyjnym (odpowiednio hemoglobina $r = 0.46$, $p = 0.033$ i $r = 0.73$, $p = 0.026$; hematokryt $r = 0.44$, $p = 0.040$ i $r = 0.72$, $p = 0.029$; liczba erytrocytów $r = 0.43$, $p = 0.044$ i $r = 0.70$, $p = 0.036$). Ponadto w grupie mężczyzn z trzema i/lub więcej oznaczeniami morfologii obserwowano istotną statystycznie ujemną korelację pomiędzy stężeniem TNF-α a parametrami morfologii krwi obwodowej (hemoglobina, hematokryt, liczba erytrocytów) z oznaczeń wykonanych przy przyjęciu pacjentów do szpitala (odpowiednio $r = -0.51$, $p = 0.016$; $r = -0.53$, $p = 0.011$; $r = -0.61$, $p = 0.002$).

Badanie zostało uzupełnione o ocenę użyteczności klinicznej oznaczonych biomarkerów przy użyciu regresji logistycznej wraz z analizą krzywych ROC. W toku analiz znaleziono model umożliwiający różnicowanie kobiet bez niedokrwistości pooperacyjnej od kobiet, które niedokrwistość rozwinęły, składający się z połączenia hepcydyny i IL-6 ($p = 0.00574$, czułość 87.5%, swoistość 71.4%, $AUC = 0.8809$). TNF-α to biomarker, który z powodzeniem może różnicować mężczyzn z niedokrwistością przy przyjęciu do szpitala od mężczyzn bez niedokrwistości pooperacyjnej ($p = 0.03237$, czułość 72.0%, swoistość 56.3%, $AUC = 0.6687$). Panele biomarkerów, na które składają się hepcydyna, IL-6 i CRP oraz IL-6 i CRP mogą różnicować mężczyzn z niedokrwistością przy przyjęciu do szpitala odpowiednio od kobiet, które niedokrwistość rozwinęły ($p = 0.00927$, czułość 72.0%, swoistość 85.7%, $AUC = 0.8285$) oraz od kobiet bez niedokrwistości pooperacyjnej ($p = 0.02763$, czułość 80.0%, swoistość 62.5%, $AUC = 0.6966$).

5.4. III artykuł oryginalny

Analiza polimorfizmów MMP-2 -735C/T (rs2285053) i MMP-9 -1562C/T (rs3918242) w ocenie ryzyka zachorowania na raka płuca

Nie było różnic istotnych statystycznie w częstotliwości występowania poszczególnych genotypów MMP-2-735C/T i MMP-9-1562C/T pomiędzy pacjentami z rakiem płuca i osobami z grupy kontrolnej, pomiędzy poszczególnymi podtypami raka płuca oraz pomiędzy pacjentami z rakiem płuca bez przerzutów, z przerzutami do węzłów chłonnych i z odległymi przerzutami. Co za tym idzie, nie występowały różnice istotne statystycznie w częstotliwości rzadszego allelu (z *ang. minor allele frequency*, MAF) pomiędzy pacjentami z rakiem płuca a zdrowymi osobami z grupy kontrolnej zarówno dla polimorfizmu MMP-2-735C/T (*MAF* = 0.1273 vs. 0.1400) jak i MMP-9-1562C/T (*MAF* = 0.1651 vs. 0.1667). Częstotliwość rzadszego allelu w przypadku obu oznaczanych polimorfizmów nie różniła się pomiędzy pacjentami z badanej populacji (grupa kontrolna + grupa badana) a średnią częstotliwością rzadszego allelu dla populacji europejskiej (odpowiednio $MAF_{\text{MMP-2-735C/T}}$ = 0.1333 vs. 0.1101 i $MAF_{\text{MMP-9-1562C/T}}$ = 0.1659 vs. 0.1666).

Porównanie częstotliwości rzadszego allelu polimorfizmów MMP-2-735C/T oraz MMP-9-1562C/T pomiędzy populacjami o różnej przynależności etnicznej, wykazało wyższą częstotliwość rzadszego allelu polimorfizmu MMP-2-735C/T w populacji wschodnioazjatyckiej (*MAF* = 0.2591) w porównaniu z populacją europejską (*MAF* = 0.1101, p = 0.000134), południowoazjatycką (*MAF* = 0.1454, p = 0.000132), afrykańską (*MAF* = 0.1164, p = 0.008623) i amerykańską (*MAF* = 0.1761, p = 0.000254). Ponadto wyższą częstotliwość rzadszego allelu polimorfizmu MMP-2-735C/T obserwowano w populacji amerykańskiej w porównaniu z populacją europejską (p = 0.006904) i afrykańską (p = 0.008623).

W analizie regresji logistycznej genotypy CC oraz CT polimorfizmu MMP-2-735C/T wykazywały odpowiednio 5.4 oraz 7.2-razy wyższe ryzyko zachorowania na raka płuca w porównaniu z genotypem TT. W przypadku polimorfizmu MMP-9-1562C/T genotypy CC i CT również wiązały się z wyższym ryzykiem zachorowania na raka płuca, jednak ryzyko to było zdecydowanie niższe (odpowiednio tylko o 1.5 i 1.6-razy wyższe). Ponadto analiza regresji logistycznej wykazała, że płeć męska, starszy wiek oraz wypalone paczkolata zwiększają ryzyko zachorowania na raka płuca.

Analizie poddano surowicze stężenia MMP-2 i MMP-9 pomiędzy poszczególnymi podtypami raka płuca oraz niepalącymi i palącymi osobami z grupy kontrolnej w zależności odpowiednio od genotypów MMP-2-735C/T i MMP-9-1562C/T. U niepalących osób z grupy kontrolnej i genotypem CT ($\bar{x}$ = 237.00 ng/mL) polimorfizmu MMP-2-735C/T obserwowano wyższe stężenie MMP-2 niż u osób z genotypem CC ($\bar{x}$ = 204.04 ng/mL, p = 0.041479). Odwrotną zależność obserwowano u pacjentów z gruczolakorakiem i genotypem CT ($\bar{x}$ = 126.37 ng/mL), u których stężenie MMP-2 było niższe niż u pacjentów z gruczolakorakiem i genotypem CC ($\bar{x}$ = 157.69 ng/mL, p = 0.013222) oraz u pacjentów z rakiem płaskonabłonkowym i genotypem CT ($\bar{x}$ = 130.43 ng/mL) w porównaniu z pacjentami z rakiem płaskonabłonkowym płuca i genotypem CC ($\bar{x}$ = 146.11 ng/mL). Stężenie MMP-2 różniło się istotnie statystycznie również pomiędzy pacjentami z innymi podtypami raka płuca i genotypem CT ($\bar{x}$ = 164.78 ng/mL) a pacjentami z gruczolakorakiem i genotypem CT ($\bar{x}$ = 126.37 ng/mL, p = 0.003790), a także pacjentami z rakiem płaskonabłonkowym płuca i genotypem CT ($\bar{x}$ = 130.43 ng/mL, p = 0.029928).

W przypadku analizy MMP-9 obserwowano wyższe stężenia u osób zdrowych palących niż u zdrowych niepalących wśród wszystkich genotypów MMP-9-1562C/T (385.67 ng/mL, 562.80 ng/mL i 648.57 ng/mL w porównaniu z 312.41 ng/mL, 452.62 ng/mL i 358.70 ng/mL odpowiednio dla genotypu CC, CT i TT). Ponadto istotnie statystycznie wyższe stężenie MMP-9 występowało u zdrowych palących osób z genotypem CT ($\bar{x}$ = 562.80 ng/mL) polimorfizmu MMP-9-1562C/T niż u osób z genotypem CC ($\bar{x}$ = 385.67 ng/mL, p = 0.000936). Stężenie MMP-9 było również istotnie statystycznie wyższe u pacjentów z genotypem CT i innymi podtypami raka płuca ($\bar{x}$ = 928.88 ng/mL) niż u pacjentów z genotypem CC ($\bar{x}$ = 821.64 ng/mL, p = 0.023315).

6. Wnioski

6.1. Artykuł przeglądowy

Markery genetyczne w diagnostyce raka płuca

1) Wysokie obciążenie mutacjami nowotworowymi pacjentów z rakiem płuca jest prawdopodobnie związane z paleniem papierosów i ekspozycją na ksenobiotyki zawarte w dymie papierosowym.
2) Wykrywanie mutacji genetycznych ma znaczenie w procesie diagnostycznym, ale też na różnych etapach procesu leczenia.
3) Markery genetyczne oraz badanie płynnej biopsji mogą być szczególnie przydatne w rozpoznawaniu raka płuca we wczesnym stadium zaawansowania choroby.
4) Markerami genetycznymi o największym potencjale włączenia do panelu badań przesiewowych są mikroRNA i ich profile ekspresji.

6.2. I artykuł oryginalny

Nowe spojrzenie na stare biomarkery zaangażowane w zmiany w mikrośrodowisku guza i ich znaczenie diagnostyczne w niedrobnokomórkowym raku płuca

1) Zaobserwowane korelacje pomiędzy stężeniami biomarkerów sugerują, że mikrośrodowisko guza płuca różni się biochemicznie w zależności od podtypu nowotworu, co może wpływać na patogenezę oraz progresję nowotworu.
2) Uzupełnienie badań markerów nowotworowych stężeniami oznaczonych parametrów panelu stanu zapalnego i panelu metabolizmu glukozy poprawiło statystyczne wskaźniki wartości diagnostycznej kombinacji biomarkerów różnicujących gruczolakoraka od raka płaskonabłonkowego płuca, jednak rezultaty wciąż nie są zadowalające, zapewniając tylko dostateczną dyskryminację modelu ($0.7 < AUC \leq 0.8$).
3) Wymodelowane kombinacje biomarkerów z powodzeniem różnicują pacjentów ze stadiami zaawansowania raka płuca IIB od IIA oraz IIIA od IIB, wpływającymi na podejmowane decyzje terapeutyczne, co mogłoby przyspieszyć wdrożenie odpowiedniego leczenia w jak najkrótszym czasie.
4) Wymodelowane kombinacje biomarkerów różnicujące gruczolakoraka od raka płaskonabłonkowego oraz stadium IIB od IIA i IIIA od IIB mogą zostać włączone

do schematu diagnostyki raka płuca jako tanie, proste i praktyczne narzędzie diagnostyczne.

6.3. II artykuł oryginalny

Hepcydyna jako biomarker diagnostyczny w niedokrwistości u pacjentów z rakiem płuca

1) Hepcydyna i IL-6 pełnią istotną rolę w rozwoju niedokrwistości u pacjentów onkologicznych.
2) Zaobserwowana dodatnia korelacja pomiędzy stężeniem hepcydyny a stężeniem hemoglobiny, wartością hematokrytu i liczbą erytrocytów z oznaczenia morfologii krwi obwodowej po zabiegu operacyjnym sugeruje, że niskie stężenie hepcydyny odpowiada nie tylko za większe ryzyko rozwinięcia niedokrwistości pooperacyjnej, ale także, że im niższe jest to stężenie, tym cięższy stopień niedokrwistości.
3) Oznaczenie stężenia hepcydyny i CRP może służyć do oceny patogenezy niedokrwistości – oceny czy niedokrwistość jest wynikiem niedoboru żelaza, stanu zapalnego, czy niedoboru żelaza i stanu zapalnego.
4) Oznaczenie stężenia hepcydyny i IL-6 przy przyjęciu pacjenta do szpitala może pomóc w wyodrębnieniu pacjentów, u których z dużym prawdopodobieństwem rozwinie się niedokrwistość pooperacyjna.
5) a) Krótkofalowo, przeciwdziałanie i leczenie niedokrwistości u pacjentów z rakiem płuca może przyspieszyć rekonwalescencję po zabiegu operacyjnym i skrócić czas hospitalizacji pacjentów (pacjenci z niedokrwistością pooperacyjną spędzają średnio od 2 do 4 dni więcej w szpitalu).

   b) Długofalowo, przeciwdziałanie i leczenie niedokrwistości u pacjentów z rakiem płuca może być kluczowym punktem poprawy ich przeżywalności.

   c) Ogólnie, przeciwdziałanie i leczenie niedokrwistości u pacjentów z rakiem płuca wydaje się korzystne zarówno dla samego pacjenta, jak i z ekonomicznego punktu widzenia.
6) Wyniki badań sugerują potrzebę włączenia hepcydyny do praktyki klinicznej, co będzie wymagało opracowania ilościowej, czułej, szybkiej, łatwej i taniej metody oznaczania tego hormonu.

6.4. III artykuł oryginalny

Analiza polimorfizmów MMP-2 -735C/T (rs2285053) i MMP-9 -1562C/T (rs3918242) w ocenie ryzyka zachorowania na raka płuca

1) Wyniki badań sugerują związek pomiędzy pochodzeniem etnicznym a ryzykiem wystąpienia mutacji w genie kodującym MMP-2 oraz tłumaczą różnice w wynikach poszczególnych badań przeprowadzonych na różnych populacjach. Pochodzenie etniczne musi być brane pod uwagę przy ocenie ryzyka zachowania na raka płuca opartego na polimorfizmie MMP-2-735C/T.
2) Różnice w stężeniach MMP-2 i MMP-9 zarówno wśród pacjentów z rakiem płuca, jak i osób zdrowych pomiędzy genotypami odpowiednio MMP-2-735C/T i MMP-9-1562C/T potwierdzają, że polimorfizm w regionie promotora genu MMP-2 i MMP-9 wpływa na poziom ich ekspresji.
3) Wyższe stężenie MMP-2 u pacjentów z rakiem płuca (związane z bardziej agresywnym przebiegiem choroby i wyższym prawdopodobieństwem wystąpienia przerzutów odległych) i genotypem MMP-2-735CC (związanym z wyższym ryzykiem zachorowania na raka płuca) sugeruje gorszą prognozę, z krótszym przeżyciem niż u pacjentów z genotypem -735CT i/lub -735TT.
4) Poszczególne genotypy polimorfizmów rs2285053 i rs3918242 wpływają na surowicze stężenia MMP-2 i MMP-9, które w przypadku podwyższonego stężenia przez dłuższy czas mogą zwiększać ryzyko nowotworzenia raka płuca oraz wpływać na bardziej agresywny przebieg choroby i gorsze przeżycie pacjentów.
5) Wyniki stężeń MMP-2 i MMP-9 w grupie osób zdrowych niepalących i palących sugerują, że palenie tytoniu jest czynnikiem wywołującym procesy przebudowy macierzy zewnątrzkomórkowej, która może inicjować procesy nowotworzenia.

6.5. Wnioski końcowe

Przeprowadzone badania są dowodem na możliwości zastosowania wybranych biomarkerów, to jest IL-6, TNF-α, MMP-2, MMP-9, białek ostrej fazy (CRP i SAA_1) oraz hepcydyny w szeroko pojętej diagnostyce raka płuca, na różnych etapach procesu diagnostycznego. Wnioski zostały wyciągnięte na podstawie uzyskanych wyników, popartych między innymi wyliczonymi statystycznymi wskaźnikami wartości diagnostycznej badań laboratoryjnych oraz w oparciu o istniejące dowody naukowe i literaturę tematu. Ze względu na stosunkowo świeżo przeprowadzoną zbiórkę materiału

biologicznego (w okresie od czerwca do grudnia 2020 roku) do badań w niniejszej rozprawie doktorskiej, a także ograniczony czas na przygotowanie rozprawy doktorskiej w ramach Szkoły Doktorskiej, nie było możliwości wykonania analizy wyjściowego stężenia biomarkerów w ocenie 5-letniego całkowitego przeżycia pacjentów oraz 5-letniego przeżycia wolnego od choroby. Taka analiza byłaby istotnym uzupełnieniem naszych badań między innymi w kontekście badania nad niedokrwistością pooperacyjną u pacjentów z rakiem płuca czy badania polimorfizmu MMP-2 -735C/T i MMP-9 -1562C/T.

7. Streszczenie

Wprowadzenie: W 2020 roku rak płuca był najczęstszą przyczyną nowotworowej śmierci na świecie, odpowiedzialną za około 20% zgonów z powodu nowotworu i jednocześnie charakteryzował się on niskim współczynnikiem 5-letniego przeżycia, wynoszącym około 22%. Obecnie stosowane narzędzia diagnostyczne, to jest RTG klatki piersiowej czy badanie cytologiczne plwociny nie są wystarczająco czułymi badaniami przesiewowymi w diagnostyce raka płuca, a dostępne w diagnostyce laboratoryjnej badania markerów nowotworowych (CEA, CYFRA 21-1, NSE, SCC-Ag, ProGRP) nie umożliwiają rozpoznania raka płuca we wczesnym stadium zaawansowania choroby. Dane epidemiologiczne raka płuca wskazują na pilną potrzebę znalezienia bardziej czułych biomarkerów, które znalazłyby zastosowanie w diagnostyce raka płuca.

Rak płuca to choroba przewlekła, wieloczynnikowa, o heterogennej grupie nowotworów, co utrudnia diagnostykę, leczenie, a także zrozumienie procesów leżących u podstaw patogenezy tego nowotworu. W niniejszych badaniach oparto się na teorii mikrośrodowiska guza płuca, na które składają się komórki nowotworowe, macierz zewnątrzkomórkowa, mikrośrodowisko stanu zapalnego z komórkami immunologicznymi, cytokinami prozapalnymi i czynnikami wzrostu, które mogą być źródłem potencjalnych biomarkerów oraz uzupełnieniem diagnostyki biochemicznej aktualnie znanych markerów nowotworowych. Zmiany zachodzące w mikrośrodowisku guza płuca oddziałują na procesy nowotworzenia, progresji nowotworowej, przerzutowania, a także wpływają na ogólnoustrojową odpowiedź chorego, w tym na rozwój przewlekłego stanu zapalnego czy zaburzenia metaboliczne. Tym samym analiza zmian zachodzących w mikrośrodowisku guza płuca może pozwolić na lepsze poznanie podłoża rozwoju raka płuca, a także dalszych procesów zachodzących w organizmie pacjenta, które predysponują do gorszego przebiegu choroby, gorszej odpowiedzi na terapię, krótszego czasu przeżycia wolnego od choroby czy całkowitego czasu przeżycia.

Cel pracy: Celem niniejszej rozprawy doktorskiej było poszerzenie aktualnej wiedzy w zakresie mechanizmów leżących u podstaw patogenezy raka płuca w oparciu o teorię mikrośrodowiska guza poprzez zbadanie zmian biochemicznych wartości potencjalnych biomarkerów, a także analiza możliwości ich zaimplementowania w diagnostyce raka płuca. Do pracy zostały wybrane biomarkery o istotnej roli w procesach zachodzących w mikrośrodowisku guza, to jest cytokiny prozapalne IL-6 i TNF-α, metaloproteinazy macierzy zewnątrzkomórkowej MMP-2 i MMP-9, parametry

metabolizmu glukozy w komórce nowotworowej, czyli glukoza, mleczan oraz dehydrogenaza mleczanowa, a także białka ostrej fazy CRP i SAA_1 będące wyznacznikiem ogólnoustrojowej odpowiedzi chorego na toczące się zmiany w środowisku nowotworowym.

Materiał i metody: Rozprawa doktorska obejmuje obserwacyjne badania retrospektywne, w których porównywano pacjentów z rakiem płuca z poszczególnymi podtypami i stopniem zaawansowania nowotworu, pacjentów z rakiem płuca podzielonych w zależności od występowania powikłania pooperacyjnego, to jest niedokrwistości pooperacyjnej oraz porównywano grupę pacjentów z rakiem płuca z grupą kontrolną. Grupę badaną stanowiło 112 pacjentów z rakiem płuca, 71 mężczyzn i 41 kobiet, 50 pacjentów z gruczolakorakiem i 35 z rakiem płaskonabłonkowym, od których przed leczeniem chirurgicznym pobrano krew do badań. Grupę kontrolną stanowiło 100 zdrowych niepalących i palących ochotników. W surowicy i osoczu pacjentów z rakiem płuca oznaczono wartości stężeń markerów nowotworowych CEA, CYFRA 21-1, NSE oraz biomarkerów mikrośrodowiska guza płuca, to jest IL-6, TNF-α, SAA_1, CRP, MMP-2, MMP-9, glukozy, mleczanu, dehydrogenazy mleczanowej, a także hormonu hepcydyny, wykorzystując testy EIA, testy ELISA oraz zautomatyzowane systemy biochemiczne. Ponadto z krwi pełnej przy użyciu gotowego zestawu komercyjnego opartego na metodzie kolumnowej zostało wyizolowane DNA, a następnie wykorzystane do badania polimorfizmu MMP-2 -735C/T oraz MMP-9 -1562C/T. Analiza statystyczna uzyskanych wyników oraz danych zebranych z dokumentacji medycznej pacjentów została przeprowadzona w pakiecie oprogramowania Statistica (TIBCO Software Inc., Palo Alto, CA, USA) w wersji 13.1 z użyciem dodatkowego zestawu medycznego (Plus Package, wersja 5.0.96).

Wyniki: Zarówno pomiędzy podtypami raka płuca, jak i pomiędzy poszczególnymi stadiami zaawansowania raka płuca występowały różnice istotne statystycznie w stężeniach glukozy. Ponadto u pacjentów z zaawansowanymi stadiami raka płuca obserwowano istotnie wyższe stężenia IL-6 oraz istotnie wyższą aktywność dehydrogenazy mleczanowej. W obrębie poszczególnych podtypów raka płuca obserwowano istotne statystycznie korelacje pomiędzy biomarkerami. Gruczolakorak charakteryzował się silnymi ujemnymi korelacjami pomiędzy wartościami stężeń IL-6 i SAA1, IL-6 i MMP-2 oraz pomiędzy MMP-2 i MMP-9, a także dodatnimi korelacjami pomiędzy IL-6 i MMP-9, SAA_1 i mleczanem oraz glukozą i mleczanem. Silna dodatnia korelacja pomiędzy wartościami stężeń IL-6 i MMP-9 występowała także wśród

pacjentów z rakiem płaskonabłonkowym, których charakteryzowały dodatnie korelacje pomiędzy IL-6 i glukozą, SAA_1 i CRP oraz SAA_1 i mleczanem.

Różnice istotne statystycznie w surowiczych stężeniach MMP-2 i MMP-9 były obserwowane także pomiędzy zdrowymi niepalącymi i palącymi osobami z grupy kontrolnej, z wyższymi wartościami stężeń u zdrowych palących niż palących. Dodatkowo analiza polimorfizmu wykazała, że surowicze stężenia MMP-2 i MMP-9 różniły się istotnie statystycznie w zależności od odpowiednio genotypu MMP-2 -735CC i -735CT oraz MMP-9 -1562CC i -1562CT zarówno wśród pacjentów z rakiem płuca i poszczególnymi podtypami raka płuca, jak i wśród zdrowych osób niepalących i palących.

Analiza użyteczności klinicznej badanych biomarkerów wykazała, że kombinacje markerów nowotworowych CEA, CYFRA 21-1 i NSE z SAA_1 i glukozą podnoszą ich wartość diagnostyczną, i skuteczniej różnicują pacjentów z gruczolakorakiem od pacjentów z rakiem płaskonabłonkowym niż pojedyncze biomarkery. Kombinacje IL-6, glukozy i dehydrogenazy mleczanowej oraz CEA, IL-6, SAA_1, MMP-9 i mleczanu najlepiej różnicują pacjentów ze stadium zaawansowania raka płuca IIB od IIA, natomiast kombinacja CEA, IL-6 i dehydrogenazy mleczanowej jest najlepsza w różnicowaniu pacjentów ze stadium IIIA od tych ze stadium IIB. Ponadto kombinacje markerów IL-6, TNF-α, CRP oraz hormonu hepcydyny są w stanie różnicować kobiety i mężczyzn z rakiem płuca bez niedokrwistości zarówno od pacjentów z niedokrwistością rozwiniętą po zabiegu operacyjnym, jak i pacjentów z niedokrwistością od momentu przyjęcia do szpitala z wysoką czułością i swoistością diagnostyczną oraz dużym AUC krzywej ROC.

Wnioski: Zaobserwowane zmiany biochemicznych wartości badanych biomarkerów sugerują, że mikrośrodowisko guza płuca różni się biochemicznie w zależności od podtypu nowotworu, co może wpływać na patogenezę oraz progresję nowotworu. Ponadto zmiany stężeń biomarkerów mikrośrodowiska płuca mają swoje podłoże genetyczne, sprawiając, że pacjenci będący nosicielami predysponującej mutacji mogą mieć większe ryzyko nasilenia procesów zachodzących w mikrośrodowisku guza płuca, a co za tym idzie większe ryzyko nowotworzenia raka płuca, a także bardziej agresywnego przebiegu choroby i gorszego przeżycia. Sprawia to, że biomarkery są istotnym źródłem informacji na temat procesów toczących się w mikrośrodowisku guza. Przeprowadzone badania użyteczności klinicznej biomarkerów są dowodem na możliwości zastosowania tych biomarkerów w praktyce klinicznej, między innymi do różnicowania pacjentów z gruczolakorakiem od pacjentów z rakiem płaskonabłonkowym

oraz pacjentów ze stadiami zaawansowania raka płuca IIB od IIA oraz IIIA od IIB, które determinują podejmowane decyzje terapeutyczne. Biomarkery jak IL-6, CRP i hormon hepcydyna mogą być także z powodzeniem wdrożone do praktyki klinicznej do oceny patogenezy niedokrwistości, a także w celu wyodrębnienia grupy pacjentów operacyjnych z ryzykiem rozwinięcia powikłania pooperacyjnego, to jest niedokrwistości pooperacyjnej, która wiąże się z niekorzystnymi rokowaniami dla pacjentów z rakiem płuca.

8. Summary

Background: Lung cancer was the leading cause of cancer death worldwide in 2020, accounting for more than 20% of all cancer deaths while also having a poor 5-year survival rate of approximately 22%. Current diagnostic tools, such as chest radiography and sputum cytology, are not sufficiently sensitive in the diagnosis of lung cancer, whereas laboratory diagnostic tumour markers (CEA, CYFRA 21-1, NSE, SCC-Ag, ProGRP) do not enable the diagnosis of lung cancer at an early stage of the disease. Epidemiological data show that there is an urgent need to find more sensitive biomarkers that could improve lung cancer detection.

Lung cancer is a chronic, multifactorial disease with a heterogeneous tumour group that hampers diagnostic and therapeutic approaches, as well as understanding of the processes that underlie its pathogenesis. The current dissertation research is based on the tumour microenvironment theory. Tumour microenvironment is a mixture of tumour cells, extracellular matrix, inflammatory microenvironment with immunological cells, proinflammatory cytokines, and growth factors that could be a source of biomarkers and fill the gap in biochemical diagnostics of currently used tumour markers. Changes in the tumour microenvironment affect cancerogenesis, tumour progression, and metastasis, as well as patients' systemic responses, such as the development of chronic inflammation or metabolic disorders. Thus, analysing changes in the lung tumour microenvironment allows for a better understanding of lung cancer pathogenesis, as well as subsequent processes in the patient's body that lead and predispose to worsening disease course, worsening treatment response, or shorter overall survival or disease-free survival.

Objective of the study: The objective of this dissertation was to broaden knowledge on the mechanisms underlying lung cancer pathogenesis based on tumour microenvironment theory by examining the biochemical changes' values of potential biomarkers and analysing the feasibility of incorporating them into lung cancer diagnostics. In this case, proinflammatory cytokines - IL-6, and TNF-α, extracellular matrix metalloproteinases - MMP-2, and MMP-9, biomarkers of glucose metabolism in cancer cells - glucose, lactate, and lactate dehydrogenase, as well as acute phase proteins - CRP, and SAA_1 that are indicators of the patient's systemic response to changes in the tumour microenvironment - were chosen.

Materials and methods: The dissertation includes observational retrospective studies in which lung cancer patients with particular histological types and stages of the

disease were compared, as well as lung cancer patients subdivided in terms of the prevalence of anaemia during hospitalization. Moreover, comparisons between the group of lung cancer patients and the control group were made. Blood samples were collected from 112 lung cancer patients, comprising 71 men and 41 women; 50 adenocarcinoma patients and 35 squamous cell carcinoma patients, prior to any surgical treatments. The control group included 100 healthy non-smokers and smokers. In the sera and plasmas of lung cancer patients were measured the concentrations of tumour markers CEA, CYFRA 21-1, NSE, and the lung cancer tumour microenvironment biomarkers IL-6, TNF-α, SAA_1, CRP, MMP-2, MMP-9, glucose, lactate, lactate dehydrogenase, and hepcidin hormone, using EIA tests, ELISA tests, and automated clinical biochemistry systems. Whole blood samples were used for DNA genomic isolation using binding column technique, and the polymorphisms MMP-2 -735C/T and MMP-9 -1562C/T were then analysed. TIBCO Software Inc. (Palo Alto, CA, USA) (2017), Statistica, version 13 with the additional Plus Package (version 5.0.96), and a significance threshold of $p < 0.05$ were used to perform statistical analyses of the obtained results and data from patients' hospital records.

Results: There were statistically significant differences in glucose concentrations between lung cancer subtypes and stages. Patients with advanced lung cancer had considerably higher levels of IL-6 and lactate dehydrogenase activity. There were statistically significant correlations between the biomarkers studied across specific lung cancer subtypes. Patients with adenocarcinoma had strong negative correlation between IL-6 and SAA_1, IL-6 and MMP-2, and MMP-2 and MMP-9, as well as positive correlations between IL-6 and MMP-9, SAA_1 and lactate, and glucose and lactate. In squamous cell carcinoma patients, there was also a strong positive link between IL-6 and MMP-9 concentrations, as well as a positive correlation between IL-6 and glucose, SAA_1 and CRP, and SAA_1 and lactate.

There were also statistically significant differences in serum MMP-2 and MMP-9 concentrations between healthy non-smokers and smokers in the control group, with smokers having greater amounts of MMP-2 and MMP-9. Furthermore, polymorphism analysis revealed that serum concentrations of MMP-2 and MMP-9 vary statistically significantly depending on genotype, MMP-2 -735CC and -735CT, and MMP-9 -1562CC and -1562CT, respectively, in lung cancer patients with specific subtypes, as well as healthy non-smokers and smokers.

The clinical utility of the biomarkers studied revealed that combining the tumour markers CEA, CYFRA 21-1, and NSE with SAA_1 and glucose improves their diagnostic value and distinguishes patients with adenocarcinoma from patients with squamous cell carcinoma more effectively than single tumour markers. Combinations of IL-6, glucose, and lactate dehydrogenase, as well as CEA, IL-6, SAA_1, MMP-9, and lactate dehydrogenase, can best distinguish patients with stage IIB lung cancer from those with stage IIA, whereas CEA, IL-6, and lactate dehydrogenase can distinguish patients with stage IIIA lung cancer from those with stage IIB. Furthermore, combinations of the biomarkers IL-6, TNF-α, CRP, and hepcidin hormone can distinguish women and men with lung cancer without anaemia from both patients with anaemia developed after surgery and patients with anaemia since admission to the hospital with high diagnostic sensitivity, specificity, and AUC of the ROC curve.

Conclusions: The observed changes in the biochemical values of the biomarkers studied indicate that the lung tumour microenvironment vary biochemically depending on the tumour subtype, which may affect pathogenesis and tumour development. Furthermore, changes in the concentrations of lung microenvironment biomarkers have a genetic basis, which means that patients who carry a predisposing mutation are at a higher risk of intensifying the processes occurring in the lung tumour microenvironment, and thus have a higher risk of lung cancer development, as well as a more aggressive course of the disease and worsen survival outcomes. As a result, biomarkers are an essential source of information on the processes occurring in the tumour microenvironment. The research on the clinical utility of biomarkers demonstrates the possibility of using these biomarkers in clinical practice, for example, to distinguish patients with adenocarcinoma from patients with squamous cell carcinoma and patients with lung cancer stages IIB from IIA and IIIA from IIB, which determine therapeutic decisions. Biomarkers such as IL-6, CRP, and the hormone hepcidin can also be successfully used in clinical practice to assess the pathogenesis of anaemia and to identify a group of surgical patients who are at risk of developing a postoperative side effect, namely postoperative anaemia, which is associated with a poor prognosis in lung cancer patients.

9. Piśmiennictwo

1. Horn, L.; Lovly, C.M. Chapter 74: Neoplasms of the Lung. In *Harrison's Principles of Internal Medicine*; Loscalzo, J., Fauci, A., Kasper, D., Hauser, S., Longo D., Jameson, J.L., Eds.; McGraw Hill: New York, NY, USA, 2018.
2. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* **2021**, *71*, 209–249, doi:10.3322/caac.21660.
3. Lung Source: Globocan 2020 Number of New Cases in 2020, Both Sexes, All Ages; 2020. Dostępne online: https://gco.iarc.fr/today/data/factsheets/cancers/15-Lung-fact-sheet.pdf
4. Didkowska, J.; Wojciechowska, U.; Mańczuk, M.; Łobaszewski, J. Lung Cancer Epidemiology: Contemporary and Future Challenges Worldwide. *Ann. Transl. Med.* **2016**, *4*, 150, doi:10.21037/atm.2016.03.11.
5. The Economist Intelligence Unit, *BREATHING IN A NEW ERA: A Comparative Analysis of Lung Cancer Policies across Europe*, 2019.
6. Krzakowski, M.; Jassem, J.; Antczak, A.; Błasińska, K.; Chorostowska-Wynimko, J.; Dziadziuszko, R.; Głogowski, M.; Grodzki, T.; Kowalski, D.; Krenke, R.; et al. Thoracic Neoplasms. *Oncology in Clinical Practice* **2022**, *18*, 1–39, doi:10.5603/OCP.2021.0022.
7. Hirsch, F.R.; Scagliotti, G.V; Mulshine, J.L.; Kwon, R.; Curran, W.J.; Wu, Y.-L.; Paz-Ares, L. Lung Cancer: Current Therapies and New Targeted Treatments. *Lancet* **2017**, *389*, 299–311, doi:10.1016/S0140-6736(16)30958-8.
8. Schwartz, A.G.; Prysak, G.M.; Bock, C.H.; Cote, M.L. The Molecular Epidemiology of Lung Cancer. *Carcinogenesis* **2006**, *28*, 507–518, doi:10.1093/carcin/bgl253.
9. Lu, T.; Yang, X.; Huang, Y.; Zhao, M.; Li, M.; Ma, K.; Yin, J.; Zhan, C.; Wang, Q. Trends in the Incidence, Treatment, and Survival of Patients with Lung Cancer in the Last Four Decades. *Cancer Manag. Res.* **2019**, *11*, 943–953, doi:10.2147/CMAR.S187317.
10. Kanty Kulpa, J.; Rychlik, U.; Stasik, Z.; Tarapacz, J.; Wójcik, E.; Niemiec, J.; Kowalik, A. Diagnostyka Laboratoryjna Chorób Nowotworowych - 12.3.15. Rak Płuca. In *Diagnostyka laboratoryjna*; Solnica, B., Ed.; PZWL Wydawnictwo Lekarskie: Warszawa, 2019; pp. 424–427, ISBN 978-83-200-5814-7.
11. Jassem, J.; Wysocki, W.M.; Mejza, F. 3.16. Nowotwory Płuc i Opłucnej. In *Interna Szczeklika 2021/2022*; Niżankowska-Mogilnicka, E., Krenke, R., Mejza, F., Eds.; Medycyna Praktyczna: Kraków, 2021; pp. 571–578, ISBN 978-83-7430-654-6.
12. Barta, J.A.; Powell, C.A.; Wisnivesky, J.P. Global Epidemiology of Lung Cancer. *Ann. Glob. Health.* 2019, *85*, doi:10.5334/aogh.2419.

13. Marino, F.Z.; Bianco, R.; Accardo, M.; Ronchi, A.; Cozzolino, I.; Morgillo, F.; Rossi, G.; Franco, R. Molecular Heterogeneity in Lung Cancer: From Mechanisms of Origin to Clinical Implications. *Int. J. Med. Sci,* **2019**, *16*, 981–989, doi:10.7150/ijms.34739.
14. Travis, W.D.; Brambilla, E.; Nicholson, A.G.; Yatabe, Y.; Austin, J.H.M.; Beasley, M.B.; Chirieac, L.R.; Dacic, S.; Duhig, E.; Flieder, D.B.; et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances since the 2004 Classification. *J. Thorac. Oncol.* 2015, *10*, 1243–1260, doi:10.1097/JTO.0000000000000630.
15. Inamura, K. Lung Cancer: Understanding Its Molecular Pathology and the 2015 WHO Classification. *Front. Oncol.* **2017**, *7*, doi:10.3389/fonc.2017.00193.
16. National Comprehensive Cancer Network, Non-Small Cell Lung Cancer - NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®), *Version 3,* 2023.
17. Mets, O.; Smithuis, R. TNM Classification 8th Edition. Dostępne online: https://radiologyassistant.nl/chest/lung-cancer/tnm-classification-8th-edition
18. Schabath, M.B.; Cote, M.L. Cancer Progress and Priorities: Lung Cancer. *Cancer Epidemiol. Biomarkers Prev.* **2019**, *28*, 1563–1579, doi:10.1158/1055-9965.EPI-19-0221.
19. Bade, B.C.; Dela Cruz, C.S. Lung Cancer 2020: Epidemiology, Etiology, and Prevention. *Clin. Chest. Med.* 2020, *41*, 1–24, doi:10.1016/j.ccm.2019.10.001.
20. Krantz, S.B.; Zeeshan, K.; Kuchta, K.M.; Hensing, T.A.; Mangold, K.A.; Zheng, S.L.; Xu, J. Germline Mutations in High Penetrance Genes Are Associated with Worse Clinical Outcomes in Patients with Non–Small Cell Lung Cancer. *JTCVS Open* **2022**, *12*, 399–409, doi:10.1016/j.xjon.2022.09.001.
21. Shields, P.G.; Harris, C.C. Molecular Epidemiology and the Genetics of Environmental Cancer. *JAMA* **1991**, *266*, 681-687, PMID: 2072479.
22. Biomarker Testing for Cancer Treatment. Dostępne online: https://www.cancer.gov/about-cancer/treatment/types/biomarker-testing-cancer-treatment
23. García-Giménez, J.L.; Seco-Cervera, M.; Tollefsbol, T.O.; Romá-Mateo, C.; Peiró-Chova, L.; Lapunzina, P.; Pallardó, F. V. Epigenetic Biomarkers: Current Strategies and Future Challenges for Their Use in the Clinical Laboratory. *Crit. Rev. Clin. Lab. Sci.* 2017, *54*, 529–550, doi:10.1080/10408363.2017.1410520.
24. Gernand, W. Wartość Diagnostyczna Badań Laboratoryjnych. In *Diagnostyka laboratoryjna*; Solnica, B., Ed.; PZWL Wydawnictwo Lekarskie: Warszawa, 2019; pp. 7–16, ISBN 978-83-200-5814-7.
25. Tumor Markers. Dostępne online: https://www.cancer.gov/about-cancer/diagnosis-staging/diagnosis/tumor-markers-fact-sheet
26. Kanty Kulpa, J.; Rychlik, U.; Stasik, Z.; Tarapacz, J.; Wójcik, E.; Niemiec, J.; Kowalik, A. Diagnostyka Laboratoryjna Chorób Nowotworowych. In *Diagnostyka laboratoryjna*;

Solnica, B., Ed.; PZWL Wydawnictwo Lekarskie: Warszawa, 2019; pp. 379–397, ISBN 978-83-200-5814-7.

27. Tumor Markers in Common Use. Dostępne online: https://www.cancer.gov/about-cancer/diagnosis-staging/diagnosis/tumor-markers-list
28. Solnica, B. 27.1. Badania Biochemiczne, Hematologiczne i Koagulologiczne. In *Interna Szczeklika 2021/2022*; Solnica, B., Ed.; Medycyna Praktyczna: Kraków, 2021, ISBN 978-83-7430-654-6.
29. Isgrò, M.A.; Bottoni, P.; Scatena, R. Neuron-Specific Enolase as a Biomarker: Biochemical and Clinical Aspects. *Adv. Exp. Med. Biol.* **2015**, *867*, 125-143, doi:10.1007/978-94-017-7215-0_9.
30. Xi, K.X.; Zhang, X.W.; Yu, X.Y.; Wang, W.D.; Xi, K.X.; Chen, Y.Q.; Wen, Y.S.; Zhang, L.J. The Role of Plasma MiRNAs in the Diagnosis of Pulmonary Nodules. *J. Thorac. Dis.* **2018**, *10*, 4032–4041, doi:10.21037/jtd.2018.06.106.
31. Chen, J.; Zhou, R. Tumor Microenvironment Related Novel Signature Predict Lung Adenocarcinoma Survival. *PeerJ.* **2021**, *9*, doi:10.7717/peerj.10628.
32. Chen, G.; Dong, Z.; Wu, D.; Chen, Y. Profiles of Immune Infiltration in Lung Adenocarcinoma and Their Clinical Significant: A Gene-Expression–Based Retrospective Study. *J. Cell Biochem.* **2020**, *121*, 4431–4439, doi:10.1002/jcb.29667.
33. Bezel, P.; Valaperti, A.; Steiner, U.; Scholtze, D.; Wieser, S.; Vonow-Eisenring, M.; Widmer, A.; Kowalski, B.; Kohler, M.; Franzen, D.P. Evaluation of Cytokines in the Tumor Microenvironment of Lung Cancer Using Bronchoalveolar Lavage Fluid Analysis. *Cancer Immunol. Immunother.* **2021**, *70*, 1867-1876, doi:10.1007/s00262-020-02798-z.
34. Dietel, M.; Jöhrens, K.; Laffert, M.; Hummel, M.; Bläker, H.; Müller, B.M.; Lehmann, A.; Denkert, C.; Heppner, F.L.; Koch, A.; et al. Predictive Molecular Pathology and Its Role in Targeted Cancer Therapy: A Review Focussing on Clinical Relevance. *Cancer Gene Ther.* **2013**, *20*, 211–221, doi:10.1038/cgt.2013.13.
35. Blohmer, J.-U.; Dunst, J.; Harrison, L.; Johnston, P.; Khayat, D.; Ludwig, H.; O'Brien, M.; Van Belle, S.; Vaupel, P. Cancer-Related Anemia: Biological Findings, Clinical Implications and Impact on Quality of Life. *Oncology* **2005**, *68* (Suppl. S1), 12–21, doi:10.1159/000083129.
36. Birgegård, G.; Aapro, M.S.; Bokemeyer, C.; Dicato, M.; Drings, P.; Hornedo, J.; Krzakowski, M.; Ludwig, H.; Pecorelli, S.; Schmoll, H.; et al. Cancer-Related Anemia: Pathogenesis, Prevalence and Treatment. *Oncology* **2005**, *68* (Suppl. S1), 3–11, doi:10.1159/000083128.
37. Ludwig, H.; Van Belle, S.; Barrett-Lee, P.; Birgegård, G.; Bokemeyer, C.; Gascón, P.; Kosmidis, P.; Krzakowski, M.; Nortier, J.; Olmi, P.; et al. The European Cancer Anaemia Survey (ECAS): A Large, Multinational, Prospective Survey Defining the Prevalence,

Incidence, and Treatment of Anaemia in Cancer Patients. *Eur. J. Cancer* **2004**, *40*, 2293–2306, doi:10.1016/j.ejca.2004.06.019.

38. Dicato, M.; Plawny, L.; Diederich, M. Anemia in Cancer. *Ann. Oncol*. **2010**, *21* (Suppl. S7), 167-172, doi:10.1093/annonc/mdq284.
39. Kanty Kulpa, J.; Rychlik, U.; Stasik, Z.; Tarapacz, J.; Wójcik, E.; Niemiec, J.; Kowalik, A. Diagnostyka Laboratoryjna Chorób Nowotworowych - 12.5. Niedokrwistość Towarzysząca Nowotworom. In *Diagnostyka laboratoryjna*; Solnica, B., Ed.; PZWL Wydawnictwo Lekarskie: Warszawa, 2019; pp. 431–437, ISBN 978-83-200-5814-7.
40. Bartsch, H.; Nair, U.; Risch, A.; Rojas, M.; Wikman, H.; Alexandrov, K. Genetic Polymorphism of CYP Genes, Alone or in Combination, as a Risk Modifier of Tobacco-Related Cancers. *Cancer Epidemiol. Biomarkers Prev.* **2000**, *9*, 3–28, PMID: 10667460.
41. Pass, H.I.; Beer, D.G.; Joseph, S.; Massion, P. Biomarkers and Molecular Testing for Early Detection, Diagnosis, and Therapeutic Prediction of Lung Cancer. *Thorac. Surg. Clin.* **2013**, *23*, 211–224, doi:10.1016/j.thorsurg.2013.01.002.
42. Kan, Z.; Jaiswal, B.S.; Stinson, J.; Janakiraman, V.; Bhatt, D.; Stern, H.M.; Yue, P.; Haverty, P.M.; Bourgon, R.; Zheng, J.; et al. Diverse Somatic Mutation Patterns and Pathway Alterations in Human Cancers. *Nature* **2010**, *466*, 869–873, doi:10.1038/nature09208.
43. Xuchao, Z.; Zhiyong, L.; Shengyue, W.; Shun, L.; Yong, S.; Ying, C.; Jianming, Y.; Weiping, L.; Yingyong, H.; Yangqiu, L.; et al. Application of Next-Generation Sequencing Technology to Precision Medicine in Cancer: Joint Consensus of the Tumor Biomarker Committee of the Chinese Society of Clinical Oncology. *Cancer Biol. Med.* **2019**, *16*, 189, doi:10.20892/j.issn.2095-3941.2018.0142.
44. Park, E.; Shim, H.S. Detection of Targetable Genetic Alterations in Korean Lung Cancer Patients: A Comparison Study of Single-Gene Assays and Targeted next-Generation Sequencing. *Cancer Res. Treat.* **2020**, *52*, 1–9, doi:10.4143/crt.2019.305.
45. Dama, E.; Melocchi, V.; Colangelo, T.; Cuttano, R.; Bianchi, F. Deciphering the Molecular Profile of Lung Cancer: New Strategies for the Early Detection and Prognostic Stratification. *J. Clin. Med.* **2019**, *8*, 108, doi:10.3390/jcm8010108.
46. Mehrad, M.; Roy, S.; Bittar, H.T.; Dacic, S. Next-Generation Sequencing Approach to Non–Small Cell Lung Carcinoma Yields More Actionable Alterations. *Arch. Pathol. Lab. Med.* **2018**, *142*, 353–357, doi:10.5858/arpa.2017-0046-OA.
47. Sands, J.M.; Nguyen, T.; Shivdasani, P.; Sacher, A.G.; Cheng, M.L.; Alden, R.S.; Jänne, P.A.; Kuo, F.C.; Oxnard, G.R.; Sholl, L.M. Next-Generation Sequencing Informs Diagnosis and Identifies Unexpected Therapeutic Targets in Lung Squamous Cell Carcinomas. *Lung Cancer* **2020**, *140*, 35–41, doi:10.1016/j.lungcan.2019.12.005.

48. Fernandes, M.G.O.; Jacob, M.; Martins, N.; Moura, C.S.; Guimarães, S.; Reis, J.P.; Justino, A.; Pina, M.J.; Cirnes, L.; Sousa, C.; et al. Targeted Gene Next-Generation Sequencing Panel in Patients with Advanced Lung Adenocarcinoma: Paving the Way for Clinical Implementation. *Cancers (Basel)* **2019**, *11*, 1229, doi:10.3390/cancers11091229.
49. Santarpia, M.; Liguori, A.; D'Aveni, A.; Karachaliou, N.; Gonzalez-Cao, M.; Daffinà, M.G.; Lazzari, C.; Altavilla, G.; Rosell, R. Liquid Biopsy for Lung Cancer Early Detection. *J. Thorac. Dis.* 2018, *10*, S882–S897, doi:10.21037/jtd.2018.03.81.
50. Müllauer, L. Next Generation Sequencing: Clinical Applications in Solid Tumours. *Memo* **2017**, *10*, 244–247, doi:10.1007/s12254-017-0361-1.
51. Calabrese, F.; Lunardi, F.; Pezzuto, F.; Fortarezza, F.; Vuljan, S.; Marquette, C.; Hofman, P. Are There New Biomarkers in Tissue and Liquid Biopsies for the Early Detection of Non-Small Cell Lung Cancer? *J. Clin. Med.* **2019**, *8*, 414, doi:10.3390/jcm8030414.
52. Crowley, E.; Di Nicolantonio, F.; Loupakis, F.; Bardelli, A. Liquid Biopsy: Monitoring Cancer-Genetics in the Blood. *Nat. Rev. Clin. Oncol.* **2013**, *10*, 472–484, doi:10.1038/nrclinonc.2013.110.
53. Sholl, L.M.; Aisner, D.L.; Allen, T.C.; Beasley, M.B.; Cagle, P.T.; Capelozzi, V.L.; Dacic, S.; Hariri, L.P.; Kerr, K.M.; Lantuejoul, S.; et al. Liquid Biopsy in Lung Cancer: A Perspective From Members of the Pulmonary Pathology Society. *Arch. Pathol. Lab. Med.* **2016**, *140*, 825–829, doi:10.5858/arpa.2016-0163-SA.
54. Lu, S.; Kong, H.; Hou, Y.; Ge, D.; Huang, W.; Ou, J.; Yang, D.; Zhang, L.; Wu, G.; Song, Y.; et al. Two Plasma MicroRNA Panels for Diagnosis and Subtype Discrimination of Lung Cancer. *Lung Cancer* **2018**, *123*, 44–51, doi:10.1016/j.lungcan.2018.06.027.
55. Aiso, T.; Ohtsuka, K.; Ueda, M.; Karita, S.; Yokoyama, T.; Takata, S.; Matsuki, N.; Kondo, H.; Takizawa, H.; Okada, A.; et al. Serum Levels of Candidate MicroRNA Diagnostic Markers Differ among the Stages of Non-small-cell Lung Cancer. *Oncol. Lett.* **2018**, *16*, 6643-6651, doi:10.3892/ol.2018.9464.

10. Źródła finansowania

Praca przeglądowa została opublikowana dzięki wsparciu finansowemu na uiszczenie opłaty publikacyjnej z Funduszu Ministerstwa Nauki i Szkolnictwa Wyższego w ramach programu „Regionalna Inicjatywa Doskonałości" na lata 2019-2022, zarejestrowanego pod numerem 016/RID/2018/19, całkowita kwota finansowania 11.998.121,30 zł.

Badania prowadzone w ramach doktoratu, których wyniki zostały opublikowane w I pracy oryginalnej, były realizowane w ramach tematu „Markery biochemiczne w diagnostyce raka płuca we wczesnym stadium zaawansowania choroby – badania wstępne" w projekcie subwencyjnym dla Młodych Naukowców (młody naukowiec: Katarzyna Wadowska, opiekun naukowy: Mariola Śliwińska-Mossoń; kwota dofinansowania 26.783,00 zł), ujętym w systemie SIMPLE Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu pod numerem STM.D010.20.129.

Badania prowadzone w ramach doktoratu, których wyniki zostały opublikowane w II pracy oryginalnej, były realizowane w ramach tematu „Stres oksydacyjny w przebiegu zespołu metabolicznego i innych chorób przewlekłych" w ramach subwencji Katedry Analityki Medycznej, Zakładu Chemii Klinicznej i Hematologii Laboratoryjnej (kierownik zadania: Mariola Śliwińska-Mossoń, kwota dofinansowania 35.712,00 zł), ujętym w systemie SIMPLE Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu pod numerem SUBZ.D010.22.039.

Badania prowadzone w ramach doktoratu, których wyniki zostały opublikowane w III pracy oryginalnej, były realizowane w ramach tematu „Analiza polimorfizmów MMP-2 -735C/T (rs2285053) i MMP-9 -1562C/T (rs3918242) w ocenie ryzyka zachorowania na raka płuca", w ramach Subwencji 2022 (kierownik projektu: Katarzyna Wadowska, opiekun naukowy: Mariola Śliwińska-Mossoń, kwota dofinansowania 50.000,00 zł), ujętym w systemie SIMPLE Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu pod numerem SUBK.D010.22.041.

11. Wykaz skrótów

AIC – kryterium informacyjne Akaikego, z ang. *Akaike information criterion*

AJCC – Amerykański Wspólny Komitet ds. Raka, z ang. *American Joint Committee on Cancer's*

ALK – z ang. *anapestic lymphoma kinase rearrangements*

AMMS – system informatyczny szpitala, Asseco Medical Management Solutions

ANOVA – jednoczynnikowa analiza wariancji, z ang. *one-way analysis of variance*

AUC – pole powierzchni pod krzywą krzywej ROC, z ang. *area under the curve*

CC – genotyp homozygotyczny CC polimorfizmu MMP-2-735C/T lub MMP-9-1562C/T

CEA (CAM5) – antygen rakowo-płodowy, z ang. *carcinoembryonic antigen*

CRP – białko C-reaktywne, z ang. *C-reactive protein*

CT – genotyp heterozygotyczny CT polimorfizmu MMP-2-735C/T lub MMP-9-1562C/T

cTNM – kliniczna ocena TNM, z ang. *clinical TNM*

CYFRA 21-1 – fragmenty cytokeratyny 19

dNTP – deoksynukleotyd, z ang. *deoxynucleotide triphosphate*

EDTA – kwas wersenowy, z ang. *ethylenediaminetetraacetic acid*

EGFR – z ang. *epidermal growth factor mutations*

EIA – test immunoenzymatyczny, z ang. *enzyme immunoassay*

ELISA – test immunoenzymatyczny fazy stałej, z ang. *enzyme-linked immunosorbent assay*

G – stopień złośliwości nowotworu, z ang. *grade*

ICGC – Międzynarodowe Konsorcjum Genomu Raka, z ang. *International Cancer Genome Consortium*

IL-6 – interleukina 6, z ang. *interleukin 6*

MAF – częstotliwość rzadszego allelu, z ang. *minor allele frequency*

MCH – średnie masa hemoglobiny w krwince czerwonej, z ang. *mean corpuscular haemoglobin*

MCHC – średnie stężenie hemoglobiny w krwince czerwonej, z ang. *mean corpuscular haemoglobin concentration*

MCV – średnia objętość krwinki czerwonej, z ang. *mean corpuscular volume*

me – wartość mediany

MMP-2 i -9 – metaloproteinaza macierzy pozakomórkowej -2 i -9, z ang. *matrix metalloproteinase -2 and -9*

NGS – sekwencjonowanie nowej generacji, z ang. *new generation sequencing*

NOS – nowotwory płuca niesklasyfikowane, z ang. *not-otherwise specified*

NSCLC – rak niedrobnokomórkowy płuca, z ang. *non-small cell lung carcinoma*

NSE – enolaza swoista dla neuronów, z ang. *neuron-specific enolase*

p – wartość p, prawdopodobieństwo testowe, z ang. *p-value, probability value*

PCR-RFLP – metoda polimorfizmu długości fragmentów restrykcyjnych w połączeniu z techniką łańcuchowej reakcji polimerazy, z ang. *polymerase-chain reaction–restriction fragment length polymorphism*

PORT – Polski Ośrodek Rozwoju Technologii

PPV – wartość predykcyjna wyniku dodatniego, z ang. *positive predictive value*

ProGRP – prekursor peptydu uwalniającego gastrynę, z ang. *pro-gastrin-releasing peptide*

pTNM – patomorfologiczna ocena TNM, z ang. *pathological TNM*

r – współczynnik korelacji r-Pearsona

RIR – test rozsądnej istotnej różnicy Tukeya

ROC – krzywa ROC, z ang. *receiver-operator characteristics*

ROS1 – z ang. *c-ros oncogene 1 rearrangements*

RTG – rentgenografia (KP – klatki piersiowej)

SAA_1 – surowiczy amyloid A_1, z ang. *serum amyloid* A_1

SCC-Ag – antygen raka płaskonabłonkowego, z ang. *squamous cell carcinoma antigen*

SCLC – rak drobnokomórkowy płuca, z ang. *small cell lung carcinoma*

TCGA – Atlas Genomu Raka, z ang. *The Cancer Genome Atlas*

TMB – obciążenie mutacjami nowotworowymi, z ang. *tumour mutational burden*

TNF-α – czynnik martwicy nowotworu α, z ang. *tumour necrosis factor* α

TNM – ocena stanu guza pierwotnego – regionalnych węzłów chłonnych – przerzutów odległych, z ang. *tumour – lymph nodes – metastasis*

TT – genotyp homozygotyczny TT polimorfizmu MMP-2-735C/T lub MMP-9-1562C/T

VATS – wideotorakoskopia, z ang. *video-assisted thoracoscopic surgery*

WHO – Światowa Organizacja Zdrowia, z ang. *World Health Organisation*

$\bar{x}$ – wartość średnia

12. Spis rysunków i tabel

12.1. Spis rysunków

Rysunek_1. *Statystyki (a) zachorowalności i (b) śmiertelności z powodu raka na świecie. [2,3] 5*

Rysunek_2. *Uproszczona grafika przedstawiająca funkcję wybranych biomarkerów (glukoza, dehydrogenaza mleczanowa, mleczan, MMP-2, MMP-9, IL-6, TNF-α, CRP i SAA_1) oraz zależności pomiędzy nimi, jakie zachodzą w mikrośrodowisku guza płuca. 16*

12.2. Spis tabel

Tabela_1. *Klasyfikacja patomorfologiczna niedrobnokomórkowego raka płuca.........................7*

Tabela_2. *Postępowanie terapeutyczne w niedrobnokomórkowym raku płuca na podstawie stopnia zaawansowania z 8. Edycji TNM...8*

Tabela_3. *Możliwości diagnostyczne markerów nowotworowych stosowanych w diagnostyce laboratoryjnej raka płuca...11*

Tabela_4. *Testy wykorzystane do wykonania oznaczeń stężeń biomarkerów..............................20*

Tabela_5. *Protokół opracowanej i zastosowanej metody PCR/RFLP do analizy polimorfizmów MMP-2-735C/T (rs2285053) i MMP-9-1562C/T (rs3918242)...23*

13. Spis załączników

Załącznik 1 – artykuł przeglądowy:

Katarzyna Wadowska, Iwona Bil-Lula, Łukasz Trembecki, Mariola Śliwińska-Mossoń: Genetic markers in lung cancer diagnosis: a review. *International Journal of Molecular Sciences*, **2020**, vol. 21, nr 13, art. 4569 [24 s.], DOI:10.3390/ijms21134569

Załącznik 2 – I artykuł oryginalny:

Katarzyna Wadowska, Piotr Błasiak, Adam Rzechonek, Iwona Bil-Lula, Mariola Śliwińska-Mossoń: New insights on old biomarkers involved in tumour microenvironment changes and their diagnostic relevance in non-small cel lung carcinoma. *Biomolecules*, **2021**, vol. 11, nr 8, art. 1208 [24 s.], DOI:10.3390/biom11081208

Załącznik 3 – II artykuł oryginalny:

Katarzyna Wadowska, Piotr Błasiak, Adam Rzechonek, Iwona Bil-Lula, Mariola Śliwińska-Mossoń: Hepcidin as a diagnostic biomarker in anaemic lung cancer patients. *Cancers*, **2023**, vol. 15, nr 1, art. 224 [24 s.], DOI:10.3390/cancers15010224

Załącznik 4 – III artykuł oryginalny:

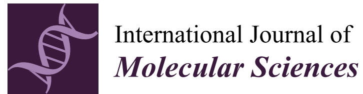
Katarzyna Wadowska, Piotr Błasiak, Adam Rzeczonek, Mariola Śliwińska-Mossoń: Analysis of MMP-2 -735C/T (rs2285053) and MMP-9 -1562C/T (rs3918242) polymorphisms in the risk assessment of developing lung cancer. *International Journal of Molecular Sciences*, **2023**, vol. 24, nr 13, art. 10576 [20 s.], DOI:10.3390/ijms241310576

Załącznik 5 – Całkowity dorobek naukowy

Załącznik 6 – Oświadczenia współautorów publikacji wchodzących w skład cyklu

13.1. Załącznik 1 – artykuł przeglądowy

Katarzyna Wadowska, Iwona Bil-Lula, Łukasz Trembecki, Mariola Śliwińska-Mossoń: Genetic markers in lung cancer diagnosis: a review. *International Journal of Molecular Sciences*, **2020**, vol. 21, nr 13, art. 4569 [24 s.], DOI:10.3390/ijms21134569


International Journal of
Molecular Sciences

Review

Genetic Markers in Lung Cancer Diagnosis: A Review

Katarzyna Wadowska [1], Iwona Bil-Lula [1], Łukasz Trembecki [2,3] and Mariola Śliwińska-Mossoń [1,*]

[1] Department of Medical Laboratory Diagnostics, Division of Clinical Chemistry and Laboratory Haematology, Wroclaw Medical University, 50-556 Wroclaw, Poland; katarzyna.wadowska@student.umed.wroc.pl (K.W.); iwona.bil-lula@umed.wroc.pl (I.B.-L.)
[2] Department of Radiation Oncology, Lower Silesian Oncology Center, 53-413 Wroclaw, Poland; lukasz.trembecki@umed.wroc.pl
[3] Department of Oncology, Faculty of Medicine, Wroclaw Medical University, 53-413 Wroclaw, Poland
* Correspondence: mariola.sliwinska-mosson@umed.wroc.pl; Tel.: +48-71-784-06-30

Received: 1 June 2020; Accepted: 25 June 2020; Published: 27 June 2020

Abstract: Lung cancer is the most often diagnosed cancer in the world and the most frequent cause of cancer death. The prognosis for lung cancer is relatively poor and 75% of patients are diagnosed at its advanced stage. The currently used diagnostic tools are not sensitive enough and do not enable diagnosis at the early stage of the disease. Therefore, searching for new methods of early and accurate diagnosis of lung cancer is crucial for its effective treatment. Lung cancer is the result of multistage carcinogenesis with gradually increasing genetic and epigenetic changes. Screening for the characteristic genetic markers could enable the diagnosis of lung cancer at its early stage. The aim of this review was the summarization of both the preclinical and clinical approaches in the genetic diagnostics of lung cancer. The advancement of molecular strategies and analytic platforms makes it possible to analyze the genome changes leading to cancer development—i.e., the potential biomarkers of lung cancer. In the reviewed studies, the diagnostic values of microsatellite changes, DNA hypermethylation, and *p53* and *KRAS* gene mutations, as well as microRNAs expression, have been analyzed as potential genetic markers. It seems that microRNAs and their expression profiles have the greatest diagnostic potential value in lung cancer diagnosis, but their quantification requires standardization.

Keywords: lung cancer; carcinogenesis; genetic markers; epigenetic markers; liquid biopsy; NGS; genetic alterations; molecular landscape; microRNA; molecular heterogeneity

1. Introduction

Lung cancer—i.e., bronchogenic malignant tumors stemming from airway epithelioma, is the most often diagnosed cancer in the world and the most frequent cause of cancer death. Every year, approximately 1.8 million new cases of lung cancer are diagnosed worldwide. In 2012, approximately 1.6 million people died of lung cancer and it is estimated that the number of lung cancer deaths will increase to 3 million in 2035 [1,2]. Lung cancer has a relatively poor prognosis, and the 5-year survival varies from 4% to 17%, depending on the stage of the disease at the time of its diagnosis [3]. The advancement of non-invasive diagnostics enhances the possibility of detecting lung cancer, however only 10–15% of new cases are diagnosed at its clinical early stage [4]. A total of 75% of patients are diagnosed with lung cancer at its advanced stage, when treatment options are limited. Nevertheless, in patients with clinical stage IA disease in the TNM (tumor-lymph nodes-metastasis) classification, the 5-year survival reaches approximately 60%, which indicates that a large number of patients suffer from undetectable metastases at this stage of the disease [5–7]. The currently used diagnostic tools—i.e., chest radiography and sputum cytology—are not sensitive enough in the diagnosis of non-small cell

lung carcinoma (NSCLC), while tumor markers, such as CEA (carcinoembryonic antigen), CYFRA 21-1, NSE (neuron-specific enolase), or SCCA (squamous cell carcinoma antigen) do not make the diagnosis possible at the early stage of lung cancer [4]. These data indicate the need to find more specific, less invasive biomarkers that could be used alternatively or complementary to radiological approaches and improve lung cancer detection and the determination of its stage [8].

Lung cancer does not result from the sudden transformation of bronchia epithelioma but from the final stage of multistage carcinogenesis, with gradually increasing genetic and epigenetic changes [7,9]. The main etiological factor is the exposure to the carcinogenic components of tobacco smoke. About 90% of lung cancer cases in men and 80% in women are caused by smoking. Exposure to the xenobiotics of the tobacco smoke is associated with the modification of genetic information [10]. Nowadays, mutations that are characteristic of lung cancer and which may enable diagnosis at the early stage of the disease are sought for.

The aim of this study was to conduct an overview of the existing knowledge of the genetic markers in lung cancer diagnosis.

2. Genetic Markers in Diagnosis of Early-Stage Lung Cancer

2.1. Carcinogenesis

The better understanding of pathogenesis and the role of genetic factors in the development of lung cancer facilitates searching for morphological and molecular abnormalities characteristic not only of invasive cancer, but also for the changes referred to as preinvasive lesions in the lungs of current and former smokers without lung cancer. Morphological abnormalities, such as hyperplasia, metaplasia, dysplasia, and carcinoma in situ (CIS), may precede or accompany invasive cancer. Hyperplasia of the bronchial epithelium and squamous metaplasia have been generally considered to be reactive changes, caused by chronic inflammation and mechanical trauma. Hyperplasia and metaplasia are believed to be reversible changes which may spontaneously regress after smoking cessation. Dysplasia and carcinoma in situ are premalignant changes that frequently precede squamous cell carcinoma of the lung [7,9,11].

The molecular basis of lung cancer is the gradual accumulation of genetic and epigenetic changes in the cell nucleus. These changes lead to the weakening of the DNA structure and its greater susceptibility to subsequent mutations. Due to a neoplastic process, cells are no longer subjected to the mechanisms controlling their division and location. This is caused by irregularities in cell cycle regulation (mutations of protooncogenes and suppressor genes), and disorders in the repair processes in damaged DNA. Further changes, such as the increased expression of growth factors, sustained angiogenesis, evading apoptosis (mutations of anti-apoptotic and proapoptotic genes), limitless replicative potential, tissue invasion, and metastasis, affect tumor progression [12].

The multistage model of carcinogenesis is associated with gradually accruing molecular changes, which are shown in Figure 1 [7]. Cancer formation requires somatic alterations or "hits" that will occur only in the cancer cells [13]. The first molecular changes occurring in bronchial epithelium are microsatellite alterations. Microsatellite alterations are extensions or deletions of small repeating DNA sequences and appear as microsatellite instability (MSI, i.e., allele shift) or loss of heterozygosity (LOH), which is the absence of a normally present allele. Three or more alterations are minimally needed for a cell to transform into cancer, whereas tumor progression and metastasis lesions acquire additional DNA alterations. Molecular changes detected frequently in dysplasia are regarded as intermediate in terms of timing, and the changes detected at CIS or invasive stages are regarded as late changes. At the dysplasia stage, DNA methylation takes place [7,14,15].

Mutations are an inherent feature of lung cancer development, and their detection has a significance in both the diagnostic and treatment stages of disease. Mutations in cancerogenesis that confer growth advantage in the cancer cells are considered driver mutations. The number of driver mutations exerting an effect on the carcinogenesis is limited. Most solid tumors exhibit between 40 and 150

non-silent mutations, and most of them are regarded as passenger mutations that do not contribute to the malignant phenotype. A broad spectrum of genomic changes seen in lung cancer is associated with mutation classification, which requires a tiered approach and may enable the differentiation of driver from passenger mutations. The profound analysis of genome changes leading to cancer development enables looking for abnormalities in specific genes, which may be specific tumor markers used in diagnostics. Genomic alterations encompass mechanistic rearrangements of DNA, resulting in single nucleotide variants (SNVs) known as point mutations and small-scale deletions/insertions (indels) or copy number variants (CNVs), which reflect large-scale mutations such as amplifications, deletions, inversions, and translocations [13,16–20].

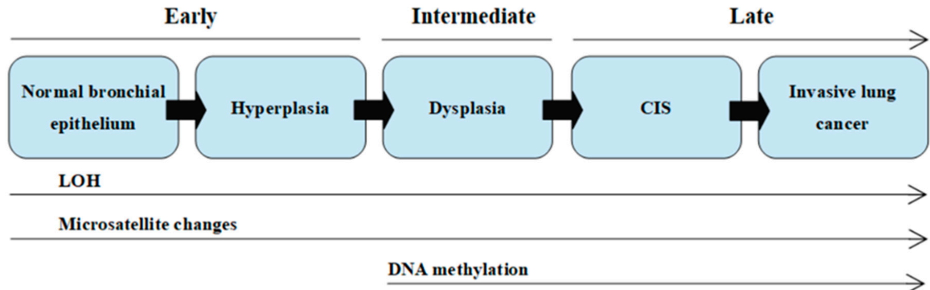


Figure 1. Scheme of the sequential changes during carcinogenesis in a simplified manner. LOH—loss of heterozygosity; CIS—carcinoma in situ. On the basis of (own modification of [7]).

2.2. Genetic Biomarkers

Ideal reliable biomarkers should have high sensitivity and specificity, a high area under the curve (AUC) in a receiver-operator characteristic (ROC) analysis, and a high positive predictive value (PPV). A biomarker is a clinical tool for early diagnosis, prognosis, and monitoring disease evolution that enables clinical decision-making [21]. Genetic markers—i.e., changes in the structure, expression, or sequence of the genetic material—can be used to diagnose and verify the genetic predisposition to cancer and monitor the course of the disease. The development and use of DNA-based molecular markers facilitate studies on genetic variations in healthy and sick individuals. A basic attribute of genetic markers is their polymorphism—i.e., the presence of more than one allele at a specific locus (biallelic or multiallelic). The analysis of polymorphic markers makes it possible to diagnose genetically based diseases, with the unknown products of specific genes or the molecular nature of the changes leading to disease development [22,23]. The molecular markers, epigenetic markers (DNA methylation, non-coding RNA analysis), seem to be most promising because of their crucial role in the cell cycle [24].

2.3. Liquid Biopsy

Potential markers may be found in various biological samples—e.g., blood, urine, tissues, bronchoalveolar lavage, as well as in saliva and sputum—but none have been moved to the clinical setting yet [23,25]. A new approach in lung cancer detection is liquid biopsy—i.e., the sampling and analysis of component isolated or purified from a non-solid biological tissue. Liquid biopsy refers to the detection of tumor components in bodily fluids, such as urine, saliva, cerebrospinal fluid, and liquid cytology specimens, but primarily in blood (plasma). The blood drawing required to collect a liquid biopsy is less invasive than a tissue biopsy, what makes it easily accessible and allows the near real-time monitoring of the cancer [26]. Furthermore, tissue samples can pinpoint the exact genomic state at any tumor location, but they cannot provide a complete understanding of the tumor's heterogeneity, while blood samples carry cell-free tumor DNA (ctDNA) from many points of tumor origin [27]. Liquid biopsy with its advantages and disadvantages is compared to conventional tissue biopsies and cytology specimens in Table 1.

The release of ~160 base pair, nucleosome-bound fragments of DNA into circulation is a product of normal cell apoptosis. Tumor cells release their contents into the circulation too, and their amount is

proportional to the overall burden of the disease. The cancer components obtained from liquid biopsy sampling are circulating tumor cells (CTCs), cell-free circulating DNA (cfDNA), and exosomes. It is also possible to isolate from blood samples such epigenetic markers as free microRNAs (miRNAs) and circulating histones and nucleosomes [8,13,24,28]. Positron emission tomography-computed tomography imaging detects tumors measuring no less than 7 to 10 mm in size and containing around 1 billion cells, while tumors containing approximately 50 million malignant cells release a sufficient amount of ctDNA to enable their detection in blood. The ctDNA released from lung cancer is detectable at levels of 0.1% to 5% of the total cfDNA. Those findings show the great potential of liquid biopsy in early stage cancer diagnosis and, hence, the selection of appropriate treatment, which explains the interest in this new technical advancement [13,29].

Table 1. Comparison of small histopathological and cytology specimens versus liquid biopsy.

Type of Specimen	ADVANTAGES	DISADVANTAGES	Examples of Molecular Markers	Ref.
Small Histopathological Specimens *,** **Cytology Specimens *,****	• small biopsies and cytology samples are the basic diagnostic specimens due to the small minority of lung cancer cases that can be surgically removed (70% of lung cancers are unresectable) • recent guidelines opt for the minimization of cytology use and replacing it with biopsies, which should be the gold standard in lung cancer sampling (more appropriate material for differential and molecular diagnostics)	• tumor biopsies are often insufficient for molecular study or impossible to obtain • small biopsy and/or cytology samples may not be representative of the total tumor because of histologic heterogeneity • in the case of biopsies, multiple sampling is the requirement—minimum of 4 biopsies • the rebiopsy is rarely performed, and in the view of intratumor heterogeneity a single-biosy-based analysis for personalized medicine may be a great limitation • limited amount of cells • in the study by Wang et al. (2015) [30], lung resection specimens had a significantly higher overall mutation rate compared to small biopsy and cytology specimens	• FISH tests for *ALK* and *ROS1* can be applied to different biological specimens—biopsies or cytological samples • determination of *EGFR* T790M in tumor tissue and in cfDNA are both valid alternatives; if *EGFR* T790M testing in plasma is negative, a new biopsy is recommended whenever possible; *EGFR* can be detected in plasma with a high prevalence, reflecting the landscape and heterogeneity of primary tumors and metastases	[24,30–34]
Liquid biopsy	• minimally invasive tool • liquid biopsy permits frequent sample collection, timely assessment of the patient's disease status • liquid biopsy offers different possible applications—response monitoring, tumor recurrence detection, determination of residual disease after full tumor resection, early detection of lung cancer, and immuno-oncology • liquid biopsy enables testing for genetic and epigenetic abnormalities specific to the tumor, and provides abilities to identify mutations in both primary and metastatic lesions—liquid biopsy represents the whole genomic picture of the tumor • a new source for cancer biomarkers	• validation and clinical usefulness are not sufficiently determined as yet • lack of standardization • detection techniques require a high sensitivity in order to detect the DNA from tumor cells • negative results require testing with conventional techniques, such as tumor biopsy • hemolysis may influence the results	• microRNAs are most commonly assessed in patient's serum or plasma; an example is a panel of circulating microRNAs in the study by Sromek et al. (2017) [35]—the combination of miR-9, miR-16, miR-205, and miR-486 can serve as potential NSCLC biomarkers with 80% sensitivity and 95% specificity	[24,35,36]

* direct comparisons between small histopathological specimens and cytology samples are limited, and both of these specimens appear to perform similarly, with the high feasibility of molecular testing; ** whenever possible, cytology should be interpreted in conjunction with the histology of small biopsies, as the 2 modalities are complementary, in order to achieve the most specific and concordant diagnosis; *ALK* - anaplastic lymphoma kinase; cfDNA - cell-free circulating DNA; *EGFR* - epidermal growth factor receptor; FISH - fluorescence in situ hybridization; NSCLC - non-small cell lung carcinoma; *ROS1* - c-ros oncogene 1.

A retrospective study reported by East Carolina University researchers compared standard molecular analysis strategies to liquid biopsy. The analysis showed that liquid biopsy can provide

results within 72 h, enabling lung cancer patients to start targeted therapy within a median of eight days from diagnosis [37].

3. Advancement of Molecular Strategies and Techniques Used to Identify Lung Cancer Genetic Markers

In recent years, the advancement of molecular strategies and analytic platforms, including genomics, epigenomics, and proteomics, has been observed, enabling the analysis of the genome changes which play a key role in the pathogenesis and progression of lung cancer. Cancer genomic research unlocks possibilities in the understanding of somatic modifications in cancer that may be used as a tool in prevention, early diagnosis, novel treatments, and resistance monitoring. Hence, genomic testing can help to identify genomic changes as potential biomarkers of lung cancer. However, the broad spectrum of genomic alterations and lack of a universal technique for their detection requires a tiered approach to their analysis (Table 2) [17,23,25,38].

Table 2. Techniques used frequently for mutation detection, based on [17].

Mutation Detection Techniques	Variant Types	
	SNVs	CNVs
Single-gene assays:		
Sanger sequencing	+	-
pyrosequencing	+	-
allele-specific PCR	+	-
single base extension	+	-
multiplex ligation-dependent probe amplification	+	copy number only
mass spectrometry	+	-
Gene-panel assays:		
amplicon-based panels	-	+
hybrid capture sequencing	-	+
next-generation sequencing	+	+
Fluorescence-based methods:		
fluorescence in situ hybridization	-	+
microarray-based CGH	-	+

Variant types are detected routinely (+) or cannot be detected (-). SNVs—single nucleotide variants, known as point mutations, small-scale deletions/insertions (indels); CNVs—copy number variants, including large-scale mutations such as amplifications, deletions, inversions, and translocations.

3.1. Genomics

In 1982, the first human, naturally occurring tumorigenic somatic mutation was discovered. That discovery and the advancement of molecular technologies gave the beginning of genomics, leading to the completion of the first human reference genome and the first human cancer genome by the Human Genome Organization (HUGO) [39]. Genomics deals with organisms' genome analysis, and its aim is to determine the DNA sequence, map the genome, and determine the dependencies and interactions within the genome. Genomics analyzes genetic material in a multi-complex way by testing many genes at the same time [23,40]. The discovery of the duplex DNA structure and its complementary rules in 1953 gave the beginning for the development of new molecular diagnostic technologies based on four major techniques [41]:

(a) Enzymatic DNA restriction—there are four classes of restriction enzymes. The most commonly exploited are the enzymes belonging to class II, which require only ions of Mg2+ to recognize their target DNA sequence and cleave it. The usage of different combinations of restriction enzymes allows us to characterize and manipulate DNA in fundamental DNA technology approaches such as cloning or mapping [42,43].

(b) Nucleic acid hybridization—in situ hybridization (ISH) uses labeled nucleic acid probes to detect specific DNA or RNA targets in tissue sections, intact cells, or chromosomes. The basic principle underlying ISH is the ability of single-stranded DNA or RNA to anneal specifically to a complementary sequence and form a double-stranded hybrid. Nucleic acid hybridization is

the foundation of Southern or Northern blot hybridization and microarray technology [44–46]. The development of microarray technology (also known as DNA microarrays, DNA chips) is connected with the transition of molecular biology into postgenomic era by enabling large-scale genotyping and gene expression profiling [47,48].

(c) Polymerase chain reaction (PCR)—PCR allows for the exponential amplification of specific targeted genetic loci in a reaction mixture containing DNA primers, deoxynucleotides (dNTPs), and DNA polymerases. PCR is a qualitative technique to amplify and copy a targeted area of extracted DNA a million to a billion-fold over [49]. In the course of time, modifications and advances in this molecular diagnostics technique enabled the relative or even absolute quantification of DNA through the usage of quantitative real-time PCR (qPCR) or partition-based PCR techniques, such as droplet digital PCR (ddPCR) [27,49]. The exploitation of PCR in quantitative DNA analysis is leading to the increased clinical usefulness of PCR for a broad range of applications; PCR is used in a variety of methods, such as allele-specific PCR-based methods or mutation screening methods, including melting curve analysis, that are used in the analysis of mutations sequences. PCR is also used in pyrosequencing and next-generation sequencing (NGS) as a pre-step that provides the sequencing of the generated PCR products [17],

(d) Fluorescence-based methods—the use of hybridizing fluorescent-labeled probes is one of the advancements in cytogenetics. Fluorescence-based methods include the fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH) techniques, which detect large-scale amplifications and deletions. FISH uses specific fluorescent probes that bind to nucleic acid sequences with a high degree of sequence complementarity, helping one to localize these DNA sequences on chromosomes. Microarray-based CGH (array CGH) enables us genome-wide screening for chromosomal imbalances on the basis of genomic DNA hybridization to complement probes that are immobilized on a slide [17,23,49].

3.2. Understanding of Molecular Pathology of Lung Cancer

The analysis of the lung cancer genome is focused on mutation detection. The identification of the cancer gene targets of each of somatic copy number alterations (SCNAs) is an important challenge. The majority of tumors still lack of an identified molecular driver that could be used as genetic biomarkers. Comprehensive testing looking for a large number of genomic alterations may provide clinically significant information that will allow us to impact the therapeutic options and patients' prognosis. Because SCNAs may overlap and target different genes in different cancer types, all SCNA candidates should be tested in an appropriate model system [39,50].

Past techniques that have been successfully used in the field of the cancer genome exploration are capillary electrophoresis-based "Sanger" sequencing, the array-based genome-wide analysis of amplifications and deletions, gene expression arrays, and retrovirus-mediated expression screening techniques. Until recently, the vast majority of techniques used widely for mutation detection were single analyte assays. Single analyte tests are still indispensable; however, the introduction of high-throughput NGS, also referred to as massively parallel sequencing, quickly captured the attention of molecular diagnosticians in the past decade. While single analyte assays are the combination of the amplification and analysis of a target of interest (DNA, RNA) in a single scope, a high-throughput NGS allows multiplex PCR with the simultaneous amplification of a pre-specified panel of genes in a single reaction [17,51,52]. There is a large number of potential applications for NGS technologies and different NGS tests depending on the application purpose. NGS enables the comprehensive genome or exome examination of any cancer through the parallel sequencing of millions of short reads of any type of nucleic acid (including micro-RNA and other non-protein coding DNA species). The scope of NGS can range from whole-genome sequencing (WGS) and whole-exome sequencing (WES), through RNA expression profiling, to targeted oncology panels covering either several or hundreds of genes:

- WGS and WES to find novel mutations in so far unreported gene loci;
- paired-end, mate-pair sequencing to identify structural variations;

- targeted sequencing for mutation discovery and validation;
- transcriptome sequencing for the quantification of gene expression and discovery of transcribed mutations;
- small RNA-sequencing to microRNA profiling;
- large scale analysis of DNA methylation and immunoprecipitation for the genomic mapping of DNA–protein interactions [39,53,54].

The NGS of WGS or WES is used for research purposes, while the sequencing of targeted gene panels is very common in clinical practice for the purpose of finding targetable genomic alterations. In clinical practice, NGS may enable the detection of multiple targets or alteration types, such as mutations, gene copy changes, and rearrangements [18]. In Table 2 is presented a list of techniques used in genomic analysis on the basis of mutation variants.

The molecular diagnostics of lung cancer, similarly to NGS applications purposes, is explored in two areas—for research purposes, to find novel druggable mutations, and in clinical practice to diagnose and select eligible patients for specific tyrosine kinase inhibitor (TKI) therapy [18,55]. Before the development of NGS, our understanding of the molecular pathology of lung cancer was based on such techniques as mismatch repair detection, the sequencing of candidate genes, single nucleotide polymorphism (SNP) arrays, and gene expression analysis. The availability of NGS has enabled the full mutation characterization of lung cancer in the International Cancer Genome Consortium and the Cancer Genome Atlas projects. These projects are engaged in the development of personalized medicine and the availability of targeted therapies for patients with adenocarcinoma (AC) and squamous cell carcinoma (SCC) in the first instance. The sequencing of the lung cancer genome may identify unknown variants along with the known mutations. Furthermore, it may allow the detection of less common oncogenic alterations with available targeted therapies. The National Comprehensive Cancer Network Guidelines recommends testing a panel of genes for NSCLC, which consists of epidermal growth factor (*EGFR*) mutations, anapestic lymphoma kinase (*ALK*) rearrangements, and c-ros oncogene 1 (*ROS1*) rearrangements. These biomarkers are considered the "must-tests" biomarkers in lung cancer patient diagnosis and are analyzed by single-gene assays such as PCR, immunohistochemistry (IHC), and FISH [20,55–60]. Sanger sequencing, qPCR, ddPCR, IHC, and FISH are regarded as the gold standard techniques of molecular analysis in clinical practice, while tumor-only sequencing, matched-tumor, and normal-tissue sequencing are the gold standards in somatic mutation detection [18,61].

In the study by Park et al. (2020) [55], the researchers have compared single-gene assays, such as real-time PCR, IHC, and FISH (considered as the gold standard for selecting eligible patients for *EGFR*-, *ALK*-, and *ROS1*-specific TKI therapy) to targeted NGS. Considering the NGS results as the final outcome, *EGFR* PCR revealed a sensitivity of 80.3% and specificity of 99.4%, *ALK* FISH showed a 71.4% sensitivity and 100% specificity, and *ROS1* FISH showed a 100% sensitivity and 99.5% specificity. These results are related to the lower sensitivity of single-gene assays in comparison to deep-targeted NGS. The data also revealed the necessity of revalidating the results of the single-gene assays, especially for negative *EGFR* assays.

3.3. Epigenomics

Epigenomics is the study of the complete set of epigenetic modifications on the cell genetic material. Epigenetic mechanisms play a critical role in the regulation of gene expression and are prompted by an environmental exposure, such as an exposure to the carcinogenic components of tobacco smoke [23,40,62]. Chromatin is composed of DNA, histone proteins, and other nuclear proteins. Under the influence of external factors, chromatin undergoes a number of reversible covalent modifications. The chromatin modification mainly comprises histone posttranslational modifications (PTMs) and DNA methylation. These chemical changes play an important role in the gene expression and regulation, which are involved in cellular processes such as differentiation and maturation. Methylation modifies the histone by strengthening the charge of the spool, which leads to the more tight packing of the

DNA around the spool and makes the DNA less accessible from being read—"turning off" the gene expression [63]. Epigenomics is currently a field of intense research and it fosters development in molecular cancer therapies. The methods of analysis depend on the nature of the biomarker and the availability of new technologies that are able to perform high-throughput experiments, such as microarrays and NGS in the measurements of DNA methylation, miRNAs, or HPLC coupled with mass spectrometry in the detection of histone PTMs [21].

DNA strands undergo various chemical reactions. DNA modifications, such as 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) have been applied to analytical techniques such as mass spectrometry, molecular imaging, pulldown assay, and DNA sequencing. DNA methylation is the addition of the methyl group (-CH_3) at the 5th carbon position of cytosine bases, which are located 5′ to a guanosine in a CpG dinucleotide. The DNA methylation of cytosine (5mC) can be mapped genome-wide with the use of several methods, such as methylation-specific restriction enzymes or affinity purification, or by using bisulfite conversion followed by sequencing (BS-Seq). The methylation-specific FISH (MeFISH) was the first visualization of 5mC. The hydroxymethylation of cytosine (5hmC) is also thought to have a role in epigenetic gene expression and has been analyzed with the usage of modified bisulfite sequencing methods, 5hmC-specific restriction enzymes, or immunoprecipitation [24,63–66]. Alternative techniques in the analysis of chemically modified DNA strands are multiplex ligation-dependent probe amplification (MLPA) assays that are based on the bisulfite conversion of DNA and qPCR-based reactions, such as methylation-sensitive high-resolution melting (MS-HRM) or pyrosequencing [21]. The analyses of histone variants and PTMs are a combination of molecular biology techniques that involve western blotting, ELISA analysis, mass spectrometry, and histone modification assays in the main. These combinations are the coupling of chromatin immunoprecipitation (ChIP) technology with DNA microarrays (ChIP-Chip) or with NGS (ChIP-Seq). The standard way to detect circulating histones is a direct measurement of histones by the use of immunoassays in plasma samples [8,63,64].

4. Genomic and Epigenomic Changes in Lung Cancer Diagnosis

Oncogenic transformation is a highly complex, multi-step process involving genomic and epigenomic alterations. Lung cancer is characterized by a high tumor mutational burden (TMB) in comparison with other cancer types, which is probably related to smoking habits and an exposure to the xenobiotics of tobacco smoke [12,67]. Kan et al. (2010) [68] studied 441 tumors by paying special attention to the rates of protein-altering mutations. Among the tumor types analyzed, they found that NSCLC is one of the tumors with the highest rate of protein-altering mutations, with rates in adenocarcinomas and squamous cell carcinomas (SCCs) of 3.5 and 3.9 per megabase (Mb), respectively. On the other side, prostate cancer was characterized by a low mutation rate of 0.33 per Mb in comparison with an average rate of 1.8 per Mb of DNA across all tumor types.

Studies that are concerned with mathematical modeling related to the clonal mutation burden in several cancer types showed that lung cancer reflects predominantly mutations accumulated at the early stages of tumorigenesis. Lung tumors are characterized by extensive genomic aberrations, including aneusomy, the gains and losses of large chromosome regions, gene rearrangements, copy number gain, or amplifications [67].

4.1. Oncogenes and Tumor Suppressor Genes in Lung Cancer

There are two classes of cancer genes: oncogenes and tumor suppressor genes. Most oncogenes began as proto-oncogenes—i.e., regulatory proteins involved in cell growth and proliferation or the inhibition of apoptosis. The activation of oncogenes leads to uncontrolled cellular proliferation, and cells undergo oncogenic transformation. The most frequently mutated proto-oncogenes in lung cancer are those from the *MYC*, *RAS*, and *HER* families. Tumor suppressor genes or antioncogenes are a group of genes controlling cell growth through inhibiting cellular proliferation and maintaining genome stability. In lung cancer, the most frequent alterations among tumor suppressor genes are the mutations of *TP53*,

RB, and *p16* [14,15,69]. Ahrendt et al. (1999) [15] used four PCR-based techniques (p53 sequencing, K-ras mutation ligation assays, K-ras-enriched PCR, p16 methylation-specific PCR, and microsatellite analysis) to examine the presence of cancer cells in bronchoalveolar lavage (BAL) fluid. They compared the results of genetic alterations obtained from different kinds of samples—tumor tissue, blood, and BAL fluid. *p53*, *KRAS* (Kirsten rat sarcoma viral oncogene) mutations, microsatellite instability, and *p16* methylation were found in 56%, 27%, 46%, and 38% of patients with NSCLC, respectively. Their results also showed a higher frequency of *p53* mutations in SCC than in adenocarcinoma, and a higher frequency of *KRAS* mutations in patients with the adenocarcinoma subtype.

In the early days of understanding lung cancer genetic aberrations, most of the discoveries were confined exclusively to adenocarcinoma, and only recently have NGS technologies allowed the better molecular characterization of other histotypes. The first recognized mutations in NSCLC were identified in *KRAS* and *TP53*. Then, in 2004 mutations in the kinase domain of *EGFR* were described that changed the lung cancer treatment paradigm. Both *KRAS* and *EGFR* mutations are identified almost exclusively in lung adenocarcinomas, similarly to rearrangements involving *ALK* and *ROS1*, which were described in 2007. *KRAS* was not only the first described mutation but also the most frequently mutated oncogene in NSCLC (20–35%). Activating *EGFR* mutations are found in about 15% of NSCLC, while *ALK* and *ROS1* alterations are quite rare (< 5% of lung cancers) but are frequent among light to never smokers. These mutations—*EGFR*, *ALK*, and *ROS*—may predict a good response to treatment with the specific tyrosine kinase inhibitor (TKI). The number of clinically relevant genomic alterations with already available or newly developed kinase inhibitors is rapidly increasing. There are a number of other important recognized oncogenic alterations in lung adenocarcinoma that may be also used as potentially targetable alterations, including B-Raf proto-oncogene (*BRAF*), Erb-B2 Receptor Tyrosine Kinase 2 (*ERBB2*), mesenchymal-epithelial transition factor (*MET*), and rearranged during transfection (*RET*). Current technologies such as the NGS approach reveal multiple gene alterations in a single tumor, however the co-occurrence of more than one oncogenic driver is infrequent [20,26,56–60]. The data collected in the study of the Lung Cancer Mutation Consortium (Sholl et al., 2015) [70] revealed that, among 1007 lung adenocarcinomas, the co-occurrence of more than one oncogenic driver was found in only 3% of patients. Originally, the molecular diagnostics of squamous cell carcinoma (SCC) was based on the absence of certain targetable genomic alterations that are commonly found in adenocarcinoma—i.e., *EGFR* and *ALK* [59]. Devarakonda et al. (2018) [71] analyzed the molecular profiles, consisting of 1538 genes, of 908 patients with NSCLC. Their results revealed differences in mutation frequency between adenocarcinoma and SCC, and as was expected, the characteristic activating mutations of adenocarcinoma such as *KRAS*, *HRAS*, *NRAS*, and *EGFR* were identified only in 3% of SCCs. The commencement of NGS technology also provided an opportunity to better understand the mutational landscape of specific genomic alterations in SCC diagnosis. Current studies led to the identification of numerous recurrent changes in SCC, such as gene amplifications (*CCND 1-3, CDK4, FGFR 1-3, MET, PDGFRA, PIK3CA, SOX2*), gene fusions (*FGFR3-TACC3*), tumor suppressor mutations (*PTEN, TP53*), and point mutations (*EPHA2, AKT1, DDR2*), often in combination and some of them with available therapies (Table 2) [59,68]. To sum up, in lung cancer the current National Comprehensive Cancer Network (NCCN), Domestic Lung Cancer Clinical Guidelines, and National Health and Family Planning Commission Diagnosis and Treatment Norms suggest that some driver gene variants, including the mutation of *EGFR*, *KRAS*, *BRAF*; the mutation or amplification of *HER2* (human epidermal growth factor receptor 2), *ALK*, *ROS1*, and *RET* rearrangements; and the *MET* copy number amplification or variable shear variations in *MET* exon 14 are the essential parts of the "core gene list" [20]. In Table 3, we list the molecular landscape of lung cancer with the currently available targeted therapies.

Table 3. Molecular landscape of non-small cell lung carcinoma (NSCLC) with the available treatment options, on the basis of [68].

Gene	Type of Genomic Aberrations	Frequency [%]	Currently Available Targeted Therapy *	Diagnostic Approaches	Ref.
			Adenocarcinomas (ADC)		
EGFR	EGFR-TKI sensitizing mutations: EGFR exon 21, EGFR exon 19, G719X, L861Q point mutations Copy number variations (gains)	30–40	pemetrexed or bevacizumab therapy, afatinib, erlotinib, gefitinib, dacomitinib, osimertinib	PCR: sanger, real-time PCR, ddPCR, and NGS; IHC	[67,72–77]
KRAS	G12C mutation in *KRAS* gene	20–30	AMG-510	PCR, DNA sequencing	[67,72–74,77]
MET	MET exon 14 mutation (MET ex14), skipping mutations, overexpression, amplifications	2–5 3–4	skipping mutations—crizotinib, tepotinib; amplifications—crizotinib, capmatinib	mutations: sanger sequencing, NGS; amplifications: FISH, PCR, real-time PCR, NGS	[67,72–74,76,78]
ALK	ALK fusions	3–7	crizotinib, alectinib, ceritinib, brigatinib, lorlatinib	FISH (the gold standard); ALK-IHC has become a widely used technique with two validated antibodies in lung cancer (D5F3, 5A4)	[67,72–74,76,77,79]
BRAF	V600E mutation in *BRAF* gene; can co-exist with *KRAS* mutation	0.5–5	trametinib, dabrafenib	PCR: sanger, real-time PCR, and NGS	[67,72–74,76,78]
ROS1	ROS fusions	2–3	crizotinib	ROS1-IHC (screening) is still evolving (the use of the D4D6 rabbit monoclonal antibody) **; FISH; NGS	[67,72–74,76,77,80]
RET	RET rearrangements, gene fusion of KIF5B-RET; point mutations	1–2	vandetanib, cabozantinib, alectinib, BLU-667, LOXO-292	RT-PCR is typically combined with FISH; FISH; NGS	[67,72–74,76,78]
NTRK	NTRK rearrangements, gene fusions of NTRK1 (NTRKA), NTRK2 (NTRKB), NTRK3 (NTRKC)	1–2	entrectinib, larotrectinib, LOXO-195, repotrectinib	NGS with a panel that includes testing for NTRK1, NTRK2, NTRK3; IHC with subsequent confirmation by FISH or NGS	[67,72–74,76,78]
HER2 ***	mutations in the kinase domain (exon 20), the most frequent is p.A775_G776insYVMA insertion amplifications, overexpressions	1–5 2–5	afatinib, dacomitinib, neratinib, trastuzumab, trastuzumab-emtansine, DS-8201a, poziotinib	mutations: PCR: sanger, real-time PCR and NGS; amplifications: FISH, NGS, real-time PCR	[72,74,76,78,81]
PTEN *PDGFRA* *PIK3CA* *TP53* *ERBB2* *TERT* *CDKN2A*	mutations copy number variations—gains losses	1.7 6–7 5 52 2–5 75 7	NA NA NA NA NA NA NA	- ****	[59,67,68]
			Squamous cell carcinoma (SCC)		
FGFR *TP53* *NF1* *DDR2* *PDGFRA* *PIK3CA* *PTEN* *SOX2* *CDKN2A*	gene fusion of FGFR3-TACC3, mutations of FGFR1, FGFR2 tumor suppressor mutations, copy number variations (gains) mutations of NF1 point mutations of DDR2 amplification amplification tumor suppressor mutations, copy number variations (losses) amplification and copy number variation (gain) copy number variation (loss)	23 79 10 2–3 4 15 10 8 65 15	NA NA NA NA NA NA NA NA NA		[59,67,68]

* platinum-based chemotherapy (+/- pembrolizumab) is still the treatment of choice for patients without targetable mutations [82]; ** screening with ROS1-IHC and subsequent confirmation of IHC-positive cases with the use of FISH; ROS1-inhibitors should only be given to patients whose tumors are double positive according to IHC and FISH; *** *HER2* may be present in SCC but outside the kinase domain, with certain clinical benefit data when treating with afatinib; **** NGS can potentially test for all molecular alterations; NA—not available.

4.2. Microsatellite Markers

Another aspect of lung cancer molecular diagnosis is the analysis of microsatellite markers, potentially used to assess clonality. Miscrosatellite instability (MSI) is an effect of defective DNA mismatch repair (MMR) and has been implicated in tumorigenesis. MSI was initially noted in the colon cancers of patients with hereditary nonpolyposis colon cancer (HNPCC). The most common mutations identified in HNPCC involve MLH1, MLH3, MSH2, MSH3, MSH6, PMS2, and POLE [83,84]. At the beginning of understanding the lung cancer molecular landscape, Sozzi and et. (1999) [85] investigated the frequency and extent of MSI and LOH in the plasma DNA of NSCLC patients with limited disease. The study focused on four markers detecting shifts or LOH—tetranucleotide repeat (D21S1245), recognized as being prone to microsatellite instability in various cancer types and dinucleotides D3S1234, D3S1300, and D3S4103 located in the introns of the *FHIT* (fragile histidine triad diadenosine triphosphatase) gene. *FHIT* is a tumor suppressor protein which participates in apoptosis induction (mediated by the activation of death receptors—DRs—and the caspase cascade signaling pathways) and the regulation of the cellular cycle. The overexpression of *FHIT* in NSCLC cells leads to the higher expression of DRs and the activation of caspase 3, caspase 8, and caspase 9. The *FHIT* gene encompasses the common fragile site FRA3B on chromosome 3 and shows a high rate of LOH in lung cancer, particularly in smokers [86,87]. It was found that 56% (49 of 87) of NSCLC tumors showed microsatellite alterations (shift or LOH) in at least one locus. MSI was recognized as the appearance of new tumoric allele(s) which are not present in normal DNA. LOH was scored if the allele signal was reduced to less than 30% of its normal intensity. A total of 30 out of 49 (61%) patients with microsatellite alterations in tumor DNA also showed microsatellite changes in their plasma DNA [85].

4.3. Epigenetic Changes in Lung Cancer

An enormous field of lung cancer molecular landscape is determined by epigenetic changes. The last two decades have seen exponential developments in the epigenetic understanding of lung cancer. The two main groups of epigenetic modifications are DNA methylation (hypermethylation or hypomethylation) and non-coding RNA expression, known as microRNAs (miRNAs). These epigenetic disruptions may represent reliable biomarkers for lung cancer risk assessment, early diagnosis, prognosis stratification, molecular classification, and the prediction of treatment efficacy. Aberrant DNA methylation is catalyzed by three DNA methyltransferaze enzymes (DNMTs) and promotes carcinogenesis through the promoter methylation of tumor suppressor genes, leading to silencing their expression. DNMT1 expression is increased in early stage lung cancer and is implicated in its pathogenesis through the silencing of such genes as *RASSF1A* and *CDKN2A*. *CDKN2A* was not only the first tumor suppressor gene to be found inactivated in lung cancer, but it shows promoter methylation in almost all cancers [24,65,88]. Moreover, *CDKN2A* is prone to hypermethylation early during lung cancer development, for which Palmisano et al. (2000) [89] provided scientific evidence. In their study, *CDKN2A* methylation was evident in two sputum samples which had been collected from subjects almost three years prior to diagnosis [65]. In lung cancer, the methylation status of over 40 genes has been assessed in tumors, cell lines, sera, and/or sputum. In more recent works, there was the demonstration of an increased frequency and level of promoter hypermethylation in a number of genes involved in cell cycle regulation, adhesion, apoptosis, and signal transduction. The lung cancer epigenetic diagnostics may be based on the assessment of typical hypermethylated genes or the assessment of the hypermethylation of genes that are methylated in other cancers than lung cancer. In lung cancer, there is a little or no methylation of genes commonly methylated in other cancers, such as *ARF*, *CDKN2B*, *CTTNB1*, *MLH1*, and *RB1*. The hypermethylated genes in lung tumors such as *APC*, *CKDN2A* (encodes p16INK4A and p14arf), *CHD13*, *RARB*, and *RASSF1A* may be considered as tumor suppressors [65,88]. Ooki et al. (2017) [90] evaluated a panel of cancer-specific methylated genes in tumors and adjacent normal lung tissue from NSCLC patients. A panel consisted of six genes—*SOX17*, *HOXA9*, *AJAP1*, *PTGDR*, *UNCX*, and *MARCH11*—and showed a high sensitivity (96.7%) and specificity (60%) [24]. Another panel of possible genes associated with NSCLC was

established by Liu et al. (2018) [91]. The combination of *PCDHGB6*, *HOXA9*, *MGMT*, and miR-126 was characterized by the highest sensitivity (85.2%) and specificity (81.5%) among such genes as *PCDHGB6*, *HOXA9*, *MGMT*, miR-126, *SOCS2*, and *NORE1A* [24]. A meta-analysis performed by Huang et al. (2016) [92] focused on generating a list of differentially methylated genes among NSCLC histotypes. Their results showed two hypomethylated genes (*CDKN2A* and *MGMT*) and three hypermethylated genes (*CDH13*, *RUNX3*, *APC*) in adenocarcinomas compared with SCC, with the higher sensitivity and specificity values of *CDH13* and *APC* [24].

4.4. MicroRNAs in Lung Cancer Diagnosis

The rapid development of genomics and epigenomics is associated with the increasing popularity of microRNA. Basic techniques used to detect and investigate the expression of miRNAs are northern blotting, RT-PCR (reverse transcriptase PCR), and microarrays [23,93]. RT-qPCR using TaqMan miRNA assays is the gold standard in miRNA quantification [94]. miRNA is not tissue-specific. However, during oncogenesis, tumor cells develop a unique genetic profile. The profiles of circulating miRNAs are different for each cancer microenvironment and tumor progression stage. The analysis of these profiles enables the better understanding of tumor etiopathogenesis and the origin of cancer. The profiles of miRNAs expression are useful in the case of poorly differentiated cancers and metastases with unknown primary origin. Some studies revealed that circulating miRNAs also play an important role in the treatment and prognosis of lung cancer [93,94]. microRNAs are a class of small, single-stranded, non-coding RNAs that post-transcriptionally regulate the translation of target genes and influence a series of cellular functions, such as proliferation, differentiation, and apoptosis; therefore, an altered expression of miRNAs in different cancer types can affect the deregulation of cellular activities. microRNAs develop a specific gene expression profile for individual tissues. microRNAs represent ideal markers which can be evaluated in biological fluids because of their high stability. microRNAs' resistance to endogenous and exogenous RNase activity, extreme temperatures, repeated freeze-thaw cycles, and pH changes enable their effective isolation in biological fluids and measurement using RT-qPCR. microRNAs' participation in both physiological (proliferation, differentiation, apoptosis) and pathological processes (inflammation, cancers) plays an important role in oncogenesis. microRNAs may also constitute useful diagnostic and prognostic markers for cancer diagnosis and treatment, as well as serve as potential therapeutic targets or tools. They may act as oncogenes or tumor suppressor genes, and are defined as oncomicroRNA and anti-oncomicroRNA. microRNAs' expression profiles in lung cancer are different in comparison with those in healthy tissues and with those in other types of cancer. This makes it possible to consider creating microRNA expression profiles that will be specific for lung cancer and will confirm the disease [93–96]. In one of these studies, Xing et al. (2010) [97] developed a panel of microRNAs that could be used as a sputum-based test for the early stage SCC of the lungs. During the study, they identified three microRNAs (miR-205, -210, and -708) that were overexpressed and three microRNAs (miR-126, -139, and -429) that were underexpressed in tumor specimens. miR-205 was the best single microRNA, resulting in a 65% sensitivity and 90% specificity, while the combination of three microRNAs—miR-205, -210, and -708—provided the best prediction and revealed a sensitivity of 73%. Yu et al. (2010) [98] also developed a panel of microRNAs, but this one could be used as highly sensitive and specific sputum markers for the early detection of lung adenocarcinoma. The researchers found three microRNAs (miR-486, miR-126, and miR-145) that were underexpressed and four microRNAs (miR-21, miR-182, miR-375, and miR-200b) that were overexpressed in tumor specimens. The combination of four microRNAs—miR-486, -21, -200b, and -375—provided the best prediction and resulted in a significantly higher sensitivity and specificity, at the level of 80.6% and 91.7%, than the sensitivity and specificity of the individual microRNAs. Another study that focused on the identification and construction of microRNA panels for lung cancer diagnosis was the study by Lu et al. (2018) [5]. The researchers constructed two panels consisting of the following microRNAs: model A—miR-17, miR-190b, miR-19a, miR-19b, miR-26b, and miR-375; and model B—miR-17, miR-190b, and miR-375. Model A was built

in order to evaluate the risk of lung cancer and was characterized by a high sensitivity (81%) and specificity (80%). All six microRNAs constituted effective predictors of lung cancer, with higher expression levels in the lung cancer group than in the asymptomatic high-risk group. Model B was built in order to estimate the probability of SCLC, with a sensitivity of 81% and a specificity of 80%. Zheng et al. (2011) [99] investigated the potential of circulating plasma microRNAs for the early detection of lung cancer. Out of the 15 selected microRNAs, 6 microRNAs (miR-155, miR-197, miR-182, miR-21, miR-128, and miR-183) were significantly elevated in the plasma of patients with lung cancer and subjected to further analysis. The combination of three microRNAs—miR-155, miR-197, and miR-182 showed an 81.33% sensitivity and 86.76% specificity. This panel was also measured in an independent set of 14 patients with lung cancer during the early and late phase of chemotherapy in order to explore its value in the monitoring of the treatment effectiveness of chemotherapy. The levels of miRNA-155, miR-197, and miR-182 were significantly reduced after treatment in the majority of responsive patients. In the study by Xi et al. (2018) [4], the researchers investigated the differences in the expression levels of 12 microRNAs between benign pulmonary nodules and malignant pulmonary nodules. The expression levels of miR-17, miR-146a, miR-200b, miR-182, miR-221, miR-205, miR-7, miR-21, miR-145, and miR-210 were significantly higher in the NSCLC patients, with a sensitivity at the level of 54.8–83.3% and a specificity in the range of 60.0–86.7%. In other study, Sromek et al. (2017) [35] found that combined miR-9, miR-16, miR-205, and miR-486 can serve as potential NSCLC biomarkers with an 80% sensitivity and 95% specificity. In the study by Hennessey et al. (2012) [100], the feasibility of using serum microRNAs as non-invasive biomarker assays in the early detection of lung cancer was examined. The results yielded five candidate microRNA pairs that were significantly differentially expressed between the NSCLC and healthy controls, with a sensitivity and specificity of at least 75%. A combination of two differentially expressed microRNAs—miR-15b and miR-27b—was able to discriminate NSCLC from healthy controls with a 100% sensitivity and 84% specificity. Heegaard et al. (2012) [101] focused on the measurement of 30 different circulating microRNAs that had been previously reported to be differently expressed in lung cancer tissue. The researchers found seven microRNAs which had statistically significant lower expressions in lung cancer patients (miR-146b, -221, -let7a, -155, -17-5p, -27a, and -106a) and one which was significantly increased (miR-29c). Despite significant differences in the microRNA expression levels between the study group and the control, the expression profiles could not distinguish the study group from the control group accurately. The accuracy of the best predictive panel of microRNAs was only 57–60%. Ulivi et al. (2019) [95] focused on early stage (IA-IIIA in the TNM classification) NSCLC to develop a microRNA panel as a potential prognostic biomarker in patients undergoing surgery. In a univariate analysis, five microRNAs—miR-26a-5p, miR-126-3p, miR-130b-3p, miR-205-5p, and miR-21-5p—were significantly associated with disease-free survival (DFS) in SCC patients, and four of these microRNAs (miR-26a-5p, miR-126-3p, miR-130b-3p, and miR-205-5p) were also associated with overall survival (OS). In adenocarcinoma patients, only miR-222-3p, miR-22-3p, and miR-93-5p were significantly associated with DFS, and miR-196-3p was associated with OS. The study showed that miR-126-3p played an independent prognostic role associated with a lower risk of relapse or death due to SCC. Previous reports have demonstrated that miR-126 may function as an important regulatory factor in the development of NSCLC. Xu et al. (2017) [102] also evaluated the predictive value of microRNAs in terms of the DFS and OS of patients with NSCLC. The researchers focused on angiogenic microRNAs, such as miR-18a, miR-19a, miR-20a, miR-92a, miR-126, miR-130a, miR-210, miR-296, and miR-378, that play an important role in tumorigenesis and angiogenesis. The comparison between microRNAs in the patients with NSCLC and the healthy controls showed that the plasma miR-18a and miR-126 expression levels were lower in the patients with NSCLC, whereas the expression levels of miR-19a, miR-20a, miR-92a, miR-130a, miR-210, miR-296, and miR-378 were higher in the patients with NSCLC. The low plasma expression levels of miR-18a, miR-20a, miR-92a, and miR-126 were correlated with a prolonged DFS, whereas high plasma miR-18a, miR-20a, miR-92a, miR-210, and miR-126 expression levels were correlated with a shorter OS. In another study, Yan et al. (2019) [103]

investigated the expression and clinical significance of miRNA-99a and miRNA-224 in the serum of patients with NSCLC. The expression level of miR-99a was remarkably lower in the NSCLC patients than in the control group, and it was significantly correlated with the pathological stage, the presence and absence of lymph node metastasis, and the tissue differentiation. The expression of miR-224 was significantly higher in the NSCLC patients in comparison to the healthy individuals, and it was also correlated with the pathological stage, the presence and absence of lymph node metastasis, and the pathological grade. Aiso et al. (2018) [94] analyzed microRNA expression before and after surgical resections of NSCLC. In the first phase of the study, the researchers evaluated the expression of miR-145, -20a, -21, and -223, which were previously reported as candidate diagnostic markers of NSCLC. In this phase, they revealed a significant reduction in the miR-145 and miR-20a sera levels in patients with NSCLC (of all groups—i.e., with stage I-II, III, or IV NSCLC) in comparison to the control group. The miR-21 differences with significant increases were observed only between the patients with stage-IV NSCLC, in comparison with the patients suffering from NSCLC of stage I-II. The miR-223 was remarkably higher in patients with stage-IV NSCLC in comparison with the control group. In the second phase of the study, the expression levels of miR-145, -20a, -21, and -223 were evaluated after tumor resection. The expression of miR-145 and miR-20a was significantly increased after resection in comparison with the pre-resection levels, and it was similar to the levels of microRNAs in the control group. An ROC (receiver operating characteristic) curve analysis revealed that miR-145 was the most suitable diagnostic marker for NSCLC that distinguishes the NSCLC patients of all stages from the healthy individuals with a high sensitivity and specificity. Szczyrek et al. (2019) [104] evaluated the diagnostic value of selected plasma microRNA (miR-27a-3p, miR-31, miR-182, miR-195) expression complementary to Drosha and Dicer in lung cancer patients. Dicer and Drosha enzymes play an essential role in microRNA biogenesis and the conversion of pri-miRNA into pre-miRNA. The expressions of miR-27a-3p, miR-21, and miR-182 were significantly higher in the study group than in the healthy volunteers, whereas the miR-195 expression was significantly lower in the lung cancer group. The increased expression of miR-27a-3p (89% sensitivity and 77% specificity), miR-31 (73% sensitivity and 61% specificity), and miR-182 (70% sensitivity and 79% specificity) reported in the study could contribute to a reduction in the activity of the Dicer and Drosha enzymes and to a reduction in the expression of microRNAs described as tumor suppressor genes.

5. Lung Cancer Genetic Heterogeneity

The use of molecular technology and analysis of the cancer genome showed that solid tumors are genetically heterogeneous among individuals with the same tumor type (interpatient heterogeneity); there is observed a diversity of tumor cells within a single tumor (intratumor heterogeneity) and diversity between the primary tumor and its metastasis (intertumor heterogeneity) [68]. This means that cancer subtypes may contain different molecular variants and that a wide range of genomic variants may exist within a single tumor subtype [19,27]. Mutations of *KRAS* and *TP53* were the earliest recognized mutations in NSCLC. In general, colon, pancreas, and lung carcinoma are associated with *KRAS* mutation, while somatic *TP53* mutations occur in almost every type of cancer, with the highest rates in ovarian, esophageal, colorectal, head and neck, larynx, and lung cancers [26,105,106]. This shows that similar driver gene mutations may be found among different kind of tumors. Hence, two cancers with the same histologic origin may contain different molecular variants, affecting the overall evolution of the tumor and its response and resistance to targeted therapies. The high heterogeneity between different histotypes of lung cancer may provide an explanation for the great variation in treatment responses, as well as strategies that could be different for a single tumor or that are similar for different tumor types [13,19,20]. The most critical issues related to therapeutic strategies (sensitivity or resistance to the specific treatment) represent the heterogeneity between intra- and intertumors, primary tumor and metastasis, tumor cells and circulating tumor cells, and inter-single cells. Recent studies have provided insights into resistance mutations against osimertinib—i.e., a third-generation *EGFR* inhibitor that is designed to target T790M—and crizotinib, which is the *ALK* inhibitor [107,108].

There are various resistance mechanisms to osimertinib, such as the resistant mutation at the cysteine residue at position 797 (C797) that abolishes the direct covalent binding of osimertinib or resistance via the activation of bypass pathways, such as *MET* amplification [107,109]. In the case report by Ou et al. (2017) [110], the detection of an *EGFR* solvent front mutation (MAF of G796S/R) has been reported after treatment with osimertinib. Solvent front mutation, in addition to the C797 mutation, is potentially the dominant mutation driving resistance to osimertinib. In the case report by Chen et al. (2016) [111], not only the occurrence of the *EGFR* C797S mutation but also L792F/Y/H in three clinical subjects with acquired resistance to osimertinib treatment was observed. The study by Kim et al. (2013) [112] concerned heterogenous acquired resistance mechanisms in ALK-rearranged NSCLC treated with crizotinib. The study demonstrated secondary *ALK* mutations, a high *ALK* gene copy number, and the activation of *EGFR* signals expressed by a high *EGFR* polysomy and L858R in EGFR exon 21 after treatment with crizotinib.

The key step in lung cancer diagnosis and treatment can be the inclusion of molecular classification into the clinical classification of lung cancer. An example is a new nomenclature for the adenocarcinoma subtype proposed by The Cancer Genome Atlas Consortium (TCGA). The TCGA's classification integrated the transcriptional subtypes with the histopathological, anatomic, and mutational categorizations and consists of three subtypes that are characterized by specific genetic alterations (Table 4) [56].

Table 4. The molecular classification of adenocarcinoma proposed by The Cancer Genome Atlas Consortium (TCGA) [97].

Name of the Unit	Abbreviation	Formerly	Mutations
Terminal respiratory unit	TRU	bronchioid	mutations in the *EGFR* gene and tumors expressing the kinase fusion;
Proximal-inflammatory	PI	squamoid	mutations in *NF1* and *TP53* genes
Proximal-proliferative	PP	magnoid	mutations of *KRAS* oncogene and inactivation of the *STK11* tumor suppressor gene

6. Summary

The early and accurate diagnosis of lung cancer is crucial for its effective treatment. Thanks to recent advancements in molecular strategies and analytic platforms, an increasing number of potential biomarkers in lung cancer diagnosis have been identified. Molecular biomarkers may be useful in diagnosis at an early and non-invasive stage of lung cancer in monitoring the course of the disease and the response to treatment, but none of them have been moved to the clinical setting yet. Data show that the genetic markers with the greatest diagnostic potential value in terms of lung cancer diagnosis are microRNAs and their expression profiles. Studies have reported that the false-positive rates of low-dose computer tomography (LDCT) could be reduced from 19.4% to 3.7% with an additional diagnosis conducted using a plasma miRNA panel. On the basis of the changes in the plasma microRNA, lung cancer could be predicted even 24 months earlier. This indicates that the microRNA panel has a potential to be used in order to detect lung cancer at early stages [5]. To exploit microRNA's potential, it is necessary to standardize the quantification of circulating microRNAs and clarify the individual or environmental factors which affect the levels of circulating microRNA [83].

Author Contributions: Conceptualization, M.Ś.-M., Ł.T.; resources, K.W.; data curation, K.W., M.Ś.-M.; writing—original draft preparation, K.W.; writing—review and editing, M.Ś.-M., I.B.-L.; visualization, K.W.; supervision, Ł.T.; project administration, M.Ś.-M., I.B.-L.; funding acquisition, "RID". All the authors read and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: The publication was prepared under the project financed from the funds granted by the Ministry of Science and Higher Education in the "Regional Initiative of Excellence" programme for the years 2019–2022, project number 016/RID/2018/19, amount of funding 11 998 121.30 PLN.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AC/ADC	Adenocarcinoma
ALK	Anaplastic lymphoma kinase
AUC	Area under the receiver operating characteristic (ROC) curve
BAL	Bronchoalveolar lavage
BRAF	B-raf proto-oncogene
BS-Seq	Bisulfite conversion followed by sequencing
CEA	Carcinoembryonic antigen
cfDNA	Cell-free circulating DNA
CGH	Comparative genomic hybridization
ChIP	Chromatin immunoprecipitation
CIS	Carcinoma in situ
CNV	Copy number variation
CTC	Circulating tumor cell
ctDNA	Cell-free tumor DNA
ddPCR	Droplet digital polymerase chain reaction
DFS	Disease free survival
DNMT	DNA methyltransferase enzyme
dNTP	Deoxynucleotide
DR	Death receptor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ERBB2	Erb-B2 receptor tyrosine kinase 2
FHIT	Fragile histidine triad diadenosine triphosphatase
FISH	Fluorescence in situ hybridization
HER2	Human epidermal growth factor receptor 2
HNPCC	Hereditary non-polyposis colon cancer
HPLC	High-performance liquid chromatography
HUGO	Human Genome Organization
IHC	Immunohistochemistry
ISH	In situ hybridization
KRAS	Kirsten rat sarcoma viral oncogene
LDCT	Low-dose computer tomography
LOH	Loss of heterogeneity
Mb	Megabase
MeFISH	Methylation-specific fluorescence in situ hybridization
MET	Mesenchymal-epithelial transition factor
MLPA	Multiplex ligation-dependent probe amplification
MSI	Microsatellite instability
NA	Not available
NGS	Next-generation sequencing
NSCLC	Non-small cell lung carcinoma/Non-small cell lung cancer
NSE	Neuron-specific enolase
OS	Overall survival
PCR	Polymerase chain reaction
PI	Proximal-inflammatory
PP	Proximal-proliferative
PPV	Positive predictive value
PTM	Posttranslational modification

qPCR	Quantitative real-time polymerase chain reaction
RET	Rearranged during transfection
RISC	RNA-induced silencing complex
ROC	Receiver operating characteristic
ROS1	C-ros oncogene 1
RT-PCR	Reverse-transcription polymerase chain reaction
SCC	Squamous cell carcinoma/Squamous cell cancer
SCCA	Squamous cell carcinoma antigen
SCLC	Small cell lung carcinoma/Small cell lung cancer
SCNA	Somatic copy number alteration
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
TCGA	The Cancer Genome Atlas
TKI	Tyrosine kinase inhibitor
TMB	Tumor mutation burden
TNM	TNM Classification of Malignant Tumors (tumor-lymph nodes-metastasis)
TRU	Terminal respiratory unit
WES	Whole-exome sequencing
WGS	Whole-genome sequencing

References

1. Didkowska, J.; Wojciechowska, U.; Mańczuk, M.; Łobaszewski, J. Lung cancer epidemiology: Contemporary and future challenges worldwide. *Ann. Transl. Med.* **2016**, *4*, 150. [CrossRef] [PubMed]
2. Rahal, Z.; El Nemr, S.; Sinjab, A.; Chami, H.; Tfayli, A.; Kadara, H. Smoking and Lung Cancer: A Geo-Regional Perspective. *Front. Oncol.* **2017**, *7*, 194. [CrossRef] [PubMed]
3. Hirsch, F.R.; Scagliotti, G.V.; Mulshine, J.L.; Kwon, R.; Curran, W.J.; Wu, Y.L.; Paz-Ares, L. Lung Cancer: Current Therapies and New Targeted Treatments. *Lancet* **2017**, *389*, 299–311. [CrossRef]
4. Xi, K.X.; Zhang, X.W.; Yu, X.Y.; Wang, W.D.; Xi, K.X.; Chen, Y.Q.; Wen, Y.S.; Zhang, L.J. The role of plasma miRNAs in the diagnosis of pulmonary nodules. *J. Thorac. Dis.* **2018**, *10*, 4032–4041. [CrossRef] [PubMed]
5. Lu, S.; Kong, H.; Hou, Y.; Ge, D.; Huang, W.; Ou, J.; Yang, D.; Zhang, L.; Wu, G.; Song, Y.; et al. Two Plasma microRNA Panels For Diagnosis and Subtype Discrimination of Lung Cancer. *Lung Cancer.* **2018**, *123*, 44–51. [CrossRef] [PubMed]
6. Jakubek, Y.; Lang, W.; Vattathil, S.; Garcia, M.; Xu, L.; Huang, L.; Yoo, S.Y.; Shen, L.; Lu, W.; Chow, C.W.; et al. Genomic Landscape Established by Allelic Imbalance in the Cancerization Field of a Normal Appearing Airway. *Cancer Res.* **2016**, *76*, 3676–3683. [CrossRef]
7. Hirsch, F.R.; Franklin, W.A.; Gazdar, A.F.; Bunn, P.A. Early Detection of Lung Cancer: Clinical Perspectives of Recent Advances in Biology and Radiology. *Clin. Cancer Res.* **2001**, *7*, 5–22.
8. Santarpia, M.; Liguori, A.; D'Aveni, A.; Karachaliou, N.; Gonzalez-Cao, M.; Daffinà, M.G.; Lazzari, C.; Altavilla, G.; Rosell, R. Liquid biopsy for lung cancer early detection. *J. Thorac. Dis.* **2018**, *10*, S882–S897. [CrossRef]
9. Gazdar, A.F.; Brambilla, E. Preneoplasia of lung cancer. *Cancer Biomark.* **2010**, *9*, 385–396. [CrossRef]
10. Jassem, E.; Szymanowska, A.; Siemińska, A.; Jassem, J. Palenie tytoniu a rak płuca. *Pneumonol. Alergol. Pol.* **2009**, *77*, 469–473.
11. Wistuba, I.I.; Behrens, C.; Milchgrub, S.; Bryant, D.; Hung, J.; Minna, J.D.; Gazdar, A.F. Sequential Molecular Abnormalities Are Involved in the Multistage Development of Squamous Cell Lung Carcinoma. *Oncogene* **1999**, *18*, 643–650. [CrossRef] [PubMed]
12. Potempa, M.; Jonczyk, P.; Zalewska-Ziob, M. Molekularne uwarunkowania raka płuca. *Onkol. Prak. Klin.* **2014**, *10*, 199–211.
13. Müllauer, L. Next generation sequencing: Clinical applications in solid tumours. *Memo* **2017**, *10*, 244–247. [CrossRef]
14. Hubers, A.J.; Prinsen, C.F.; Sozzi, G.; Witte, B.I.; Thunnissen, E. Molecular sputum analysis for the diagnosis of lung cancer. *Br. J. Cancer* **2013**, *109*, 530–537. [CrossRef] [PubMed]

15. Ahrendt, S.A.; Chow, J.T.; Xu, L.; Yang, S.C.; Eisenberger, C.F.; Esteller, M.; Herman, J.G.; Wu, L.; Decker, A.; Jen, J.; et al. Molecular Detection of Tumor Cells in Bronchoalveolar Lavage Fluid From Patients With Early Stage Lung Cancer. *J. Natl. Cancer Inst.* **1999**, *91*, 332–339. [CrossRef] [PubMed]
16. Kadara, H.; Scheet, P.; Wistuba, I.I. Early Events in the Molecular Pathogenesis of Lung Cancer. *Cancer Prev. Res. (Phila.)* **2016**, *9*, 518–527. [CrossRef]
17. Starostik, P. Clinical mutation assay of tumors: New developments. *Anti-Cancer Drugs.* **2017**, *28*, 1–10. [CrossRef]
18. Schwartzberg, L.; Kim, E.S.; Liu, D.; Schrag, D. Precision Oncology: Who, How, What, When, and When Not? *Am. Soc. Clin. Oncol. Educ. Book* **2017**, *37*, 160–169. [CrossRef]
19. Ou, S.I.; Nagasaka, M.; Zhu, V.W. Liquid Biopsy to Identify Actionable Genomic Alterations. *Am. Soc. Clin. Oncol. Educ. Book* **2018**, *38*, 978–997. [CrossRef]
20. Zhang, X.; Liang, Z.; Wang, S.; Lu, S.; Song, Y.; Cheng, Y.; Ying, J.; Liu, W.; Hou, Y.; Li, Y.; et al. Application of next-generation sequencing technology to precision medicine in cancer: Joint consensus of the Tumor Biomarker Committee of the Chinese Society of Clinical Oncology. *Cancer Biol. Med.* **2019**, *16*, 189–204.
21. García-Giménez, J.L.; Seco-Cervera, M.; Tollefsbol, T.O.; Romá-Mateo, C.; Peiró-Chova, L.; Lapunzina, P.; Pallardó, F.V. Epigenetic biomarkers: Current strategies and future challenges for their use in the clinical laboratory. *Crit. Rev. Clin. Lab. Sci.* **2017**, *54*, 529–550. [CrossRef] [PubMed]
22. Lis, P.; Niczyj-Raucy, M.; Lis, M. The molecular basis of cancer and genetic methods of its diagnosis. *Nat. J. (Opole)* **2011**, *44*, 92–119.
23. Pass, H.I.; Beer, D.G.; Joseph, S.; Massion, P. Biomarkers and Molecular Testing for Early Detection, Diagnosis, and Therapeutic Prediction of Lung Cancer. *Thorac. Surg. Clin.* **2013**, *23*, 211–224. [CrossRef] [PubMed]
24. Calabrese, F.; Lunardi, F.; Pezzuto, F.; Fortarezza, F.; Vuljan, S.E.; Marquette, C.; Hofman, P. Are There New Biomarkers in Tissue and Liquid Biopsies for the Early Detection of Non-Small Cell Lung Cancer? *J. Clin. Med.* **2019**, *8*, 414. [CrossRef]
25. Hassanein, M.; Callison, J.C.; Callaway-Lane, C.; Aldrich, M.C.; Grogan, E.L.; Massion, P.P. The state of molecular biomarkers for the early detection of lung cancer. *Cancer Prev. Res. (Phila.)* **2012**, *5*, 992–1006. [CrossRef]
26. Sholl, L. Molecular diagnostics of lung cancer in the clinic. *Transl. Lung Cancer Res.* **2017**, *6*, 560–569. [CrossRef]
27. Liquid Biopsy and Droplet Digital PCR Offer Improvements for Lung Cancer Testing. Available online: http://archive.vn/2020.06.26-095203/https://www.mlo-online.com/continuing-education/article/13017057/liquid-biopsy-and-droplet-digital-pcr-offer-improvements-for-lung-cancer-testing (accessed on 25 April 2020).
28. Crowley, E.; Di Nicolantonio, F.; Loupakis, F.; Bardelli, A. Liquid biopsy: Monitoring cancer-genetics in the blood. *Nat. Rev. Clin. Oncol.* **2013**, *10*, 472–484. [CrossRef]
29. Sholl, L.M.; Aisner, D.L.; Allen, T.C.; Beasley, M.B.; Cagle, P.T.; Capelozzi, V.L.; Dacis, S.; Hariri, L.P.; Kerr, K.M.; Lantuejoul, S.; et al. Liquid Biopsy in Lung Cancer: A Perspective From Members of the Pulmonary Pathology Society. *Arch. Pathol. Lab. Med.* **2016**, *140*, 825–829. [CrossRef]
30. Wang, S.; Yu, B.; Ng, C.; Mercorella, B.; Selinger, C.; O'Toole, S.; Cooper, W. The suitability of small biopsy and cytology specimens for *EGFR* and other mutation testing in non-small cell lung cancer. *Transl. Lung Cancer Res.* **2015**, *4*, 119–125.
31. Bubendorf, L.; Lantuejoul, S.; de Langen, A.; Thunnissen, E. Non-small cell lung carcinoma: Diagnostic difficulties in small biopsies and cytological specimens. *Eur. Respir. Rev.* **2017**, *26*, 170007. [CrossRef]
32. Sigel, C.; Moreira, A.; Travis, W.; Zakowski, M.; Thornton, R.; Riely, G.; Rekhtman, N. Subtyping of Non-small Cell Lung Carcinoma: A Comparison of Small Biopsy and Cytology Specimens. *J. Thorac. Oncol.* **2011**, *6*, 1849–1856. [CrossRef] [PubMed]
33. Travis, W.; Brambilla, E.; Noguchi, M.; Nicholson, A.; Geisinger, K.; Yatabe, Y.; Ishikawa, Y.; Wistuba, I.; Flieder, D.; Franklin, W.; et al. Diagnosis of Lung Cancer in Small Biopsies and Cytology: Implications of the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society Classification. *Arch. Pathol. Lab. Med.* **2013**, *137*, 668–684. [CrossRef] [PubMed]
34. McLean, A.; Barnes, D.; Troy, L. Diagnosing Lung Cancer: The Complexities of Obtaining a Tissue Diagnosis in the Era of Minimally Invasive and Personalised Medicine. *J. Clin. Med.* **2018**, *7*, 163. [CrossRef] [PubMed]

35. Sromek, M.; Glogowski, M.; Chechlinska, M.; Kulinczak, M.; Szafron, L.; Zakrzewska, K.; Owczarek, J.; Wiśniewski, P.; Wlodarczyk, R.; Talarek, L.; et al. Changes in plasma miR-9, miR-16, miR-205 and miR-486 levels after non-small cell lung cancer resection. *Cell Oncol. (Dordr.)* **2017**, *40*, 529–536. [CrossRef]
36. Johann, D.; Steliga, M.; Shin, I.; Yoon, D.; Arnaoutakis, K.; Hutchins, L.; Liu, M.; Liem, J.; Walker, K.; Pereira, A.; et al. Liquid biopsy and its role in an advanced clinical trial for lung cancer. *Exp. Biol. Med.* **2018**, *243*, 262–271. [CrossRef]
37. Mellert, H.; Jackson, L.; Pestano, G. Performance verification of a plasma-based PD-L1 test that reliably measures mRNA expression from patients with NCSLC. *J. Clin. Oncol.* **2018**, *36*, 156. [CrossRef]
38. Stratton, M.R.; Campbell, P.J.; Futreal, P.A. The cancer genome. *Nature* **2009**, *458*, 719–724. [CrossRef]
39. Daniels, M.G.; Bowman, R.V.; Yang, I.A.; Govindan, R.; Fong, K.M. An emerging place for lung cancer genomics in 2013. *J. Thorac. Dis.* **2013**, *5*, S491–S497.
40. McDermott, U.; Downing, J.R.; Stratton, M.R. Genomics and the continuum of cancer care. *N. Engl. J. Med.* **2011**, *364*, 340–350. [CrossRef]
41. Kumar, R.; Shah, P.; Pandey, D.; Kumar, A. Molecular Diagnostic Technology. In *Biotechnology in Medicine and Agriculture Principles and Practices*; Kumar, A., Pareek, A., Gupta, S.M., Eds.; I K International Publishing House: New Delhi, India, 2012; pp. 368–402.
42. Bal, J.; Wiszniewska, J.; Wiszniewski, W. Metody analizy genomu. In *Genetyka Medyczna i Molekularna*; Bal, J., Ed.; PWN: Warszawa, Poland, 2017; pp. 101–118.
43. DI Felice, F.; Micheli, G.; Camilloni, G. Restriction enzymes and their use in molecular biology: An overview. *J. Biosci.* **2019**, *44*, 38. [CrossRef]
44. Cowlen, M.S. Nucleic Acid Hybridization and Amplification In Situ. In *Molecular Diagnostics. Pathology and Laboratory Medicine*; Coleman, W.B., Tsongalis, G.J., Eds.; Humana Press: Totowa, NJ, USA, 1997; pp. 163–191.
45. Netzer, K.O. Hybridization Methods (Southern and Northern Blotting). In *Techniques in Molecular Medicine*; Hildebrandt, F., Igarashi, P., Springer Lab Manual, Eds.; Springer: Berlin, Germany, 1999; pp. 126–147.
46. Blohm, D.H.; Guiseppi-Elie, A. New developments in microarray technology. *Curr. Opin. Biotech.* **2001**, *12*, 41–47. [CrossRef]
47. Jarząb, B.; Gubała, E.; Lange, D. Mikromacierze DNA i profil ekspresji genów raka brodawkowatego tarczycy. *Endokrynol. Pol.* **2005**, *3*, 294–301.
48. Mirski, T.; Bartoszcze, M.; Bielawska-Drózd, A.; Gryko, R.; Kocik, J.; Niemcewicz, M.; Chomiczewski, K. Microarrays—New Possibilities for Detecting Biological Factors Hazardous for Humans and Animals, and for Use in Environmental Protection. *Ann. Agric. Environ. Med.* **2016**, *23*, 30–36. [CrossRef] [PubMed]
49. Sridhar, K.; Singh, A.; Butzmann, A.; Jangam, D.; Ohgami, R.S. Molecular genetic testing methodologies in hematopoietic diseases: Current and future methods. *Int. J. Lab. Hematol.* **2019**, *41*, 102–116. [CrossRef] [PubMed]
50. Beroukhim, R.; Mermel, C.H.; Porter, D.; Wei, G.; Raychaudhuri, S.; Donovan, J.; Barretina, J.; Boehm, J.S.; Dobson, J.; Urashima, M.; et al. The landscape of somatic copy-number alteration across human cancers. *Nature* **2010**, *463*, 899–905. [CrossRef]
51. Kuo, F.C.; Mar, B.G.; Lindsley, R.C.; Lindeman, N.I. The relative utilities of genome-wide, gene panel, and individual gene sequencing in clinical practice. *Blood* **2017**, *130*, 433–439. [CrossRef]
52. Vendrell, J.A.; Grand, D.; Rouquette, I.; Costes, V.; Icher, S.; Selves, J.; Larrieux, M.; Barbe, A.; Brousset, P.; Solassol, J. High-throughput detection of clinically targetable alterations using next-generation sequencing. *Oncotarget* **2017**, *8*, 40345–40358. [CrossRef]
53. Yang, M.; Kim, J.W. Principles of Genetic Counseling in the Era of Next-Generation Sequencing. *Ann. Lab. Med.* **2018**, *38*, 291–295. [CrossRef]
54. Liu, Z.; Zhu, L.; Roberts, R.; Tong, W. Toward Clinical Implementation of NextGeneration Sequencing-Based Genetic Testing in Rare Diseases: Where Are We? *Trends Genet.* **2019**, *35*, 852–867. [CrossRef]
55. Park, E.; Shim, H.S. Detection of Targetable Genetic Alterations in Korean Lung Cancer Patients: A Comparison Study of Single-Gene Assays and Targeted Next-Generation Sequencing. *Cancer Res. Treat* **2020**, *52*, 543–551. [CrossRef]
56. Dama, E.; Melocchi, V.; Colangelo, T.; Cuttano, R.; Bianchi, F. Deciphering the Molecular Profile of Lung Cancer: New Strategies for the Early Detection and Prognostic Stratification. *J. Clin. Med.* **2019**, *18*, 108. [CrossRef] [PubMed]

57. Dietel, M.; Jöhrens, K.; Laffert, M.; Hummel, M.; Bläker, H.; Müller, B.M.; Lehmann, A.; Denkert, C.; Heppner, F.L.; Koch, A.; et al. Predictive molecular pathology and its role in targeted cancer therapy: A review focussing on clinical relevance. *Cancer Gene Ther.* **2013**, *20*, 211–221. [CrossRef] [PubMed]
58. Mehrad, M.; Roy, S.; Bittar, H.T.; Dacic, S. Next-Generation Sequencing Approach to Non-Small Cell Lung Carcinoma Yields More Actionable Alterations. *Arch. Pathol. Lab. Med.* **2018**, *142*, 353–357. [CrossRef] [PubMed]
59. Sands, J.M.; Nguyen, T.; Shivdasani, P.; Sacher, A.G.; Cheng, M.L.; Alden, R.S.; Jänne, P.A.; Kuo, F.C.; Oxnard, G.R.; Sholl, L.M. Next-generation sequencing informs diagnosis and identifies unexpected therapeutic targets in lung squamous cell carcinomas. *Lung Cancer* **2020**, *140*, 35–41. [CrossRef]
60. Fernandes, M.G.O.; Jacob, M.; Martins, N.; Moura, C.S.; Guimarães, S.; Reis, J.P.; Justino, A.; Pina, M.J.; Cirnes, L.; Sousa, C.; et al. Targeted Gene Next-Generation Sequencing Panel in Patients with Advanced Lung Adenocarcinoma: Paving the Way for Clinical Implementation. *Cancers* **2019**, *11*, 1229. [CrossRef] [PubMed]
61. Zhong, Q.; Wagner, U.; Kurt, H.; Molinari, F.; Cathomas, G.; Komminoth, P.; Barman-Aksözen, J.; Schneider-Yin, X.; Rey, J.P.; Vassella, E.; et al. Multi-laboratory proficiency testing of clinical cancer genomic profiling by next-generation sequencing. *Pathol. Res. Pract.* **2018**, *214*, 957–963. [CrossRef]
62. McBride, C.M.; Koehly, L.M. Imagining roles for epigenetics in health promotion research. *J. Behav. Med.* **2017**, *40*, 229–238. [CrossRef]
63. Sueoka, T.; Koyama, K.; Hayashi, G.; Okamoto, A. Chemistry-Driven Epigenetic Investigation of Histone and DNA Modifications. *Chem. Rec.* **2018**, *18*, 1727–1744. [CrossRef]
64. Kelsey, G.; Stegle, O.; Reik, W. Single-cell epigenomics: Recording the past and predicting the future. *Science* **2017**, *358*, 69–75. [CrossRef]
65. Tsou, J.A.; Hagen, J.A.; Carpenter, C.L.; Laird-Offringa, I.A. DNA methylation analysis: A powerful new tool for lung cancer diagnosis. *Oncogene* **2002**, *21*, 5450–5461. [CrossRef]
66. Clark, S.J.; Lee, H.J.; Smallwood, S.A.; Kelsey, G.; Reik, W. Single-cell epigenomics: Powerful new methods for understanding gene regulation and cell identity. *Genome Biol.* **2016**, *18*, 17. [CrossRef] [PubMed]
67. Zito Marino, F.; Bianco, R.; Accardo, M.; Ronchi, A.; Cozzolino, I.; Morgillo, F.; Rossi, G.; Franco, R. Molecular heterogeneity in lung cancer: From mechanisms of origin to clinical implications. *Int. J. Med. Sci.* **2019**, *16*, 981–989. [CrossRef] [PubMed]
68. Kan, Z.; Jaiswal, B.S.; Stinson, J.; Janakiraman, V.; Bhatt, D.; Stern, H.M.; Yue, P.; Haverty, P.M.; Bourgon, R.; Zheng, J.; et al. Diverse somatic mutation patterns and pathway alterations in human cancers. *Nature* **2010**, *466*, 869–873. [CrossRef] [PubMed]
69. Kopczyński, P.; Krawczyński, M.R. The Role of Oncogenes and Tumor Suppressor Genes in Oncogenesis. *Now. Lekarskie* **2012**, *81*, 679–681.
70. Sholl, L.M.; Aisner, D.L.; Varella-Garcia, M.; Berry, L.D.; Dias-Santagata, D.; Wistuba, I.I.; Chen, H.; Fujimoto, J.; Kugler, K.; Franklin, W.A.; et al. Multi-institutional Oncogenic Driver Mutation Analysis in Lung Adenocarcinoma: The Lung Cancer Mutation Consortium Experience. *J. Thorac. Oncol.* **2015**, *10*, 768–777. [CrossRef]
71. Devarakonda, S.; Rotolo, F.; Tsao, M.S.; Lanc, I.; Brambilla, E.; Masood, A.; Olaussen, K.A.; Fulton, R.; Sakashita, S.; McLeer-Florin, A.; et al. Tumor Mutation Burden as a Biomarker in Resected Non-Small-Cell Lung Cancer. *J. Clin. Oncol.* **2018**, *36*, 2995–3006. [CrossRef]
72. NCCN. National Comprehensive Cancer Network Guidelines. Available online: http://archive.today/2020.06.26-094846/https://www.nccn.org/professionals/physician_gls/default.aspx (accessed on 10 May 2020).
73. Kris, M.G.; Johnson, B.E.; Berry, L.D.; Kwiatkowski, D.J.; Iafrate, A.J.; Wistuba, I.I.; Varella-Garcia, M.; Franklin, W.A.; Aronson, S.L.; Su, P.-F.; et al. Using multiplexed assays of oncogenic drivers in lung cancers to select targeted drugs. *JAMA* **2014**, *311*, 1998–2006. [CrossRef]
74. Lindeman, N.I.; Cagle, P.T.; Aisner, D.L.; Arcila, M.E.; Beasley, M.B.; Bernicker, E.H.; Colasacco, C.; Dacic, S.; Hirsch, F.R.; Kerr, K.; et al. Updated Molecular Testing Guideline for the Selection of Lung Cancer Patients for Treatment With Targeted Tyrosine Kinase Inhibitors: Guideline From the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *Arch. Pathol. Lab. Med.* **2018**, *142*, 321–346.
75. Kim, E.Y.; Lee, S.H.; Kim, A.; Kim, T.; Chang, Y.S. Tumor clonal status predicts clinical outcomes of lung adenocarcinoma with EGFR-TKI sensitizing mutation. *J. Cancer* **2019**, *10*, 5549–5556. [CrossRef]

76. Garrido, P.; Conde, E.; de Castro, J.; Gómez-Román, J.; Felip, E.; Pijuan, L.; Isla, D.; Sanz, J.; Paz-Ares, L.; López-Ríos, F. Updated guidelines for predictive biomarker testing in advanced non-small-cell lung cancer: A National Consensus of the Spanish Society of Pathology and the Spanish Society of Medical Oncology. *Transl. Oncol.* **2020**, *22*, 989–1003. [CrossRef]
77. Popper, H.; Tímár, J.; Ryska, A.; Olszewski, W. Minimal requirements for the molecular testing of lung cancer. *Transl. Lung Cancer Res.* **2014**, *3*, 301–304. [PubMed]
78. Planchard, D. Other Oncogenic Drivers (BRAF, MET, RET, HER2, NTRK). Available online: http://splf.fr/wp-content/uploads/2018/10/S14-3.pdf (accessed on 22 May 2020).
79. Gainor, J.F.; Dardaei, L.; Yoda, S.; Friboulet, L.; Leshchiner, I.; Katayama, R.; Dagogo-Jack, I.; Gadgeel, S.; Schultz, K.; Singh, M.; et al. Molecular Mechanisms of Resistance to First- and Second-Generation ALK Inhibitors in ALK-Rearranged Lung Cancer. *Cancer Discov.* **2016**, *6*, 1118–1133. [CrossRef] [PubMed]
80. Bebb, D.G.; Agulnik, J.; Albadine, R.; Banerji, S.; Bigras, G.; Butts, C.; Couture, C.; Cutz, J.C.; Desmeules, P.; Ionescu, D.N.; et al. Crizotinib inhibition of ROS1-positive tumours in advanced non-small-cell lung cancer: A Canadian perspective. *Curr. Oncol.* **2019**, *26*, e551–e557. [CrossRef] [PubMed]
81. Lee, K.; Jung, H.A.; Sun, J.M.; Lee, S.H.; Ahn, J.S.; Park, K.; Ahn, M.J. Clinical Characteristics and Outcomes of Non-small Cell Lung Cancer Patients with *HER2* Alterations in Korea. *Cancer Res. Treat.* **2020**, *52*, 292–300. [CrossRef] [PubMed]
82. Janke, F.; Bozorgmehr, F.; Wrenger, S.; Dietz, S.; Heussel, C.; Heussel, G.; Silva, C.; Rheinheimer, S.; Feisst, M.; Thomas, M.; et al. Novel Liquid Biomarker Panels for A Very Early Response Capturing of NSCLC Therapies in Advanced Stages. *Cancers* **2020**, *12*, 954. [CrossRef] [PubMed]
83. Shen, C.; Wang, X.; Tian, L.; Che, G. Microsatellite alteration in multiple primary lung cancer. *J. Thorac. Dis.* **2014**, *6*, 1499–1505. [PubMed]
84. The Cancer Genome Atlas Network; (Muzny, D.M.; Bainbridge, M.N.; Chang, K.; Dinh, H.H.; Drummond, J.A.; Fowler, G.; Kovar, C.L.; Lewis, L.R.; Morgan, M.B.; Newsham, I.F.; et al.). Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **2012**, *487*, 330–337. [CrossRef]
85. Sozzi, G.; Musso, K.; Ratcliffe, C. Detection of Microsatellite Alternations in Plasma DNA of Non-Small Cell Lung Cancer Patients: A Prospect for Early Diagosis. *Clin. Cancer Res.* **1999**, *5*, 2689–2692.
86. Pęcherzewska, R.; Nawrot, B. *FHIT*—Tumor Suppressor Protein Involved in Induction of Apoptosis and Cell Cycle Regulation. *Postepy Biochem.* **2009**, *55*, 66–75.
87. Łaczmańska, I.; Ślęzak, R. Etiologia i znaczenie kliniczne miejsc kruchych w chromosomach człowieka. *Laboratory Diagnostics* **2010**, *46*, 81–86.
88. Duruisseaux, M.; Esteller, M. Lung cancer epigenetics: From knowledge to applications. *Semin. Cancer Biol.* **2018**, *51*, 116–128. [CrossRef] [PubMed]
89. Palmisano, W.A.; Divine, K.K.; Saccomanno, G.; Gilliland, F.D.; Baylin, S.B.; Herman, J.G.; Belinsky, S.A. Predicting lung cancer by detecting aberrant promoter methylation in sputum. *Cancer Res.* **2000**, *60*, 5954–5958. [PubMed]
90. Ooki, A.; Maleki, Z.; Tsay, J.J.; Goparaju, C.; Brait, M.; Turaga, N.; Nam, H.S.; Rom, W.N.; Pass, H.I.; Sidransky, D.; et al. A Panel of Novel Detection and Prognostic Methylated DNA Markers in Primary Non-Small Cell Lung Cancer and Serum DNA. *Clin. Cancer Res.* **2017**, *23*, 7141–7152. [CrossRef]
91. Liu, F.; Zhang, H.; Lu, S.; Wu, Z.; Zhou, L.; Cheng, Z.; Bai, Y.; Zhao, J.; Zhang, Q.; Mao, H. Quantitative assessment of gene promoter methylation in non-small cell lung cancer using methylation-sensitive high-resolution melting. *Oncol. Lett.* **2018**, *15*, 7639–7648. [CrossRef] [PubMed]
92. Huang, T.; Li, J.; Zhang, C.; Hong, Q.; Jiang, D.; Ye, M.; Duan, S. Distinguishing Lung Adenocarcinoma from Lung Squamous Cell Carcinoma by Two Hypomethylated and Three Hypermethylated Genes: A Meta-Analysis. *PLoS ONE* **2016**, *11*, e0149088. [CrossRef]
93. Filip, A. MikroRNA: Nowe mechanizmy regulacji ekspresji genów. *Postępy Biochemii.* **2007**, *53*, 413–419.
94. Aiso, T.; Ohtsuka, K.; Ueda, M.; Karita, S.; Yokoyama, T.; Takata, S.; Matsuki, N.; Kondo, H.; Takizawa, H.; Okada, A.A.; et al. Serum levels of candidate microRNA diagnostic markers differ among the stages of non-small-cell lung cancer. *Oncol. Lett.* **2018**, *16*, 6643–6651. [CrossRef]
95. Ulivi, P.; Petracci, E.; Marisi, G.; Baglivo, S.; Chiari, R.; Billi, M.; Canale, M.; Pasini, L.; Racanicchi, S.; Vagheggini, A.; et al. Prognostic Role of Circulating miRNAs in Early-Stage Non-Small Cell Lung Cancer. *J. Clin Med.* **2019**, *23*, 131. [CrossRef]

96. Bianchi, F.; Nicassio, F.; Marzi, M.; Belloni, E.; Dall'olio, V.; Bernard, L.; Pelosi, G.; Maisonneuve, P.; Veronesi, G.; Di Fiore, P.P. A serum circulating miRNA diagnostic test to identify asymptomatic high-risk individuals with early stage lung cancer. *EMBO. Mol. Med.* **2011**, *3*, 495–503. [CrossRef]
97. Xing, L.; Todd, N.W.; Yu, L.; Frang, H.; Jiang, F. Early Detection of Squamous Cell Lung Cancer in Sputum by a Panel of microRNA Markers. *Mod. Pathol.* **2010**, *23*, 1157–1164. [CrossRef]
98. Yu, L.; Todd, N.W.; Xing, L.; Xie, Y.; Zhang, H.; Liu, Z.; Fang, H.; Zhang, J.; Katz, R.L.; Jiang, F. Early detection of lung adenocarcinoma in sputum by a panel of microRNA markers. *Int. J. Cancer* **2010**, *127*, 2870–2878. [CrossRef] [PubMed]
99. Zheng, D.; Haddadin, S.; Wang, Y.; Gu, L.Q.; Perry, M.C.; Freter, C.E.; Wang, M.X. Plasma microRNAs as novel biomarkers for early detection of lung cancer. *Int. J. Clin. Exp. Pathol.* **2011**, *4*, 575–586. [PubMed]
100. Hennessey, P.T.; Sanford, T.; Choudhary, A.; Mydlarz, W.W.; Brown, D.; Adai, A.T.; Ochs, M.F.; Ahrendt, S.A.; Mambo, E.; Califano, J.A. Serum microRNA biomarkers for detection of non-small cell lung cancer. *PLoS ONE* **2012**, *7*, e32307. [CrossRef]
101. Heegaard, N.H.; Schetter, A.J.; Welsh, J.A.; Yoneda, M.; Bowman, E.D.; Harris, C.C. Circulating micro-RNA expression profiles in early stage nonsmall cell lung cancer. *Int. J. Cancer* **2012**, *130*, 1378–1386. [CrossRef]
102. Xu, X.; Zhu, S.; Tao, Z.; Ye, S. High circulating miR-18a, miR-20a, and miR-92a expression correlates with poor prognosis in patients with non-small cell lung cancer. *Cancer Med.* **2018**, *7*, 21–31. [CrossRef]
103. Yan, H.Z.; Wang, W.; Du, X.; Jiang, X.D.; Lin, C.Y.; Guo, J.L.; Zhang, J. The Expression and Clinical Significance of miRNA-99a and miRNA-224 in Non-Small-Cell Lung Cancer. *Eur. Rev. Med. Pharmacol. Sci.* **2019**, *23*, 1545–1552.
104. Szczyrek, M.; Kuźnar-Kamińska, B.; Grenda, A.; Krawczyk, P.; Sawicki, M.; Głogowski, M.; Balicka, G.; Rolska-Kopińska, A.; Nicoś, M.; Jakimiec, M.; et al. Diagnostic Value of Plasma Expression of microRNAs Complementary to Drosha and Dicer in Lung Cancer Patients. *Eur. Rev. Med. Pharmacol. Sci.* **2019**, *23*, 3857–3866.
105. Beganoyic, S. Clinical Significance of the *Kras* Mutation. *Bosn. J. Basic Med. Sci.* **2009**, *9*, S17–S20. [CrossRef]
106. Olivier, M.; Hollstein, M.; Hainaut, P. *TP53* mutations in human cancers: Origins, consequences, and clinical use. *CHS. Perspect. Biol.* **2010**, *2*, a001008. [CrossRef]
107. Ramón y Cajal, S.; Sesé, M.; Capdevila, C.; Aasen, T.; De Mattos-Arruda, L.; Diaz-Cano, S.; Hernández-Losa, J.; Castellví, J. Clinical implications of intratumor heterogeneity: Challenges and opportunities. *J. Mol. Med.* **2020**, *98*, 161–177.
108. Lin, J.; Shaw, A. Resisting Resistance: Targeted Therapies in Lung Cancer. *Trends Cancer* **2016**, *2*, 350–364. [CrossRef] [PubMed]
109. Ricordel, C.; Friboulet, L.; Facchinetti, F.; Soria, J. Molecular mechanisms of acquired resistance to third-generation EGFR-TKIs in *EGFR* T790M-mutant lung cancer. *Ann. Oncol.* **2018**, *29*, i28–i37. [CrossRef] [PubMed]
110. Ou, S.; Cui, J.; Schrock, A.; Goldberg, M.; Zhu, V.; Albacker, L.; Stephens, P.; Miller, V.; Ali, S. Emergence of novel and dominant acquired *EGFR* solvent-front mutations at Gly796 (G796S/R) together with C797S/G and L792F/H mutations in one *EGFR* (L858R/T790M) NSCLC patient who progressed on osimertinib. *Lung Cancer* **2017**, *108*, 228–231. [CrossRef] [PubMed]
111. Chen, K.; Zhou, F.; Shen, W.; Jiang, T.; Wu, X.; Tong, X.; Shao, Y.; Qin, S.; Zhou, C. Novel Mutations on *EGFR* Leu792 Potentially Correlate to Acquired Resistance to Osimertinib in Advanced NSCLC. *J. Thorac. Oncol.* **2017**, *12*, e65–e68. [CrossRef] [PubMed]
112. Kim, S.; Kim, T.; Kim, D.; Go, H.; Keam, B.; Lee, S.; Ku, J.; Chung, D.; Heo, D. Heterogeneity of Genetic Changes Associated with Acquired Crizotinib Resistance in ALK-Rearranged Lung Cancer. *J. Thorac. Oncol.* **2013**, *8*, 415–422. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

13.2. Załącznik 2 – I artykuł oryginalny

Katarzyna Wadowska, Piotr Błasiak, Adam Rzechonek, Iwona Bil-Lula, Mariola Śliwińska-Mossoń: New insights on old biomarkers involved in tumour microenvironment changes and their diagnostic relevance in non-small cel lung carcinoma. *Biomolecules*, **2021**, vol. 11, nr 8, art. 1208 [24 s.], DOI:10.3390/biom11081208

Article

New Insights on Old Biomarkers Involved in Tumor Microenvironment Changes and Their Diagnostic Relevance in Non-Small Cell Lung Carcinoma

Katarzyna Wadowska [1,*], Piotr Błasiak [2,3], Adam Rzechonek [2,3], Iwona Bil-Lula [1] and Mariola Śliwińska-Mossoń [1]

[1] Department of Medical Laboratory Diagnostics, Division of Clinical Chemistry and Laboratory Haematology, Wroclaw Medical University, Borowska 211A, 50-556 Wroclaw, Poland; iwona.bil-lula@umed.wroc.pl (I.B.-L.); mariola.sliwinska-mosson@umed.wroc.pl (M.Ś.-M.)
[2] Department and Clinic of Thoracic Surgery, Wroclaw Medical University, Grabiszyńska 105, 53-439 Wroclaw, Poland; piotr.blasiak@umed.wroc.pl (P.B.); adam.rzechonek@umed.wroc.pl (A.R.)
[3] Department of Thoracic Surgery, Lower Silesian Center for Lung Diseases, Grabiszyńska 105, 53-439 Wroclaw, Poland
* Correspondence: katarzyna.wadowska@student.umed.wroc.pl; Tel.: +48-71-784-06-26

Abstract: Background: Lung cancer is a multifactorial disease with a heterogeneous tumor group that hampers diagnostic and therapeutic approaches, as well as understanding of the processes that underlie its pathogenesis. Current research efforts are focused on examining alterations in the tumor microenvironment, which may affect the pathogenesis and further malignant progression in lung cancer. The aim of this study was to investigate changes in the levels of biomarkers involved in the lung tumor microenvironment and their diagnostic utility in differentiating lung cancer subtypes and stages. Methods: This study comprised 112 lung cancer patients, 50 with adenocarcinoma, 35 with squamous cell carcinoma, 13 with other non-small cell lung carcinoma subtypes, and 14 with other lung neoplasms than non-small cell lung carcinoma. Tumor markers (CEA, CYFRA 21-1, and NSE) were measured in the patients' sera and plasmas, along with IL-6, TNF-α, SAA_1, CRP, MMP-2, MMP-9, glucose, lactate, and LDH, utilizing enzyme-linked immunosorbent assays, enzyme immunoassays, and automated clinical chemistry and turbidimetry systems. The results were statistically analyzed across patient groups based on the subtype and stage of lung cancer. Results: Glucose concentrations showed statistically significant ($p < 0.05$) differences both between lung cancer subtypes and stages, with the highest levels in patients with other lung neoplasms (me = 130.5 mg/dL) and in patients with stage IIB lung cancer (me = 132.0 mg/dL). In patients with advanced lung cancer, IL-6 and LDH had considerably higher concentration and activity. There was also a significant positive correlation between IL-6 and MMP-9 in adenocarcinoma and SqCC, with correlation coefficients of 0.53 and 0.49, respectively. The ROC analyses showed that the best single biomarkers for distinguishing adenocarcinoma from squamous cell carcinoma are glucose, CRP, and CYFRA 21-1; however, their combination did not significantly improve sensitivity, specificity, and the AUC value. The combinations of IL-6, glucose, LDH and CEA, IL-6, SAA1, MMP-9, and lactate can distinguish patients with stage IIB lung cancer from those with stage IIA with 100% sensitivity, 100% specificity, and with an AUC value of 0.8333 and 1.0000, respectively, whereas the combination of CEA, IL-6, and LDH can identify patients with stage IIIA lung cancer from those with stage IIB with 72.73% sensitivity, 94.44% specificity, and an AUC value of 0.8686. Conclusion: There is a link between biomarkers of tumor microenvironment changes and tumor markers, and combinations of these markers may be clinically useful in the differential diagnosis of adenocarcinoma and squamous cell carcinoma, as well as lung cancer stages IIB and IIA, and IIIA and IIB.

Keywords: lung cancer; non-small cell lung carcinoma; adenocarcinoma of lung; squamous cell carcinoma of lung; tumor microenvironment; malignant progression; biochemical tumor markers; diagnostic biomarkers; differential diagnoses; ROC analyses

Citation: Wadowska, K.; Błasiak, P.; Rzechonek, A.; Bil-Lula, I.; Śliwińska-Mossoń, M. New Insights on Old Biomarkers Involved in Tumor Microenvironment Changes and Their Diagnostic Relevance in Non-Small Cell Lung Carcinoma. *Biomolecules* **2021**, *11*, 1208. https://doi.org/10.3390/biom11081208

Academic Editor: Vladimir N. Uversky

Received: 29 June 2021
Accepted: 10 August 2021
Published: 13 August 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Cancer is one of the multifactorial diseases that is thought to be caused by complex interactions between genetic and environmental factors. The precise pathogenesis of lung cancer has not yet been fully understood [1–3]. Based on current knowledge, lung neoplasm results from the final stage of multi-stage bronchial cells carcinogenesis, with progressively increasing genetic and epigenetic changes due to exposure to environmental factors and patients' individual predispositions to lung cancer [4,5]. Recent studies on gene and protein expression focused on early diagnosis of the type and stage of lung cancer in relevance to treatment options, simultaneously providing information on the initiation, progression of tumorigenesis, and differences between subtypes [6]. Growing evidence shows that lung cancer represents a group of histologically, cellularly, and molecularly heterogeneous tumors within the same histological type, which influences response and resistance to targeted therapies as well as diagnostic processes [7].

Non-small cell lung carcinoma (NSCLC) is the most common type of lung cancer, accounting for up to 85% of cases. The tissue composition and molecular landscape of NSCLCs are both heterogeneous, affecting clinical decision making in lung cancer treatment. NSCLC must be divided into two types: adenocarcinoma and squamous cell carcinoma (SqCC), each of which has a unique set of clinically actionable mutations [8–12]. Lung tumor cells with acquired somatic mutations influence cytokine and chemokine secretion, modifying the chemoattractant properties of the tumor microenvironment (TME). Changes in the TME, remodeling of the extracellular matrix (ECM), pro- and anti-inflammatory processes, and cell metabolism are affected by neoplastic progression, but they also affect further progression and metastasis of the tumor. These changes can be examined by evaluating particular inflammatory and metabolic markers, which, in turn, may be considered diagnostic biomarkers [13–18].

Diagnosis of lung cancer requires improvement. Most patients are diagnosed with locally advanced stage of pulmonary neoplasm or metastatic disease and have a 1-year survival rate of about only 20%. In addition, the 5-year survival rate of patients with lung cancer is approximately 15%, which in combination with the fact that lung cancer is the most prevalent cancer in the world, accounting for 2.09 million cases, makes it the most common cause of cancer death (1.76 million deaths in 2018) [19–21]. These data suggest an urgent need for early detection of lung neoplasm and to differentiate subtypes with available target therapies. However, early detection of neoplastic changes requires adequate diagnostic methods characterized by high sensitivity, specificity, and non-invasiveness [22].

Widely described cancer antigens, such as cytokeratin 19 fragment (CYFRA 21-1), carcinoembryonic antigen (CEA), and neuron-specific enolase (NSE), have confirmed usefulness as a tumor biomarker panel in the diagnosis of lung cancer, but they lack high sensitivity and specificity [4,23,24]. Combining tumor markers with serum proteins appears to be an approach that has the potential to increase the diagnostic value of tumor markers. The study of the relationship between interleukin-6 (IL-6) and tumor markers in breast and colorectal cancers is an example of such a combination, as well as one of the research hotspots [25,26]. We decided to take this study a step further and investigate the relationship between tumor markers with pro- and anti-inflammatory cytokines (IL-6, tumor necrosis factor-α—TNF-α), as well as matrix metalloproteinases-2 and -9 (MMP-2, MMP-9), both of which have been shown to be essential in the tumor microenvironment. Furthermore, we chose to look at easily accessible markers such as acute-phase proteins (C-reactive protein—CRP, serum amyloid A_1—SAA_1) that are linked to the expression of pro- and anti-inflammatory cytokines, as well as metabolic markers associated with the Warburg effect, i.e., glucose metabolism in cancer cells (glucose, lactate dehydrogenase—LDH, and lactate). Figure 1 shows the relationship between the chosen biomarkers and their roles in the tumor microenvironment as well as cancer progression.

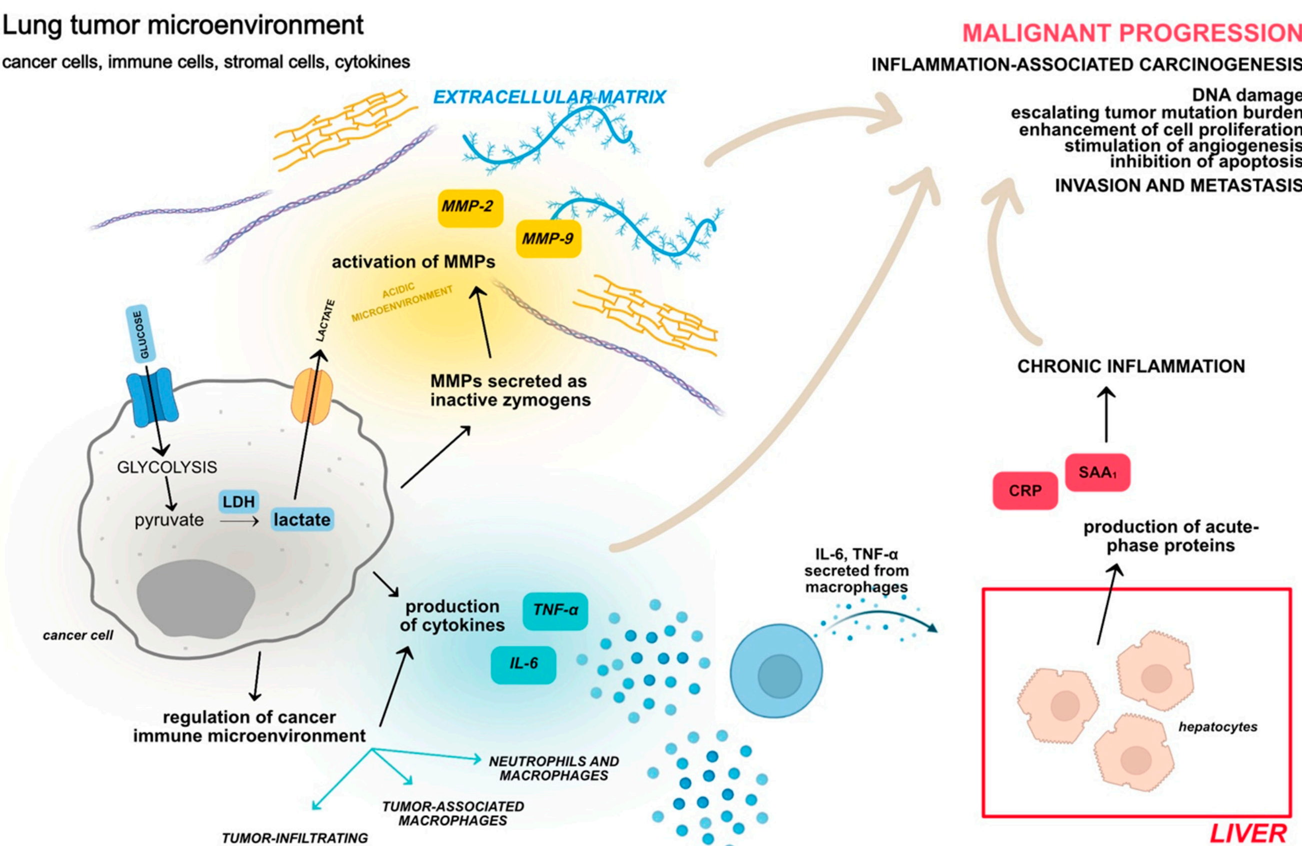


Figure 1. A simplified diagram depicting the role of glucose, LDH, lactate, MMP-2, MMP-9, TNF-α, IL-6, SAA1, and CRP in lung cancer progression. Created with BioRender.com and Affinity Designer. TME is a complex mixture of tumor cells, extracellular matrix, and an inflammatory microenvironment including immune cells and cytokines that plays an essential part in tumorigenesis and progression [14,17,27]. The unrestricted proliferation of cancer cells influences TME, manifesting itself, among other things, in increased glucose uptake. Glucose is glycolyzed after it enters the cell through the glucose transporters (GLUT). Glucose is converted to pyruvate during glycolysis, and this conversion provides the carbon needed to produce precursors for nucleotide, protein, and lipid synthesis in lung tumor cells. In subsequent steps, LDH reduces pyruvate to lactate under anaerobic conditions. Even in the presence of oxygen, lung cancer cells metabolize glucose via lactic acid fermentation, and lactate is secreted into the extracellular space by monocarboxylate transporters (MCT), causing the acidification of the microenvironment [28–32]. The acidic microenvironment activates MMPs, which are secreted as an inactive zymogens by tumor cells. MMPs are metastasis-associated proteins that alter cellular signals, regulate the expression of cytokines and growth factors, and cause the degradation of extracellular matrix and cellular membrane components, resulting in cancer cells invasion and metastasis. Lactate excretion also affects the function of immune cells and triggers immune escape of tumor cells. Immune cells within the TME are divided into tumor-antagonizing and tumor-promoting immune cells and regulated by the cancer cells. Effector T cells, natural killer (NK) cells, M1-polarized macrophages, and N1-polarized neutrophils secrete pro-inflammatory cytokines, chemokines, and reactive oxygen/nitrogen species to induce the cytotoxicity in cancer cells and recruit other cells with antitumor activity [33]. Many immune cells act through the secretion of TNF-α and IL-6, which have a synergistic relationship. TNF-α expression can cause a 60-fold increase in IL-6 production, while IL-6 is able to induce expression of TNF-α in NSCLC cells. These macrophage-secreted cytokines regulate liver cell production of the non-specific acute-phase proteins SAA_1 and CRP, which are linked to inflammation [34–40]. Elevated IL-6 expression is associated to the transition from acute to chronic inflammation, which contributes to the development of inflammation-associated carcinogenesis. Chronic inflammatory mechanisms may contribute to tumor formation, growth, and metastasis via DNA damage, enhanced cell proliferation, angiogenesis stimulation, and apoptosis inhibition [41]. LDH—lactate dehydrogenase; MMP-2—matrix metalloproteinase-2; MMP-9—matrix metalloproteinase-9; TNF-α—tumor necrosis factor-α; IL-6—interleukin-6; CRP—C-reactive protein; SAA_1—serum amyloid A_1; TME—tumor microenvironment; GLUT—glucose transporter; MCT—monocarboxylate transporter; NK—natural killer.

The aim of this study was to see if there were any changes in the levels of evaluated biomarkers between lung cancer subtypes and stages. We decided to investigate the relationship between the assessed tumor and the inflammatory and metabolic markers to see if combining them could improve their diagnostic value in patients with lung adenocarcinoma and squamous cell carcinoma, as well as chosen lung cancer stages IIB and IIA, IIIA and IIB, resulting in better therapeutic decision making.

2. Materials and Methods

2.1. Patients

The research group consists of 112 consecutive patients recruited by the Department of Thoracic Surgery at the Lower Silesian Center for Lung Diseases in Wroclaw, Poland. Before collecting the blood samples, the experiment was approved by the local Ethics Committee at Wroclaw Medical University, and all patients signed written informed consent to participate in the study. Prior to any surgical treatment, venous blood samples were collected into tubes with ethylenediaminetetraacetic acid (EDTA) anticoagulant and tubes with clot activator from all patients. Blood samples were centrifuged at $2000\times g$ for 8–10 min at room temperature to separate plasma and serum, which were then stored at −80 °C until use. The average storage time was less than 6 months.

The research group was surgically treated, either with a thoracotomy or with video-assisted thoracoscopic (VAT) surgery. The majority of the patients (61 out of 112, 54.46%) underwent lobectomy, with the remainder undergoing wedge resection (28 out of 112, 25.00%), biopsy (9 out of 112, 8.04%), segmentectomy (7 out of 112, 6.25%), bilobectomy (5 out of 112, 4.46%), and pulmonectomy (2 out of 112, 1.79%). A histopathological examination was performed on the tumor tissue obtained, which provided us with the necessary information about the diagnosis of lung cancer. The information about the studied group was completed by clinical and pathological data obtained from hospital medical reports using the Asseco Medical Management Solutions (AMMS) IT system. The characteristics of the studied group are shown in Table 1.

Table 1. Patients' characteristics according to the type of lung cancer.

	Non-Small Cell Lung Carcinoma			**Non-NSCLC**	**Overall**
	Adenocarcinoma	**Squamous Cell Carcinoma (SqCC)**	**Other NSCLCs' Subtypes**		
N (%)	50 (44.64%)	35 (31.25%)	13 (11.61%)	14 (12.50%)	112 (100%)
Age					
Mean ± SD	67 ± 8	68 ± 8	68 ± 12	65 ± 5	67 ± 8
Range	39–81	47–82	40–84	54–71	39–84
Median	69	69	73	67	68
Gender					
Male	29 (58.00%)	27 (77.14%)	8 (61.54%)	7 (50.00%)	71 (63.39%)
Female	21 (42.00%)	8 (22.86%)	5 (38.46%)	7 (50.00%)	41 (36.61%)
Surgery					
Lobectomy	30 (60.00%)	19 (54.29%)	7 (53.85%)	5 (35.71%)	61 (54.46%)
Wedge resection	9 (18.00%)	9 (25.71%)	4 (30.77%)	6 (42.86%)	28 (25.00%)
Biopsy	6 (12.00%)	-	-	3 (21.43%)	9 (8.04%)
Segmentectomy	5 (10.00%)	1 (2.86%)	1 (7.69%)	-	7 (6.25%)
Bilobectomy	-	4 (11.43%)	1 (7.69%)	-	5 (4.46%)
Pulmonectomy	-	2 (5.71%)	-	-	2 (1.79%)
Stage					
IA1	2 (4.00%)	3 (8.57%)	-	-	5 (4.46%)
IA2	6 (12.00%)	6 (17.14%)	3 (23.08%)	-	15 (13.39%)
IA3	3 (6.00%)	1 (2.86%)	1 (7.69%)	-	5 (4.46%)
IB	12 (24.00%)	4 (11.43%)	2 (15.38%)	-	18 (16.07%)
IIA	3 (6.00%)	1 (2.86%)	1 (7.69%)	1 (7.14%)	6 (5.36%)

Table 1. *Cont.*

	Non-Small Cell Lung Carcinoma			Non-NSCLC	Overall
	Adenocarcinoma	Squamous Cell Carcinoma (SqCC)	Other NSCLCs' Subtypes		
IIB	7 (14.00%)	12 (34.29%)	2 (15.38%)	-	21 (18.75%)
IIIA	9 (18.00%)	3 (8.57%)	-	-	12 (10.71%)
IIIB	4 (8.00%)	3 (8.57%)	2 (15.38%)	2 (14.29%)	11 (9.82%)
IVA	4 (8.00%)	2 (5.71%)	2 (15.38%)	6 (42.86%)	14 (12.50%)
IVB	-	-	-	-	-
Grading					
G1	1	-	-	-	1
G2	19	25	1	-	45
G3	19	-	1	-	20
NA	11	10	11	14	46
Smoking history					
Current	15 (30.00%)	10 (28.57%)	3 (23.08%)	3 (21.43%)	31 (27.68%)
Former	23 (46.00%)	21 (60.00%)	6 (46.15%)	5 (35.71%)	55 (49.11%)
Passive	1 (2.00%)	-	-	1 (7.14%)	2 (1.79%)
Never	2 (4.00%)	-	-	-	2 (1.79%)
NA	9 (18.00%)	4 (11.43%)	4 (30.77%)	5 (35.71%)	22 (19.64%)
Pack-years					
Mean ± SD	30 ± 17	36 ± 20	40 ± 26	38 ± 24	34 ± 19
Range	0–60	3–106	20–90	0–68	0–106
Median	30	38	35	36	30

SD—standard deviation; NA—not available; G1—grade 1, well differentiated; G2—grade 2, moderately differentiated; G3—grade 3, poorly differentiated.

Based on the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology, we divided the study group by histopathological type into NSCLC and non-NSCLC patients (one with small cell lung carcinoma [SCLC], four with benign pulmonary nodules, two with mesothelioma, and seven with metastases of breast, colorectal, stomach, or esophageal cancer to the lungs), and then further subdivided the NSCLC group into adenocarcinoma = 50, SqCC = 35, and others (five patients with large cell neuroendocrine carcinoma, four patients with typical carcinoid, two with pleomorphic carcinoma, and two with not otherwise specified carcinoma). We also used the American Joint Committee on Cancer's (AJCC) 8th TNM Staging System to divide the study group by lung cancer stage. These groups were compared among themselves in the statistical analyses.

2.2. Methods

Serum blood samples were used to measure levels of CEA, NSE, IL-6, TNF-α, SAA1, MMP-2, and MMP-9 by the enzyme-linked immunosorbent assay (ELISA). The assay was performed according to the manufacturer's instruction (CEA ELISA/Catalog Number: EIA 1868, DRG Instrument GmbH, Marburg, Germany; Human Enolase 2/Neuron-specific Enolase Quantikine ELISA Kit/Catalog Number: DENL20, R&D Systems, Inc., Minnesota, MN, USA; Human IL-6 DuoSet ELISA/Catalog Number: DY206, R&D Systems, Inc., Minnesota, MN, USA; Human TNF-alpha DuoSet ELISA/Catalog Number: DY210, R&D Systems, Inc., Minnesota, MN, USA; Human Serum Amyloid A1 DuoSet ELISA/Catalog Number: DY3019-05, R&D Systems, Inc., Minnesota, MN, USA; Total MMP-2 Quantikine ELISA Kit/Catalog Number: MMP200, R&D Systems, Inc., Minnesota, MN, USA; and Human MMP-9 Quantikine ELISA Kit/Catalog Number: DMP900, R&D Systems, Inc., Minnesota, MN, USA). CEA was immobilized with monoclonal anti-CEA antibody and detected by monoclonal anti-CEA antibody conjugated to horseradish peroxidase (HRP). NSE/MMP-2/MMP-9 was immobilized with specific for human enolase 2/total MMP-2/human MMP-9 monoclonal antibody and was detected using polyclonal antibody specific for human enolase 2/total MMP-2/for human MMP-9 conjugated to HRP. IL-6/TNF-α/SAA_1 was im-

mobilized by mouse anti-human IL-6 capture antibody/mouse anti-human TNF-α capture antibody/mouse anti-human serum amyloid A1 capture antibody and was detected using biotinylated goat anti-human IL-6 detection antibody/biotinylated goat anti-human TNF-α detection antibody/biotinylated mouse anti-human serum amyloid A1 detection antibody along with streptavidin conjugated to HRP. In each assay, the reaction was developed using a tetramethylbenzidine (TMB) substrate solution. The substrate reaction was stopped, and the extinction was measured at 450 nm with the correction read at 540 nm using an ELISA reader. The kits provided standards ranging from 0 ng/mL to 100 ng/mL for CEA, 0 ng/mL to 20 ng/mL for NSE, 0 pg/mL to 600 pg/mL for IL-6, 0 pg/mL to 1000 pg/mL for TNF-α, 0 ng/mL to 100 ng/mL for SAA_1, 0 ng/mL to 32 ng/mL for MMP-2, and 0 ng/mL to 20 ng/mL for MMP-9. The standard curve was used to calculate the concentrations of measured biomarkers and was linearized by plotting the logs of the standards' concentrations versus the logs of the measured absorbances. If the measurements were out of the range, the samples were diluted, and the concentration read from the standard curve was multiplied by the dilution factor.

The concentrations of CYFRA 21-1 were determined using an enzyme immunoassay (EIA), which is a non-competitive immunoassay. The assay was carried out in accordance with the manufacturer's instructions (CYFRA 21-1 EIA/Catalog Number: 211-10, Fujirebio Diagnostics AB, Göteborg, Sweden), with two monoclonal antibodies (Anti-CYFRA 21-1 monoclonal antibody from mouse and Biotin Anti-CYFRA 21-1 monoclonal antibody from mouse) directed against two separate antigenic determinants of soluble fragments of cytokeratin 19. The assay procedure was similar to that of ELISA, and the kit contained standards ranging from 0 ng/mL to 45.4 ng/mL. The methods for calculating the results remained the same.

Plasma samples were used to measure concentrations of CRP, glucose, lactate, and LDH. The assay was performed by commercial test kits according to the manufacturer's instruction (C-REACTIVE PROTEIN/Catalog Number: 31321, BioSystems S.A., Barcelona, Spain; GLUCOSE-HK/Catalog Number: 11538, BioSystems S.A., Barcelona, Spain; LACTATE/Catalog Number: 11736, BioSystems S.A., Barcelona, Spain; and LACTATE DEHYDROGENASE, LDH/Catalog Number: 11580, BioSystems S.A., Barcelona, Spain) using BioSystems a15 analyzer.

2.3. Statistical Analysis

Prior to all statistical analyses, the estimated concentrations of the evaluated biomarkers were logarithmically transformed to achieve normal distribution. The Shapiro-Wilk test was used to determine if the data obtained for each biomarker had a normal distribution across all analyzed groups.

The analysis of variance (ANOVA) method was used to compare the concentrations of biomarkers in four groups of lung cancer patients (adenocarcinoma—ADC, squamous cell carcinoma—SqCC, other NSCLCs—Other, and not NSCLCs) as well as seven groups of lung cancer stage. Before choosing the ANOVA test for the analyses, the Brown–Forsythe test was used to verify the equality of the variables in groups. In the analysis, the F Welch's test was performed. In the lack of a normal distribution for the biomarker, the Kruskal–Wallis one-way ANOVA on ranks was performed to compare the concentrations. After determining whether there are any differences in biomarker levels across groups, post hoc tests were conducted to determine which groups vary statistically significantly. Tukey's honestly significant difference (HSD) test was used in the post hoc analysis.

The pairwise correlations were computed using Pearson correlation in variates to evaluate the associations between the levels of all analyzed proteins in lung cancer patients with adenocarcinoma, SqCC, other NSCLCs, other lung neoplasms than NSCLC groupings, and overall.

The final step of statistical analysis was to evaluate the diagnostic utility of our biomarkers in distinguishing adenocarcinoma and SqCC, as well as lung cancer stages IIB and IIA, and IIIA and IIB. The ability of the assessed biomarkers to differentiate chosen

groups was estimated using logistic regression models. To begin, the generalized linear model (GLM) was used to exclude out the effect of patients' age, gender, and smoking status on biomarker differences across groups. The sensitivity, specificity, and area under the curve (AUC) for each individual marker were calculated using the receiver operating characteristic (ROC) curve analysis. Following that, the generalized linear model with Akaike information criterion (AIC) estimation was employed to find the optimal model and the combination of biomarkers with the best diagnostic value. The ROC analysis was not performed for patients with other subtypes of NSCLC and other lung cancers than NSCLC due to the smaller sample size. In all these analyses, a *p*-value of 0.05 or less was considered statistically significant.

All statistical analyses were carried out using TIBCO Software Inc. (Palo Alto, CA, USA) (2017). Statistica (data analysis software system), version 13 (http://statistica.io, accessed on 10 May 2021 and 30 July 2021) with the additional Plus Package (version 5.0.96), was used.

3. Results

3.1. Patients' Characteristic

Tumor (CEA, CYFRA 21-1, and NSE), inflammatory markers (IL-6, TNF-α, SAA_1, CRP, MMP-2, and MMP-9), and metabolic markers (glucose, lactate, and LDH) were measured in the sera and plasmas of 112 lung cancer patients, who were classified into four groups based on subtype and seven groups based on stage. The research included 41 women (21 with adenocarcinoma, 8 with SqCC, 5 with other NSCLCs, and 7 with other lung neoplasms than NSCLCs) and 71 men (29 with adenocarcinoma, 27 with SqCC, 8 with other NSCLCs, and 7 with other lung neoplasms than NSCLCs). The majority of the patients were diagnosed at an early stage of lung cancer, with 43 (38.39%) diagnosed at stage I and 27 (24.11%) diagnosed at stage II.

We examined differences in biomarker concentrations across histopathological groups of lung cancer patients in terms of gender before comparing measured biomarkers across this groups. There were statistically significant differences in CRP concentration between female and male adenocarcinoma patients (p = 0.0411; meanF = 4.42 mg/L, meanM = 23.93 mg/L; raw mean difference [D] = 19.51 mg/L), MMP-2 concentration in SqCC patients (p = 0.0065; meanF = 114.10 ng/mL, meanM = 151.53 ng/mL; D = 37.43 ng/mL), and SAA_1 concentration in other NSCLCs (p = 0.0068; meanF = 13.51 µg/mL, meanM = 298.14 µg/mL; D = 284.63 µg/mL). The only statistically significant difference between male and female in the analysis of the entire research group was the level of CRP (p = 0.0080; meanF = 10.24 mg/L, meanM = 29.58 mg/L; D = 19.34 mg/L). The average age (±standard deviation [SD]) of lung cancer patients was 67 ± 8, and no statistically significant differences between the groups were found. The average age of adenocarcinoma patients was 67 ± 8, that of SqCC patients 68 ± 8, that of other NSCLC patients 68 ± 12, and that of patients with other lung neoplasms than NSCLC was 66 ± 6.

3.2. Tumor Markers

The only statistically significant difference in tumor marker concentrations in lung cancer patients was in the levels of CYFRA 21-1 between SqCC (me = 5.49 ng/mL) and other NSCLCs (me = 2.47 ng/mL; p = 0.0426). Figure 2 demonstrates the statistically significant distribution of CYFRA 21-1 concentrations in groups divided by lung cancer subtype. Table 2 compiles descriptive statistics for all tumor markers analyzed based on lung cancer type, subtype, and stage.

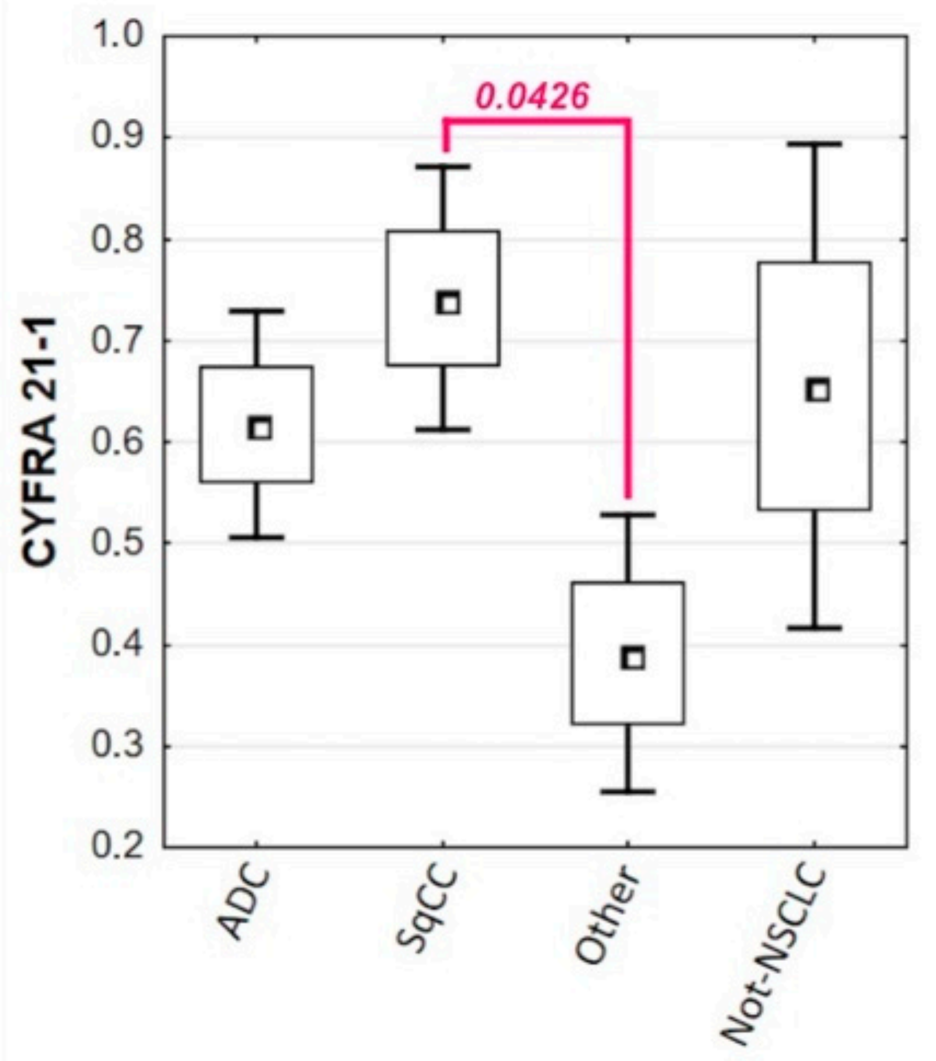


Figure 2. Distribution of CYFRA 21-1 levels in lung cancer subtypes with the statistically significant difference between patients with SqCC and other NSCLCs (p = 0.0426).

3.3. Inflammatory Markers

There were no statistically significant differences in any inflammatory marker between lung cancer types and subtypes. The concentrations of IL-6 between lung cancer stages were the only statistically significant difference in the analysis of variances (p = 0.0175). Tukey's HSD analysis, on the other hand, did not reveal which groups significantly differ statistically. The graph displaying the distribution of IL-6 concentrations among groups indicates a peak in IL-6 concentrations in patients with stage IIB (me = 44.14 pg/mL), IIIA (me = 47.36 pg/mL), and IIIB (me = 37.00 pg/mL) lung cancer. Figure 3 depicts the distribution of IL-6 marker concentrations among lung cancer stages. Table 3 collects descriptive statistics of inflammatory markers according to lung cancer type, subtype, and stage.

Table 2. Levels of the tumor markers according to lung cancer type, subtype, and stage.

	Non-Small Cell Lung Carcinoma			Non-NSCLC	Overall
	ADC	SqCC	Other		
CEA [ng/mL]					
Mean ± SEM	4.72 ± 1.04	3.43 ± 0.76	2.80 ± 0.31	3.97 ± 1.65	3.96 ± 0.54
Range	1.26–36.08	1.14–26.28	1.83–4.65	1.78–20.38	1.14–36.08
Median	2.81	2.41	2.29	2.22	2.41
CYFRA 21-1 [ng/mL]					
Mean ± SEM	6.32 ± 1.77	7.63 ± 1,14	2.78 ± 0.40	7.20 ± 2.59	6.46 ± 0.88
Range	1.15–63.89	1.17–27.94	1.22–4.65	0.72–33.60	0.72–63.89
Median	3.89	5.49	2.47	4.27	4.13
NSE [ng/mL]					
Mean ± SEM	8.51 ± 2.07	7.38 ± 1.58	11.92 ± 5.13	7.03 ± 1.20	8.37 ± 1.21
Range	1.60–99.94	2.18–38.36	0.98–64.63	2.83–17.13	0.98–99.94
Median	4.45	4.24	4.37	5.31	4.42

	IA	IB	IIA	IIB	IIIA	IIIB	IVA
CEA [ng/mL]							
Mean ± SEM	2.71 ± 0.24	2.96 ± 0.55	6.36 ± 3.60	3.07 ± 0.36	6.92 ± 2.99	4.48 ± 2.00	5.07 ± 2.39
Range	1.26–5.95	1.88–10.21	1.83–24.35	1.81–8.31	1.87–36.08	1.78–20.38	1.14–26.28
Median	2.35	2.37	2.94	2.48	3.37	2.29	2.21
CYFRA 21-1 [ng/mL]							
Mean ± SEM	4.50 ± 0.77	3.28 ± 0.67	4.57 ± 1.33	7.86 ± 1.58	5.00 ± 0.70	5.43 ± 1.31	15.59 ± 5.75
Range	1.17–13.22	1.15–10.21	1.44–7.85	1.73–27.94	1.27–9.03	2.38–13.49	0.72–63.89
Median	3.2	2.23	4.49	4.13	4.53	4.23	11.7
NSE [ng/mL]							
Mean ± SEM	10.03 ± 2.97	11,48 ± 5.45	6.50 ± 2.30	4.91 ± 0.75	5.06 ± 0.77	8.35 ± 2.98	10.29 ± 2.80
Range	0.98–64.63	2.25–99.94	2.33–17.13	2.45–17.21	1.60–10.66	1.87–37.26	2.18–35.79
Median	4.44	3.93	4.22	3.51	4.93	5.37	5.74

SEM—standard error of the mean.

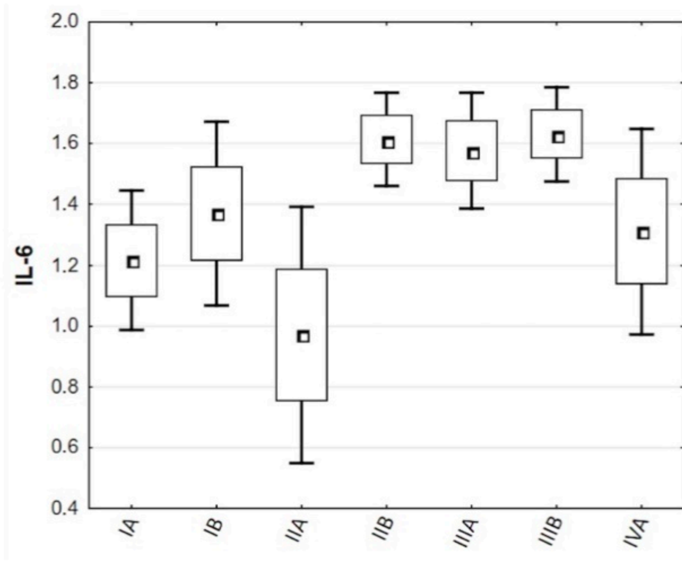


Figure 3. Distribution of IL-6 concentrations among lung cancer stages.

Table 3. Levels of the inflammatory markers according to lung cancer type, subtype, and stage.

	Non-Small Cell Lung Carcinoma			Non-NSCLC	Overall
	ADC	**SqCC**	**Other**		
IL-6 [pg/mL]					
Mean ± SEM	41.79 ± 5.39	42.04 ± 5.61	42.34 ± 12.13	33.65 ± 8.82	40.92 ± 3.43
Range	0.21–215.95	1.50–141.29	0.32–131.09	0.37–126.81	0.21–215.95
Median	34.74	38.45	31.43	25.63	34.34
TNF-α [pg/mL]					
Mean ± SEM	4.09 ± 0.82	15.47 ± 12.40	13.95 ± 8.55	27.97 ± 21.28	11.77 ± 4.79
Range	0.57–33.16	0.67–436.81	1.20–111.55	1.44–302.38	0.57–436.81
Median	2.13	2.33	2.63	2.83	2.37
SAA1 [μg/mL]					
Mean ± SEM	94.45 ± 17.74	119.66 ± 24.43	203.26 ± 61.77	82.01 ± 31.02	112.59 ± 13.70
Range	0.03–515.75	0.32–635.87	0.21–636.77	1.26–395.53	0.03–636.77
Median	12.16	83.17	208.23	32.62	45.63
CRP [mg/L]					
Mean ± SEM	21.65 ± 6.17	22.43 ± 5.28	29.54 ± 11.62	27.25 ± 12.40	23.39 ± 3.77
Range	0.22–217.67	0.40–109.66	0.60–114.20	0.60–153.93	0.22–217.67
Median	2.15	8.16	13.7	3.8	4.38
MMP-2 [ng/mL]					
Mean ± SEM	151.15 ± 5.08	142.97 ± 5.24	165.10 ± 19.10	152.64 ± 12.71	150.40 ± 3.88
Range	90.70–237.57	81.92–209.82	82.89–364.63	59.16–231.50	59.16–364.63
Median	144.09	141.97	158.76	144.39	143.95
MMP-9 [ng/mL]					
Mean ± SEM	1174.24 ± 97.77	1020.80 ± 110.68	916.97 ± 143.82	1190.65 ± 178.72	1098.48 ± 62.19
Range	73.03–2753.98	295.32–3327.25	104.94–1870.75	413.85–2371.49	73.03–3327.25
Median	1026.56	820.38	899.82	995.46	968.05

	IA	**IB**	**IIA**	**IIB**	**IIIA**	**IIIB**	**IVA**
IL-6 [pg/mL]							
Mean ± SEM	32.68 ± 8.91	40.47 ± 7.30	15.72 ± 6.29	54.42 ± 8.68	47.56 ± 8.86	50.37 ± 9.04	37.80 ± 9.36
Range	0.32–215.95	0.21–112.70	1.50–36.19	6.38–141.29	7.40–120.61	18.77–100.23	0.36–126.81
Median	21.1	39.37	8.52	44.14	47.36	37	28.86
TNF-α [pg/mL]							
Mean ± SEM	4.21 ± 1.00	5.31 ± 1.84	3.73 ± 1.02	24.85 ± 20.66	2.44 ± 0.37	17.18 ± 10.05	24.72 ± 21.37
Range	0.57–21.40	1.26–33.16	1.36–7.61	1.21–436.81	1.30–5.84	0.67–111.55	1.20–302.38
Median	2.1	2.68	2.95	2.33	2.18	2.2	2.79
SAA_1 [μg/mL]							
Mean ± SEM	90.66 ± 22.79	133.35 ± 37.96	131.89 ± 61.54	95.38 ± 33.32	95.07 ± 36.25	98.03 ± 42.02	199.94 ± 48.35
Range	0.03–386.11	0.08–636.77	1.32–336.87	0.50–635.87	0.59–309.17	5.79–395.53	3.25–548.31
Median	35.56	101.97	75.59	7.58	10.09	13.3	211.35
CRP [mg/L]							
Mean ± SEM	12.94 ± 5.44	21.36 ± 9.16	20.15 ± 11.38	17.11 ± 6.93	22.77 ± 9.02	33.84 ± 13.96	50.94 ± 16.92
Range	0.60–93.34	0.25–114.20	0.60–64.00	0.60–109.66	0.22–81.32	1.08–153.93	0.60–217.67
Median	2.44	2.4	4.55	2.84	2.8	21.02	24.52
MMP-2 [ng/mL]							
Mean ± SEM	148.52 ± 7.97	161.15 ± 14.29	131.11 ± 5.22	143.06 ± 6.32	142.62 ± 11.87	140.81 ± 12.38	162.71 ± 8.96
Range	81.92–237.57	101.88–364.63	113.46–145.31	90.70–209.82	94.93–224.22	59.16–200.94	118.06–227.51
Median	152.7	142.99	133.87	143.99	132.16	144.27	153.14
MMP-9 [ng/mL]							
Mean ± SEM	996.67 ± 148.87	1103.62 ± 143.04	960.91 ± 276.48	1178.16 ± 145.49	1191.19 ± 231.05	1074.92 ± 149.41	1055.56 ± 139.83
Range	73.03–2753.98	396.52–2594.84	508.99–2279.45	387.99–2712.13	285.38–3327.25	647.48–2367.12	463.48–2283.19
Median	734.26	1018.32	660.99	973.91	1033.8	899.82	995.19

SEM—standard error of the mean.

3.4. Metabolic Markers

Glucose concentrations showed statistically significant differences both between lung cancer subtypes and stages. When compared to patients with adenocarcinoma (me = 100.0 mg/dL), SqCC (me = 119.0 mg/dL), and other NSCLCs (me = 105.0 mg/dL), patients with other lung neoplasms had the highest glucose values (me = 130.5 mg/dL). Furthermore, individuals with stage IA lung cancer had significantly lower glucose levels (me = 101.0 mg/dL) than patients with stage IIB lung cancer (me = 132.0 mg/dL; $p = 0.0163$). There were also statistically significant differences in LDH levels between lung cancer stages. Patients in stage IVA had considerably higher LDH activity (me = 317.07 U/L) than patients in stage IIIA (me = 159.60 U/L). Table 4 collects descriptive statistics of metabolic markers in relation to lung cancer type, subtype, and stage, whereas Figure 4 depicts statistically significant differences in glucose concentrations and LDH activity in lung cancer patients.

Table 4. Levels of the metabolic markers according to lung cancer type, subtype, and stage.

	Non-Small Cell Lung Carcinoma			**Non-NSCLC**	**Overall**
	ADC	**SqCC**	**Other**		
Glucose [mg/dL]					
Mean ± SEM	111.42 ± 4.84	127.80 ± 6.48	109.38 ± 6.91	129.43 ± 7.94	118.55 ± 3.29
Range	75.00–251.00	70.00–268.00	83.00–180.00	82.00–184.00	70.00–268.00
Median	100	119	105	130.5	105.5
Lactate [mmol/L]					
Mean ± SEM	2.17 ± 0.16	2.25 ± 0.18	2.73 ± 0.75	2.44 ± 0.34	2.29 ± 0.13
Range	0.60–6.90	0.80–5.00	0.50–9.70	1.30–5.70	0.50–9.70
Median	2.05	2.2	1.8	1.95	2
LDH [U/L]					
Mean ± SEM	288.24 ± 31.26	280.66 ± 36.06	311.43 ± 47.53	316.08 ± 51.09	292.04 ± 19.82
Range	97.80–1217.00	111.86–1217.50	113.28–777.48	131.40–824.56	97.80–1217.50
Median	213.06	205.57	261.55	250.08	218.67

	IA	**IB**	**IIA**	**IIB**	**IIIA**	**IIIB**	**IVA**
Glucose [mg/dL]							
Mean ± SEM	105.08 ± 4.36	116.06 ± 7.10	105.17 ± 8.06	138.76 ± 10.83	126.50 ± 11.21	102.64 ± 6.46	119.50 ± 7.65
Range	70.00–163.00	90.00–197.00	83.00–127.00	89.00–268.00	91.00–209.00	82.00–145.00	89.00–180.00
Median	101	103.5	104	132	105.5	94	110
Lactate [mmol/L]							
Mean ± SEM	2.36 ± 0.36	2.38 ± 0.18	2.92 ± 0.53	2.08 ± 0.26	1.79 ± 0.29	1.82 ± 0.31	3.04 ± 0.50
Range	0.80–9.70	1.20–3.70	1.80–5.00	1.00–5.50	0.60–4.00	0.50–4.10	1.10–6.90
Median	1.9	2.3	2.25	1.6	1.4	1.8	2.7
LDH [U/L]							
Mean ± SEM	274.49 ± 41.33	226.19 ± 28.61	288.31 ± 56.60	351.66 ± 55.88	187.11 ± 23.35	332.96 ± 74.99	401.87 ± 71.10
Range	136.25–1217.00	113.28–558.59	144.61–513.24	116.77–1217.50	97.80–360.97	111.86–824.56	174.03–1149.40
Median	223.92	188.43	266.85	277.85	159.6	205.57	317.07

SEM—standard error of the mean.

3.5. Correlation

The pairwise correlation revealed a significant positive correlation between IL-6 and MMP-9 in adenocarcinoma and SqCC, with correlation coefficients of 0.53 and 0.49, respectively. There was also a significant positive correlation between acute-phase protein concentrations, SAA_1, and CRP in patients with all types of lung neoplasm except adenocarcinoma, with correlation coefficients of 0.44 in SqCC, 0.72 in other NSCLC, and 0.80 in other lung neoplasms. Table 5 collects pairwise relationships, which are also graphically displayed as heatmaps in Figure 5.

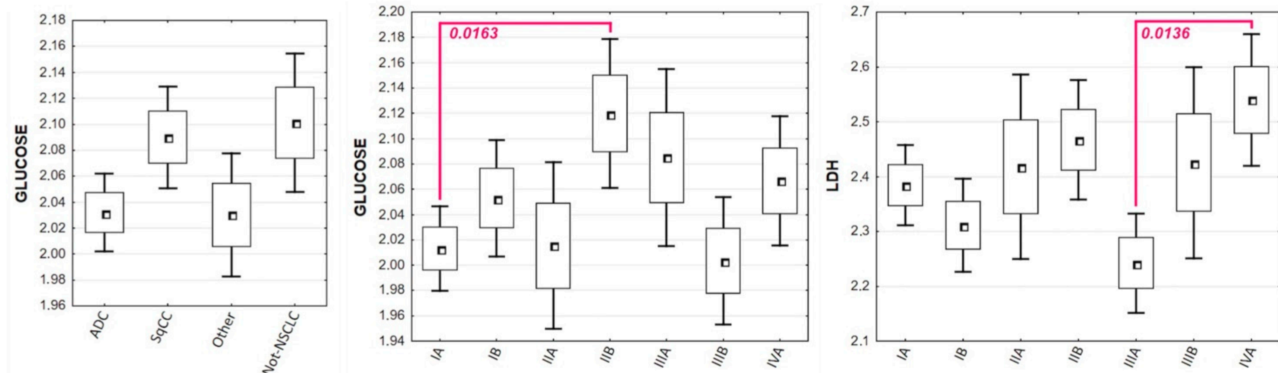


Figure 4. Distribution of glucose levels in lung cancer subtypes and stages, with a statistically significant difference between patients with stage IA and stage IIB lung cancer ($p = 0.0163$), and the activity of LDH between lung cancer stages, with a statistically significant difference between patients with stage IIIA and stage IVA lung cancer ($p = 0.0136$).

Table 5. The pairwise correlation of tumor, inflammatory, and metabolic marker combinations among the lung cancer groups studied.

Marker's Combination	Non-Small Cell Lung Carcinoma			Non-NSCLC
	ADC	SqCC	Other	
CEA + CYFRA 21-1	NS	$p = 0.048$; r = 0.36	NS	NS
CEA + LAC	$p = 0.024$; r = −0.39	NS	NS	NS
CYFRA 21-1 + IL-6	$p = 0.014$; r = 0.43	$p = 0.012$; r = 0.44	NS	NS
CYFRA 21-1 + CRP	NS	$p = 0.023$; r = 0.41	NS	NS
NSE + SAA_1	NS	$p = 0.034$; r = −0.38	NS	NS
NSE + GLU	NS	NS	$p = 0.007$; r = 0.82	NS
IL-6 + SAA_1	$p = 0.002$; r = −0.53	NS	NS	NS
IL-6 + MMP-2	$p = 0.010$; r = −0.44	NS	NS	NS
IL-6 + MMP-9	$p = 0.002$; r = 0.53	$p = 0.006$; r = 0.49	NS	NS
IL-6 + GLU	NS	$p = 0.041$; r = 0.37	NS	NS
SAA_1 + CRP	NS	$p = 0.014$; r = 0.44	$p = 0.028$; r = 0.72	$p = 0.003$; r = 0.80
SAA_1 + GLU	NS	NS	NS	$p = 0.019$; r = −0.69
SAA_1 + LAC	$p = 0.011$; r = 0.44	$p = 0.045$; r = 0.36	NS	NS
CRP + GLU	NS	NS	NS	$p = 0.004$; r = −0.79
MMP-2 + MMP-9	$p = 0.000$; r = −0.61	NS	NS	NS
MMP-2 + LDH	NS	NS	NS	$p = 0.012$; r = −0.72
GLU + LAC	$p = 0.045$; r = 0.35	NS	NS	NS

NS—not significant.

3.6. Diagnostic Value of Biomarkers for Adenocarcinoma and Squamous Cell Carcinoma

Prior to examining the diagnostic value of individual and combined biomarkers, logistic regression analysis was used to analyze and exclude the potential effects of age, gender, and smoking status on biomarker concentrations. The only statistically significant difference between patients with adenocarcinoma and SqCC was in the levels of glucose. There were no differences in the statistical significance of the remaining biomarkers between adenocarcinoma and SqCC patients, both unadjusted and adjusted for age, gender, and smoking status. These results suggest that differences in the levels of these biomarkers in our case are not due to confounding variables such as age and gender. All calculations are collected and presented in the Table 6.

Subsequently, ROC analysis with the calculation of AUC was performed to examine the ability of each of the 12 biomarkers to distinguish NSCLC patients with adenocarcinoma from patients with SqCC (results are collected in Table 7), and patients with lung cancer stages IIB and IIA (Table 8), and IIIA and IIB (Table 9). Figure 6 graphically presents the calculations of biomarkers diagnostic value in differential diagnoses.

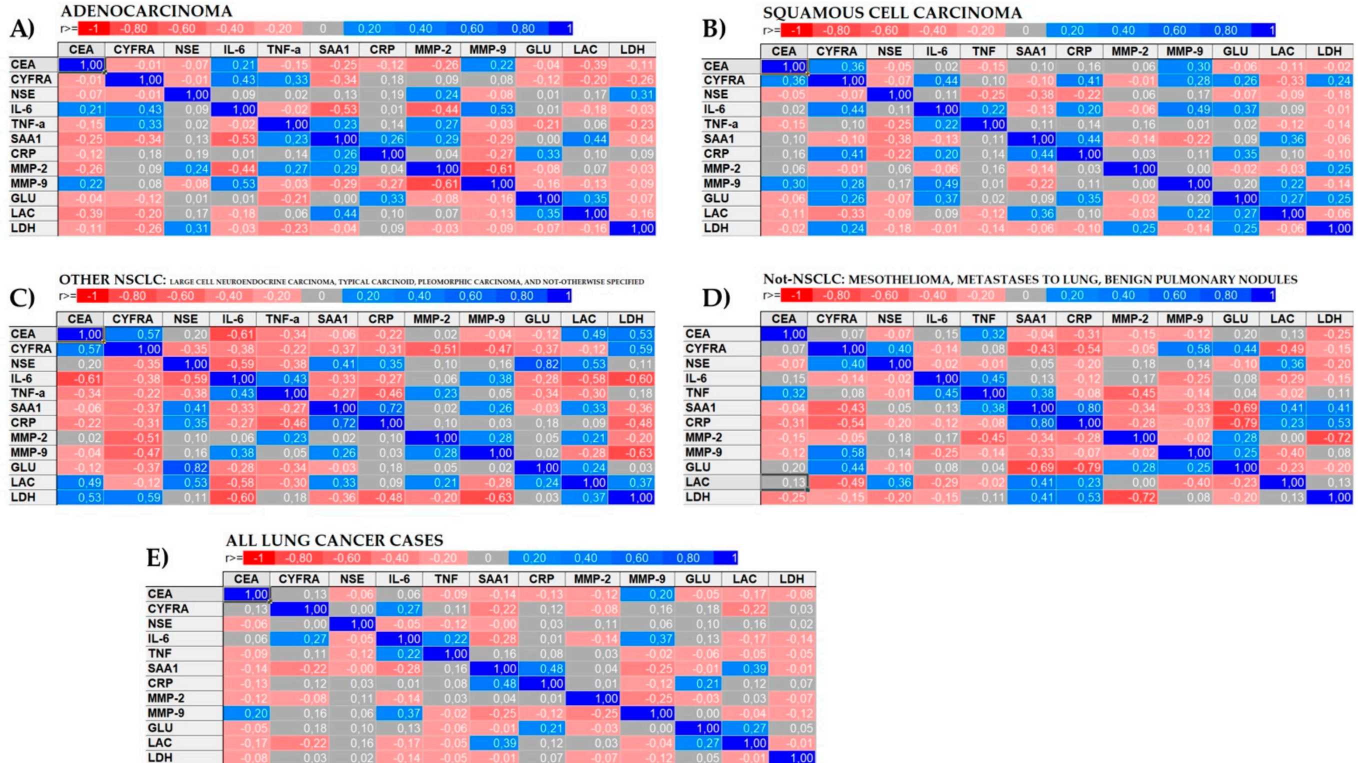


Figure 5. Heatmaps of the pairwise correlations between tumor, inflammatory, and metabolic marker concentrations in patients with (**A**) adenocarcinoma, (**B**) squamous cell carcinoma, (**C**) non-small cell lung carcinomas other than adenocarcinoma and squamous cell carcinoma, (**D**) lung neoplasms other than non-small cell lung carcinoma, and (**E**) in the entire research group without division into subgroups based on histopathological diagnosis. Correlation coefficients receive a range between −1 (red) and 1 (blue), showing a negative and positive relationship between biomarkers, respectively.

Table 6. Logistic regression analyses on the influence of confounding variables on biomarkers in distinguishing adenocarcinoma from squamous cell carcinoma patients.

	Unadjusted			Adjusted by Age, Gender, and Smoking Status		
Biomarker	**OR**	**95% CI**	***p*-Value**	**OR**	**95% CI**	***p*-Value**
CEA	1.28	−0.75–3.20	0.22	1.75	−0.37–6.08	0.08
CYFRA 21-1	2.78	−2.47–0.41	0.16	1.93	−1.61–1.62	0.99
NSE	∞	−1.08–1.60	0.70	1.56	−1.64–1.69	0.98
IL-6	∞	−1.04–0.77	0.77	1.75	−1.13–0.86	0.79
TNF-α	∞	−1.32–0.85	0.68	2.25	−1.56–0.93	0.62
SAA_1	0.71	−0.73–0.14	0.18	0.99	−0.83–0.15	0.17
CRP	1.33	−1.04–0.09	0.10	2.55	−0.78–0.60	0.80
MMP-2	1.46	−2.11–6.77	0.30	2.02	−0.65–10.84	0.08
MMP-9	0.00	−0.95–2.22	0.43	1.75	−0.97–3.08	0.31
GLU	2.82	−8.61–−0.52	0.03	5.18	−8.93–0.18	0.06
LAC	∞	−2.45–1.71	0.72	1.72	−3.47–1.52	0.44
LDH	∞	−1.84–1.91	0.97	2.57	−1.65–2.44	0.70

CI—confidence interval; NA—not available; OR—odds ratio.

Table 7. The diagnostic efficiency of single biomarkers to distinguish NSCLC patients with adenocarcinoma and squamous cell carcinoma.

Biomarker	*p*-Value	ADC vs. SqCC			SqCC vs. ADC		
		Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC
CEA	0.3131	48.7%	68.8%	0.569	71.9%	33.3%	0.431
CYFRA 21-1	0.1256	91.4%	12.9%	0.391	45.2%	85.7%	0.609
NSE	0.7077	34.0%	77.1%	0.524	8.6%	98.0%	0.476
IL-6	0.7968	92.0%	14.3%	0.483	68.6%	46.0%	0.517
TNF-α	0.6799	10.0%	97.1%	0.474	60.0%	50.0%	0.526
SAA1	0.1364	6.0%	97.1%	0.407	88.6%	34.0%	0.593
CRP	0.0762	4.2%	100.0%	0.388	64.7%	68.8%	0.612
MMP-2	0.3604	34.0%	80.0%	0.558	14.3%	88.0%	0.442
MMP-9	0.2822	30.0%	85.7%	0.568	88.6%	16.0%	0.432
GLU	0.0048	100.0%	2.9%	0.326	74.3%	70.0%	0.674
LAC	0.6378	82.0%	25.7%	0.469	42.9%	70.0%	0.531
LDH	0.9281	28.0%	85.7%	0.494	94.3%	20.0%	0.506

AUC—area under the curve.

Table 8. The diagnostic efficiency of single biomarkers to distinguish patients with stage IIB and IIA lung cancer.

Biomarker	*p*-Value	IIA vs. IIB			IIB vs. IIA		
		Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC
CEA	0.4412	83.3%	61.1%	0.606	27.8%	83.3%	0.394
CYFRA 21-1	0.4163	75.0%	47.1%	0.382	47.1%	100.0%	0.618
NSE	0.8207	50.0%	76.2%	0.536	100.0%	16.7%	0.464
IL-6	0.0000	∞	∞	0.151	57.1%	100.0%	0.849
TNF-α	0.6987	50.0%	81.0%	0.556	66.7%	50.0%	0.444
SAA1	0.3079	83.3%	52.4%	0.635	4.8%	100.0%	0.365
CRP	0.7968	33.3%	84.2%	0.461	73.7%	50.0%	0.539
MMP-2	0.1448	100.0%	14.3%	0.349	47.6%	100.0%	0.651
MMP-9	0.3411	100.0%	14.3%	0.373	85.7%	16.7%	0.627
GLU	0.0192	∞	∞	0.246	52.4%	100.0%	0.754
LAC	0.0223	100.0%	52.4%	0.726	4.8%	100.0%	0.274
LDH	0.6877	66.7%	42.9%	0.448	19.0%	100.0%	0.552

AUC—area under the curve.

Table 9. The diagnostic efficiency of single biomarkers to distinguish patients with stage IIIA and IIB lung cancer.

Biomarker	*p*-Value	IIB vs. IIIA			IIIA vs. IIB		
		Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC
CEA	0.0760	88.9%	18.2%	0.308	81.8%	61.1%	0.692
CYFRA 21-1	0.3837	41.2%	100.0%	0.596	63.6%	52.9%	0.404
NSE	0.6977	100.0%	16.7%	0.456	50.0%	81.0%	0.544
IL-6	0.8809	23.8%	91.7%	0.516	50.0%	66.7%	0.484
TNF-α	0.5366	47.6%	83.3%	0.563	75.0%	38.1%	0.437
SAA1	0.8276	81.0%	41.7%	0.518	25.0%	90.5%	0.482
CRP	0.9395	94.7%	25.0%	0.509	33.3%	89.5%	0.491
MMP-2	0.7835	57.1%	66.7%	0.532	33.3%	90.5%	0.468

Table 9. *Cont.*

Biomarker	*p*-Value	IIB vs. IIIA			IIIA vs. IIB		
		Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC
MMP-9	0.9118	19.0%	91.7%	0.512	8.3%	100.0%	0.488
GLU	0.4663	71.4%	50.0%	0.577	33.3%	76.2%	0.423
LAC	0,6246	100.0%	25.0%	0.554	66.7%	42.9%	0.446
LDH	0.0036	81.0%	83.3%	0.762	91.7%	9.5%	0.238

AUC—area under the curve.

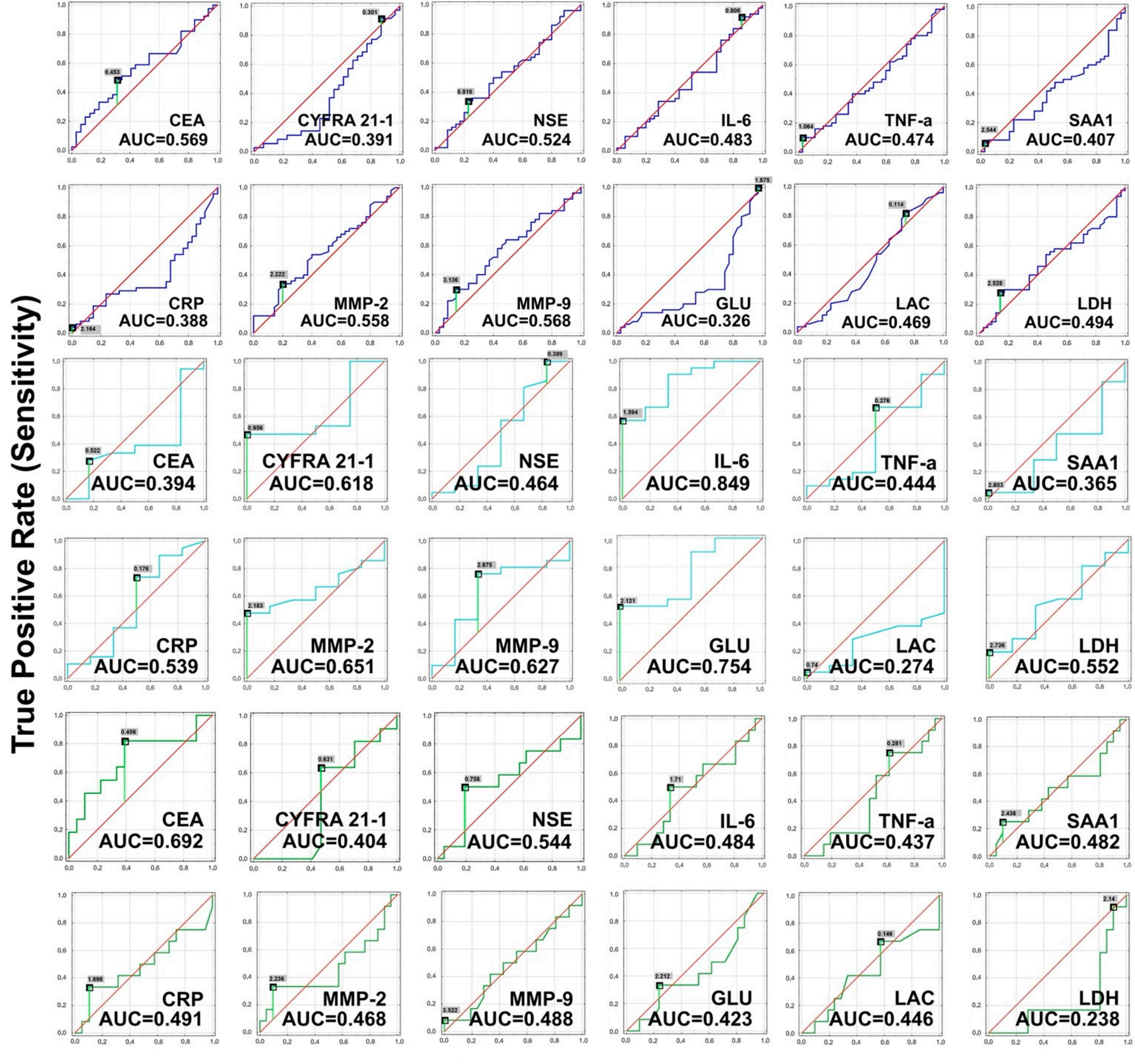


Figure 6. Receiver operating characteristic (ROC) curves evaluating the ability of single biomarkers to distinguish NSCLC patients with adenocarcinoma and squamous cell carcinoma (navy line), lung cancer stage IIB and IIA (teal line), and IIIA and IIB (green line).

CEA with AUC = 0.569, 48.7% sensitivity, and 68.8% specificity has the highest diagnostic values as single biomarker for distinguishing adenocarcinoma from SqCC patients. Glucose with AUC = 0.674, CRP with AUC = 0.612, and CYFRA 21-1 with AUC = 0.609 can identify SqCC from adenocarcinoma patients.

Lactate is the best single biomarker in the differential diagnosis of patients with stage IIA and IIB lung cancer, with AUC = 0.726, 100.0% sensitivity, and 52.4% specificity, whereas IL-6 with AUC = 0.849 may successfully distinguish patients with stage IIB from patients with stage IIA lung cancer.

LDH, on the other hand, is the best single biomarker in the differential diagnosis of stage IIB and IIIA lung cancer patients, with AUC = 0.762, 81.0% sensitivity, and 83.3% specificity. CEA (AUC = 0.692, 81.8% sensitivity, and 61.1% specificity) is the biomarker with the highest diagnostic value for distinguishing lung cancer stages IIIA and IIB.

The modeling of the combinations of markers into the panel of markers was the next step in the search for the best diagnostic tool for differential diagnosis of lung adenocarcinoma and SqCC, and lung cancer stages IIB and IIA, IIIA and IIB. For this aim, all biomarkers were mixed together, yielding thousands of combinations via GLM with the Akaike criterion included. The best model has the lowest AIC value and must fulfill two requirements: (1) the panel of biomarkers should be well-matched to the data, and (2) it should be as simple as feasible. This provided the best models, which comprised three or four biomarkers.

Tables 10–12 compile constructed models of biomarkers with the best AUC values for discriminating lung adenocarcinoma from SqCC (Table 10), stage IIB from IIA (Table 11), and stage IIIA from IIB (Table 12). Figure 7 uses ROC curves to graphically visualize these models.

Table 10. The diagnostic efficiency of multi-biomarker models to distinguish NSCLC patients with adenocarcinoma from patients with squamous cell carcinoma.

Marker Combinations	AIC	*p*-Value	OR	log OR	Sensitivity	Specificity	AUC
CYFRA 21-1 + NSE + SAA_1	82.96	0.0088	6.56	1.88	75.76%	67.74%	0.7565
CYFRA 21-1 + NSE + SAA_1 + GLU	83.59	0.0046	4.83	1.57	69.70%	67.74%	0.7693
CEA + CYFRA 21-1 + SAA_1 + GLU	84.25	0.0061	3.18	1.16	69.70%	58.06%	0.7517
CEA + CYFRA 21-1 + NSE + SAA_1	84.55	0.0069	7.64	2.03	75.76%	70.97%	0.7605
CEA + CYFRA 21-1 + SAA_1	85.09	0.0090	2.77	1.02	66.67%	58.06%	0.7243

AIC—Akaike information criterion; AUC—area under the curve; OR—odds ratio.

Table 11. The diagnostic efficiency of multi-biomarker models to distinguish stage IIB NSCLC patients from stage IIA NSCLC patients.

Marker Combinations	AIC	*p*-Value	OR	log OR	Sensitivity	Specificity	AUC
IL-6 + GLU + LDH	8.23	0.0000	∞	∞	100.00%	100.00%	0.8333
CEA + IL-6 + SAA_1 + MMP-9 + LAC	12.00	0.0001	∞	∞	100.00%	100.00%	1.0000
GLU + LAC	21.95	0.0018	40.00	3.69	95.24%	66.67%	0.9365
IL-6 + GLU	22.14	0.0033	17.00	2.83	89.47%	66.67%	0.8947
CEA + SAA_1 + LAC	26.90	0.0442	17.00	2.83	94.44%	50.00%	0.8425

AIC—Akaike information criterion; AUC—area under the curve; OR—odds ratio.

Table 12. The diagnostic efficiency of multi-biomarker models to distinguish stage IIIA NSCLC patients from stage IIB NSCLC patients.

Marker Combinations	AIC	*p*-Value	OR	log OR	Sensitivity	Specificity	AUC
TNF-α + LAC + LDH	31.75	0.0032	9.00	2.20	54.55%	88.24%	0.8609
CEA + IL-6 + LDH	34.14	0.0063	45.33	3.81	72.73%	94.44%	0.8686
CEA + LDH	34.49	0.0067	9.60	2.26	54.55%	88.89%	0.7929
CYFRA 21-1 + NSE + LAC + LDH	36.27	0.0057	9.00	2.20	54.54%	88.24%	0.8449
CEA + CYFRA 21-1 + NSE + SAA_1	37.37	0.0380	8.17	2.10	63.64%	82.35%	0.8288

AIC—Akaike information criterion; AUC—area under the curve; OR—odds ratio.

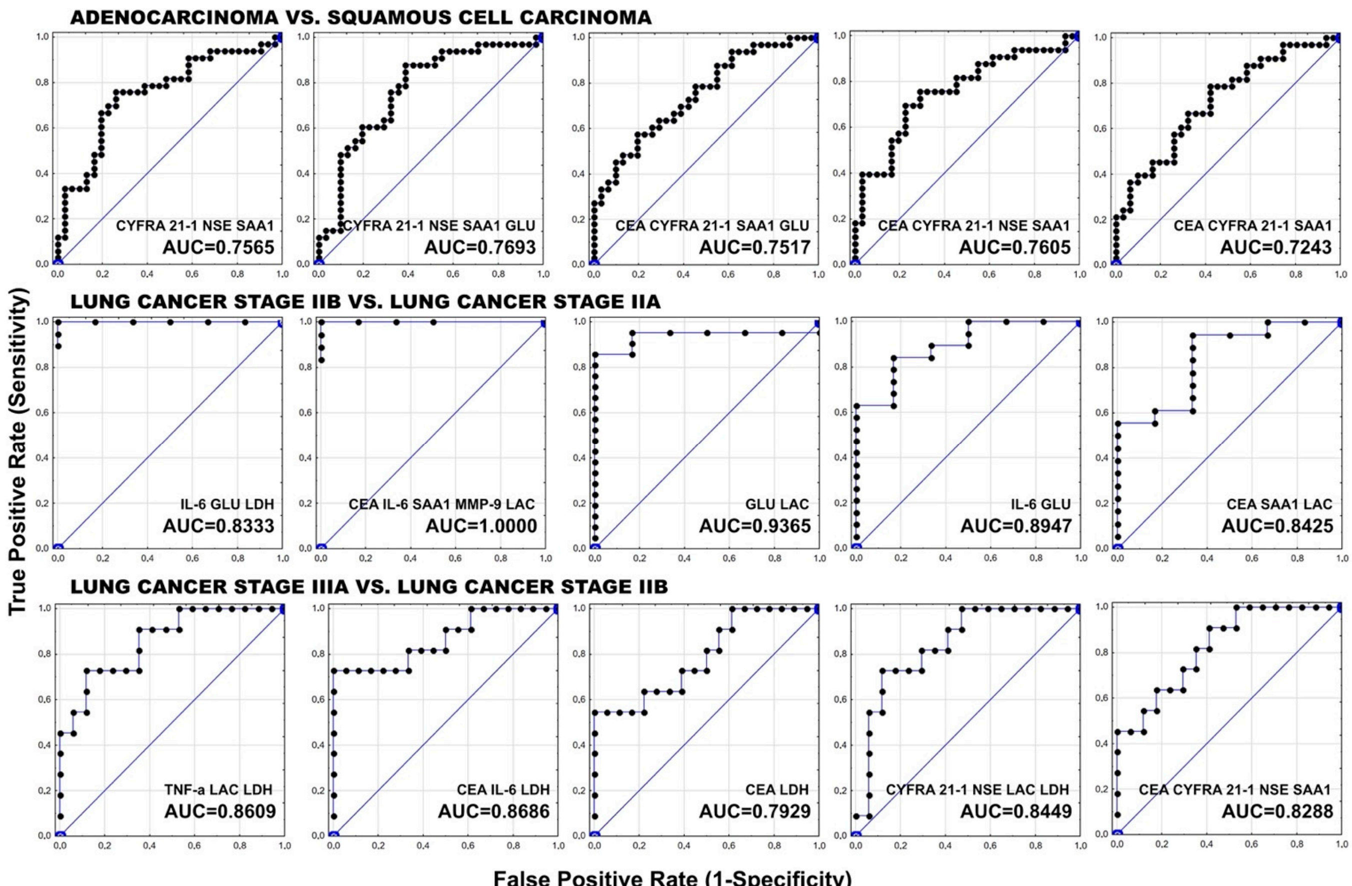


Figure 7. Receiver operating characteristic (ROC) curves evaluating the ability of multi-biomarker models to distinguish NSCLC patients with adenocarcinoma and squamous cell carcinoma, lung cancer stage IIB and IIA, and IIIA and IIB.

When distinguishing lung adenocarcinoma and SqCC, the combination of CEA, CYFRA 21-1, NSE, and SAA_1 has the highest sensitivity of 75.76%, a specificity of 70.97%, and an AUC value of 0.7605. The combination of these biomarkers with the exclusion of CEA results in a somewhat lower AUC value (0.7565) with the same sensitivity (75.76%) but slightly lower specificity (67.74%). The inclusion of glucose to this panel enhances the AUC value from 0.7565 to 0.7693 while lowering the sensitivity from 75.76% to 69.70% and maintaining the specificity at the same level (67.74%). Even though the model containing CYFRA 21-1, NSE, SAA_1, and glucose has the highest AUC, the combination of CYFRA 21-1, NSE, and SAA_1 has the lowest AIC.

The combinations of IL-6, glucose, and LDH, as well as CEA, IL-6, SAA_1, MMP-9, and lactate, have 100% sensitivity and 100% specificity in distinguishing patients with stage IIB lung cancer from those with stage IIA, with the combination of IL-6, glucose, and LDH having a lower AUC value (0.8333 in comparison to 1.0000). The simple combination of glucose and lactate is also a useful diagnostic tool for distinguishing between lung cancer stages IIB and IIA, and is characterized by 95.24% sensitivity, 66.67% specificity, and an AUC value of 0.9365.

When distinguishing stage IIIA from IIB of lung cancer, the combination of CEA, IL-6, and LDH has the highest sensitivity of 72.73%, a specificity of 94.44%, and an AUC value of 0.8686, whereas a model with the lowest AIC value consists of TNF-α, lactate, and LDH, characterized by lower sensitivity (54.55%) and slightly lower specificity (88.24%) and AUC (0.8609).

4. Discussion

Lung cancer is a major problem in modern medicine. Despite numerous studies, our understanding of lung carcinogenesis and tumor progression remains unclear. Lung cancer is a multifactorial disease with a group of heterogeneous tumors, which complicates diagnostic and therapeutic approaches, as well as comprehension of the processes that underlie its pathogenesis. Current research efforts are specifically interested in the examination of changes in the TME at the molecular level [1,3,14]. In this retrospective study, we focused on nine biomarkers related to the processes occurring in the tumor microenvironment. We examined serum levels of pro- and anti-inflammatory cytokines (IL-6, TNF-α), which regulate immune responses, cell proliferation, and differentiation; matrix metalloproteinases (MMP-2, MMP-9), which remodel and degrade ECM and mediate cell–cell adhesion; acute-phase proteins (CRP, SAA_1), which are linked to the expression of pro- and anti-inflammatory cytokines and play a role, among others, in the recruitment of immune cells to inflammatory sites; and metabolic markers (glucose, lactate, and LDH), which are associated with glucose metabolism in cancer cells, known as the Warburg effect. It is worth noting that our study group is representative and reflects the features of lung cancer patients as a whole: (1) NSCLC was the most often diagnosed type of lung cancer, (2) with an adenocarcinoma subtype predominating over SqCC, (3) women were mostly diagnosed with adenocarcinoma rather than SqCC, (4) and patients have been diagnosed in their sixth decade of life [8,9,20,21,42].

Chen et al. [43], in their work, referred to the "seed and soil" hypothesis by Stephen Paget (1889) in relation to the tumor microenvironment. The presence of tumor cells affects processes and changes in the TME, which in turn can affect tumor progression, invasion, and metastasis. Furthermore, changes in the TME might cause a systemic response of the host, manifested, for example, by the development of chronic inflammation, as seen by increased levels of acute-phase proteins, CRP, and SAA_1. In another study, Mansuet-Lupo et al. [15] discovered that neutrophil and macrophage densities are related to oncogenic driver genes, varying among lung cancer subtypes. They discovered a high density of neutrophils and macrophages in tumors with wild-type epidermal growth factor receptor (EGFR) and a high density of neutrophils in tumors with b-raf proto-oncogene (BRAF) mutation, which affects the ability of tumor cell mutational status to change the pattern of cytokines and chemokines released by tumor cells and, thus, influences the TME's chemoattractant properties. As a result, differences in the mutational status of lung cancer subtypes were expected to cause changes in the levels of cytokines IL-6 and TNF-α.

As normal tissue turns cancerous, matrix metalloproteinases are activated, and pro- and anti-inflammatory cytokines are excreted, resulting in extracellular matrix degradation and regulation of pathways activating inflammatory processes as well as pathways regulating proliferation, apoptosis, survival, and invasion. One example is IL-6's involvement in the activation of the Janus kinases-signal transducer and activator of transcription proteins (JAK-STAT), mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin (PI3K/AKT/mTOR) pathways [1]. Increasing cytokine and matrix metalloproteinase levels in the TME promote carcinogenesis and malignant progression, promoting subsequent microenvironmental changes. These markers are released into the blood at some level, allowing them to be measured in serum samples from patients. Although, no statistically significant changes in IL-6 and TNF-α levels are found between any group of lung cancer patients (adenocarcinoma, SqCC, other NSCLC, and other neoplasms than NSCLC), there are slightly higher levels of IL-6 in SqCC patients. Although IL-6 and TNF-α concentrations were not significantly different between our groups, their involvement in tumor growth should not be overlooked. Their function is shown by increased levels of acute-phase proteins and metabolic markers, which may be successfully measured in patients' sera.

Particularly intriguing is the correlation between IL-6 and MMP-9 in patients with adenocarcinoma and SqCC, with correlation coefficients of 0.53, 0.49, respectively, which undoubtedly influences TME processes. Nie et al. [2] found that IL-6 serum concentrations

of ≥4 pg/mL are associated with significantly poorer survival in both Americans and Caucasians with lung cancer. According to studies, IL-6 levels are higher in NSCLC patients compared to healthy controls, as well as in patients with metastatic NSCLC compared to undisseminated disease. Moreover, if IL-6 concentrations of ≥4 pg/mL are associated with poorer survival in lung cancer patients, and median values of this cytokine in patients with adenocarcinoma, SqCC, other NSCLC, and other lung neoplasms than NSCLC are, respectively, 34.74 pg/mL, 38.45 pg/mL, 31.43 pg/mL, and 25.63 pg/mL, we can conclude that IL-6 concentrations in our study group are elevated, and our patients are burdened with poorer outcomes. MMP-9, similar to IL-6, is produced by various tumor cells and inflammatory cells—neutrophils, eosinophils, monocytes, lymphocytes, and alveolar macrophages [44]. Animal studies showed that MMP-9 overexpression contributes to cancer development and progression. For example, cancer cells were less capable of colonizing the lung of MMP-9-deficient mice than the lung of wild-type mice, and MMP-9 null mice develop fewer cancers than wild-type mice [45]. Studies documented that MMP-9 was also involved in other steps of cancer development, including decreasing cancer cell apoptotic potential, promoting angiogenesis, and regulating immune responses to cancer, by altering cellular signals and regulating cytokines, growth factors, and angiogenic factors via complex cell–cell and cell–matrix interactions in the microenvironment [45–47]. There are reports of significant positive correlation between matrix metalloproteinases expression and metastatic capacities of cancer cells. Elevated IL-6 levels above the reference threshold and a correlation between this cytokine and the metastatic biomarker MMP-9 indicate a higher risk of lung cancer aggressiveness. The correlation between that cytokine and enzyme is an intriguing point in the ongoing exploration of their role in lung cancer, but this time together rather than separately.

Our research on the correlation between biomarkers in accordance with lung cancer type and subtype confirms the concept of tumor microenvironment that varies between types and subtypes. Moreover, evaluation of specific inflammatory and metabolic markers involved in TME alterations is the starting step for considering their diagnostic usefulness. The correlation of IL-6 and MMP-9, as well as other correlations in adenocarcinoma and SqCC and differences in the biomarker concentrations among groups, allowed us to observe subtype characteristics that might possibly be used to differentiate these two most common subtypes of lung cancer.

We looked at the relationship between well-known tumor markers and pro- and anti-inflammatory cytokines, matrix metalloproteinases, acute-phase proteins, and the Warburg effects' markers, as well as their ability to distinguish adenocarcinoma from SqCC. Before modeling a biomarker panel to identify adenocarcinoma from SqCC, we evaluated each subtype's patients' age, gender, smoking status, and lung cancer stage as potential confounding factors that could interact with and influence biomarker usage. The best markers for differentiating adenocarcinoma from SqCC are glucose (AUC = 0.674), CRP (AUC = 0.612), and CYFRA 21-1 (AUC = 0.609), and there are no significant variations in their utility when corrected for age and gender.

All of the single biomarkers exhibit poor diagnostic sensitivity, specificity, and AUC in distinguishing adenocarcinoma from SqCC. We investigated a number of patterns and biomarker combinations that might efficiently, with the best sensitivity and specificity, diagnose adenocarcinoma and SqCC patients. However, none of the panels have an AUC greater than 0.80. The combination of CEA, CYFRA 21-1, NSE, and SAA_1 has the highest diagnostic value among all available combinations (75.76% sensitivity, 70.97% specificity, and an AUC = 0.7605). In this panel, higher concentrations of CEA and NSE in adenocarcinoma and CYFRA 21-1 in SqCC are seen, which have histological explanations and overlap with the general picture of these lung cancer subtypes. The inclusion of SAA1 in a panel of markers distinguishing adenocarcinoma from SqCC patients is explained not only by histological, cellular, and molecular factors, but also by tumor localization in the lungs. Adenocarcinoma usually originates peripherally in the lungs, as opposed to SqCC, which grows centrally in the lung, where inflammation and irritation, particularly from

cigarette smoke, may exert different biological characteristics than in the peripheral part of the lung. It causes differences in the levels of inflammatory markers between adenocarcinoma and SqCC [48,49]. Increased IL-6 and TNF-α concentrations in the TME result in the overproduction of acute-phase proteins, CRP, and SAA1. Despite the confirmation of these biomarkers' involvement in lung cancer pathogenesis, we cannot consider the combination of CEA, CYFRA 21-1, NSE, and SAA1 as a panel that would successfully identify lung cancer subtypes and hence be included in the clinical diagnostic scheme of lung cancer patients. Combining biomarkers slightly improved the diagnostic characteristics of models distinguishing adenocarcinoma from SqCC, but the results are still unsatisfactory.

Knowing that changes in the TME, that is, remodeling of the extracellular matrix (ECM), pro- and anti-inflammatory processes, and cell metabolism, are affected by neoplastic progression, we conducted to examine the diagnostic potential of the studied biomarkers in relation to lung cancer stage. A peak in IL-6 concentrations in patients with stage IIB, IIIA, and IIIB lung cancer, with values of 44.14 pg/mL, 47.36 pg/mL, and 37.00 pg/mL, respectively, was one of the most notable changes in biomarker concentrations in relation to lung cancer stage. We also observed a characteristic increase in metabolic processes in advanced lung cancer stages, as evidenced by greater lactate concentrations and LDH activity in stages III and IV, as well as lower glucose levels due to increased glucose uptake. These alterations suggested that it could be useful in the differential diagnosis of certain lung cancer stages.

We determined that constructing a simple, easy, and sufficient tool capable of successfully differentiating patients with stage IIB and IIA lung cancer, as well as stage IIIA and IIB, would be clinically significant. Our choices are motivated by the first appearance of metastases (to lymph nodes) at stage IIB, as well as differences in treatment options between stages II and III, owing to the higher heterogeneity of stage III and the lack of a single therapy recommendation for all patients [50,51].

We found two combinations that can distinguish lung cancer patients with stage IIB from those with IIA with 100% sensitivity and 100% specificity, wherein IL-6, glucose, and LDH has an AUC = 0.8333 and the combination of CEA, IL-6, SAA1, MMP-9, and lactate an AUC = 1.0000. The combination of CEA, IL-6, and LDH, on the other hand, may distinguish patients with stage IIIA and stage IIB lung cancer with high sensitivity and diagnostic specificity. Increased levels of pro- and anti-inflammatory markers, as well as higher production of matrix metalloproteinases, are correlated with metastatic capacities of cancer cells, as seen by higher levels of these markers in more advanced stages of lung cancer.

In our study, we focused on the correlation of biomarkers involved in TME changes caused by ECM remodeling, pro- and anti-inflammatory processes, and cell metabolism, and we discovered some interrelationships that may influence lung cancer pathogenesis and malignant progression in relation to lung cancer subtype and stage. We also found a link between cytokines, matrix metalloproteinases, acute-phase proteins, Warburg effect markers, and tumor markers that have been proved to be clinically relevant in the detection of lung cancer. As a consequence, we discovered a few panels of markers that could separate lung adenocarcinoma from SqCC, as well as a few panels that could successfully differentiate patients with stages IIB and IIA as well as IIIA and IIB lung cancer.

We should consider the role of CEA, IL-6, SAA_1, MMP-9, LAC, and LDH in the perspective of having readily accessible and inexpensive diagnostic tools that can be employed effectively in the differential diagnosis of lung cancer stages IIB and IIA, and IIIA and IIB, and improve therapeutic decision making. An undeniable advantage of such panels as the combinations of CEA, IL-6, SAA_1, MMP-9, and lactate, IL-6, glucose, and LDH, or CEA, IL-6, and LDH is the ability of its detection in patients' blood and use in a large group of NSCLC patients whose tumor tissue is not easily accessible, particularly in peripherally situated adenocarcinomas. Our panels of markers could be integrated into the scheme of lung cancer diagnostic procedures as a low-cost, simple, and practical diagnostic tools. In the future, performing multidimensional assessment of known markers may improve diagnostic and prognostic algorithms.

Author Contributions: Conceptualization, M.Ś.-M., P.B. and A.R.; methodology, K.W., M.Ś.-M. and P.B.; data analysis, K.W.; investigation, K.W. and M.Ś.-M.; resources, K.W.; data curation, P.B. and A.R.; writing—original draft preparation, K.W.; writing—review and editing, M.Ś.-M. and K.W.; visualization, K.W.; supervision, M.Ś.-M.; project administration, I.B.-L. and M.Ś.-M.; funding acquisition, M.Ś.-M. and I.B.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the Ministry of Health subvention according to number of STM.D010.20.129 from the IT Simple system of Wroclaw Medical University.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee at Wroclaw Medical University (protocol code 106/2020 and date of approval 25 February 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: Authors declare no conflict of interest.

Abbreviations

AD	adenocarcinoma
AIC	Akaike information criterion
AJCC	American Joint Committee on Cancer
ALK	anaplastic lymphoma kinase
AMMS	Asseco Medical Management Solutions
ANOVA	analysis of variance
AUC	area under the curve
BRAF	b-raf proto-oncogene
CEA	carcinoembryonic antigen
CI	confidential interval
CRP	C-reactive protein
CYFRA 21-1	cytokeratin 19
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
GLM	generalized linear model
GLU	glucose
GLUT	glucose transporter
HRP	horseradish peroxidase
HSD	honestly significant difference (Tukey's honestly significant difference test)
IL-6	interleukin-6
JAK-STAT	Janus kinases-signal transducer and activator of transcription proteins
LAC	lactate
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
MCT	monocarboxylate transporter
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
NA	not available
NCCN	National Comprehensive Cancer Network
NK	natural killer
NN	not NSCLC/not non-small cell lung carcinoma
NS	not significant
NSCLC	non-small cell lung carcinoma
NSE	neuron-specific enolase

OR	odds ratio
Ot	other NSCLC/other non-small cell lung carcinoma
PI3K/AKT/mTOR	phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin
ROC	receiver operating characteristic
SAA_1	serum amyloid A_1
SCLC	small cell lung carcinoma
SD	standard deviation
SEM	standard error of the mean
SqCC	squamous cell carcinoma
TMB	tetramethylbenzidine
TME	tumor microenvironment
TNM	TNM Classification of Malignant Tumors (tumor—lymph node—metastasis)
TNF-α	tumor necrosis factor-α
VAT	video-assisted thoracoscopy

References

1. Kaanane, H.; Senhaji, N.; Berradi, H.; Benchakroun, N.; Benider, A.; Karkouri, M.; El Attar, H.; Casa, I.; Khyatti, M.; Nadifi, S. Association of Variants in IL6-Related Genes with Lung Cancer Risk in Moroccan Population. *Lung* **2019**, *197*, 601–608. [CrossRef] [PubMed]
2. Nie, W.; Xue, L.; Sun, G.; Ning, Y.; Zhao, X. Interleukin-6 -634C/G polymorphism is associated with lung cancer risk: A meta-analysis. *Tumor Biol.* **2014**, *35*, 4581–4587. [CrossRef]
3. Multifactorial Disease. In *Encyclopedia of Public Health*; Springer: Dordrecht, The Netherlands, 2008; Available online: http://archive.today/pLSkJ (accessed on 18 February 2021).
4. Wadowska, K.; Bil-Lula, I.; Trembecki, Ł.; Śliwińska-Mossoń, M. Genetic Markers in Lung Cancer Diagnosis: A Review. *Int. J. Mol. Sci.* **2020**, *21*, 4569. [CrossRef] [PubMed]
5. Gazdar, A.F.; Brambilla, E. Preneoplasia of lung cancer. *Cancer Biomark.* **2011**, *9*, 385–396. [CrossRef]
6. Ruiying, C.; Zeyun, L.; Yongliang, Y.; Zijia, Z.; Ji, Z.; Xin, T.; Xiaojian, Z. A comprehensive analysis of metabolomics and transcriptomics in non-small cell lung cancer. *PLoS ONE* **2020**, *15*, e0232272. [CrossRef] [PubMed]
7. Marino, F.Z.; Bianco, R.; Accardo, M.; Ronchi, A.; Cozzolino, I.; Morgillo, F.; Rossi, G.; Franco, R. Molecular heterogeneity in lung cancer: From mechanisms of origin to clinical implications. *Int. J. Med. Sci.* **2019**, *16*, 981–989. [CrossRef]
8. Collisson, E.A.; Campbell, J.D.; Brooks, A.N.; Berger, A.H.; Lee, W.; Chmielecki, J.; Beer, D.G.; Cope, L.; Creighton, C.J.; Danilova, L.; et al. Comprehensive molecular profiling of lung adenocarcinoma: The cancer genome atlas research network. *Nature* **2014**, *511*, 543–550.
9. The Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. *Nature* **2012**, *489*, 519–525. [CrossRef]
10. Inamura, K. Lung cancer: Understanding its molecular pathology and the 2015 WHO classification. *Front Oncol.* **2017**, *7*, 193. [CrossRef]
11. de Sousa, V.M.L.; Carvalho, L. Heterogeneity in Lung Cancer. *Pathobiology* **2018**, *85*, 96–107. [CrossRef]
12. Travis, W.D.; Brambilla, E.; Nicholson, A.G.; Yatabe, Y.; Austin, J.H.M.; Beasley, M.B.; Chirieac, L.R.; Dacic, S.; Duhig, E.; Flieder, D.B.; et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances since the 2004 Classification. *J. Thorac. Oncol.* **2015**, *10*, 1243–1260. [CrossRef] [PubMed]
13. Wang, L.; Zhu, B.; Zhang, M.; Wang, X. Roles of immune microenvironment heterogeneity in therapy-associated biomarkers in lung cancer. *Semin. Cell Dev. Biol.* **2017**, *64*, 90–97. [CrossRef] [PubMed]
14. Chen, J.; Zhou, R. Tumor microenvironment related novel signature predict lung adenocarcinoma survival. *PeerJ.* **2021**, *4*, e10628. [CrossRef]
15. Mansuet-Lupo, A.; Alifano, M.; Pécuchet, N.; Biton, J.R.; Becht, E.; Goc, J.; Germain, C.; Ouakrim, H.; Régnard, J.F.; Cremer, I.; et al. Intratumoral immune cell densities are associated with lung adenocarcinoma gene alterations. *Am. J. Respir. Crit. Care Med.* **2016**, *194*, 1403–1412. [CrossRef] [PubMed]
16. Sarode, P.; Schaefer, M.B.; Grimminger, F.; Seeger, W.; Savai, R. Macrophage and Tumor Cell Cross-Talk Is Fundamental for Lung Tumor Progression: We Need to Talk. *Front. Oncol.* **2020**, *10*, 324. [CrossRef]
17. Chen, G.; Dong, Z.; Wu, D.; Chen, Y. Profiles of immune infiltration in lung adenocarcinoma and their clinical significant: A gene-expression–based retrospective study. *J. Cell Biochem.* **2020**, *121*, 4431–4439. [CrossRef] [PubMed]
18. Qu, Y.; Cheng, B.; Shao, N.; Jia, Y.; Song, Q.; Tan, B.; Wang, J. Prognostic value of immune-related genes in the tumor microenvironment of lung adenocarcinoma and lung squamous cell carcinoma. *Aging (Albany N. Y.)* **2020**, *12*, 4757–4777. [CrossRef] [PubMed]
19. Śliwińska-Mossoń, M.; Wadowska, K.; Trembecki, Ł.; Bil-Lula, I. Markers Useful in Monitoring Radiation-Induced Lung Injury in Lung Cancer Patients: A Review. *J. Pers. Med.* **2020**, *10*, 72. [CrossRef] [PubMed]
20. Duma, N.; Santana-Davila, R.; Molina, J.R. Non–Small Cell Lung Cancer: Epidemiology, Screening, Diagnosis, and Treatment. *Mayo Clin. Proc.* **2019**, *94*, 1623–1640. [CrossRef]

21. Barta, J.A.; Powell, C.A.; Wisnivesky, J.P. Global epidemiology of lung cancer. *Ann. Glob. Health* **2019**, *85*, 8. [CrossRef]
22. Santarpia, M.; Liguori, A.; D'Aveni, A.; Karachaliou, N.; Gonzalez-Cao, M.; Daffinà, M.G.; Lazzari, C.; Altavilla, G.; Rosell, R. Liquid biopsy for lung cancer early detection. *J. Thorac. Dis.* **2018**, *10* (Suppl. 7), S882–S897. [CrossRef]
23. Chen, F.; Li, J.; Qi, X.; Qi, J. Diagnostic value of CYFRA 21-1 and carcinoembryonic antigen in diagnosis of operable lung cancer from benign lung disease. *J. Cancer Res. Ther.* **2018**, *4*, S400–S404.
24. Xi, K.X.; Zhang, X.W.; Yu, X.Y.; Wang, W.D.; Xi, K.X.; Chen, Y.Q.; Wen, Y.S.; Zhang, L.J. The role of plasma miRNAs in the diagnosis of pulmonary nodules. *J. Thorac. Dis.* **2018**, *10*, 4032–4041. [CrossRef]
25. Tang, H.; Bai, Y.; Shen, W.; Wei, Y.; Xu, M.; Zhou, X.; Zhao, J. Clinical significance of combined detection of interleukin-6 and tumour markers in lung cancer. *Autoimmunity* **2018**, *51*, 191–198. [CrossRef]
26. Ma, R.; Xu, H.; Wu, J.; Sharma, A.; Bai, S.; Dun, B.; Jing, C.; Cao, H.; Wang, Z.; She, J.X.; et al. Identification of serum proteins and multivariate models for diagnosis and therapeutic monitoring of lung cancer. *Oncotarget* **2017**, *8*, 18901–18913. [CrossRef]
27. Bezel, P.; Valaperti, A.; Steiner, U.; Scholtze, D.; Wieser, S.; Vonow-Eisenring, M.; Widmer, A.; Kowalski, B.; Kohler, M.; Franzen, D.P. Evaluation of cytokines in the tumor microenvironment of lung cancer using bronchoalveolar lavage fluid analysis. *Cancer Immunol. Immunother.* **2021**, *70*, 1867–1876. [CrossRef]
28. Liberti, M.V.; Locasale, J.W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem. Sci.* **2016**, *41*, 211–218. [CrossRef]
29. Vanhove, K.; Graulus, G.J.; Mesotten, L.; Thomeer, M.; Derveaux, E.; Noben, J.P.; Guedens, W.; Adriaensens, P. The Metabolic Landscape of Lung Cancer: New Insights in a Disturbed Glucose Metabolism. *Front. Oncol.* **2019**, *9*, 1215. [CrossRef] [PubMed]
30. Bamji-Stocke, S.; van Berkel, V.; Miller, D.M.; Frieboes, H.B. A review of metabolism-associated biomarkers in lung cancer diagnosis and treatment. *Metabolomics* **2018**, *14*, 81. [CrossRef] [PubMed]
31. Deng, T.; Zhang, J.; Meng, Y.; Zhou, Y.; Li, W. Higher pretreatment lactate dehydrogenase concentration predicts worse overall survival in patients with lung cancer. *Medicine* **2018**, *97*, e12524. [CrossRef] [PubMed]
32. Feng, Y.; Xiong, Y.; Qiao, T.; Li, X.; Jia, L.; Han, Y. Lactate dehydrogenase A: A key player in carcinogenesis and potential target in cancer therapy. *Cancer Med.* **2018**, *7*, 6124–6136. [CrossRef]
33. Lei, X.; Lei, Y.; Li, J.K.; Du, W.X.; Li, R.G.; Yang, J.; Li, J.; Li, F.; Tan, H.B. Immune cells within the tumor microenvironment: Biological functions and roles in cancer immunotherapy. *Cancer Lett.* **2020**, *470*, 126–133. [CrossRef]
34. Kanoh, Y.; Abe, T.; Masuda, N.; Akahoshi, T. Progression of non-small cell lung cancer: Diagnostic and prognostic utility of matrix metalloproteinase-2, C-reactive protein and serum amyloid A. *Oncol. Rep.* **2013**, *29*, 469–473. [CrossRef]
35. Fisher, D.T.; Appenheimer, M.M.; Evans, S.S. The two faces of IL-6 in the tumor microenvironment. *Semin. Immunol.* **2014**, *26*, 38–47. [CrossRef] [PubMed]
36. Gong, K.; Guo, G.; Beckley, N.; Zhang, Y.; Yang, X.; Sharma, M.; Habib, A.A. Tumor necrosis factor in lung cancer: Complex roles in biology and resistance to treatment. *Neoplasia* **2021**, *23*, 189–196. [CrossRef]
37. Vietri, L.; Fui, A.; Bergantini, L.; d'Alessandro, M.; Cameli, P.; Sestini, P.; Rottoli, P.; Bargagli, E. Serum amyloid A: A potential biomarker of lung disorders. *Respir. Investig.* **2020**, *58*, 21–27. [CrossRef] [PubMed]
38. Popko, K.; Gorska, E.; Demkow, U. Influence of interleuking-6 and G174C polymorphism in IL-6 gene on obesity and energy balance. *Eur. J. Med. Res.* **2010**, *15* (Suppl. 2), 123–127. [CrossRef]
39. Kuliczkowski, W.; Radomski, M.; Gąsior, M.; Urbaniak, J.; Kaczmarski, J.; Mysiak, A.; Negrusz-Kawecka, M.; Bil-Lula, I. MMP-2, MMP-9, and TIMP-4 and Response to Aspirin in Diabetic and Nondiabetic Patients with Stable Coronary Artery Disease: A Pilot Study. *Biomed. Res. Int.* **2017**, *2017*, 9352015. [CrossRef]
40. Śliwinska-Mosson, M.; Milnerowicz, H.; Jablonowska, M.; Milnerowicz, S.; Nabzdyk, S.; Rabczynski, J. The effect of smoking on expression of IL-6 and antioxidants in pancreatic fluids and tissues in patients with chronic pancreatitis. *Pancreatology* **2012**, *12*, 295–304. [CrossRef] [PubMed]
41. Şahin, F.; Aslan, A. Relationship between Inflammatory and Biological Markers and Lung Cancer. *J. Clin. Med.* **2018**, *7*, 160. [CrossRef]
42. De Groot, P.M.; Wu, C.C.; Carter, B.W.; Munden, R.F. The epidemiology of lung cancer. *Transl. Lung Cancer Res.* **2018**, *7*, 220–233. [CrossRef] [PubMed]
43. Chen, J.L.; Wu, J.N.; Lv, X.D.; Yang, Q.C.; Chen, J.R.; Zhang, D.M. The value of red blood cell distribution width, neutrophil-to-lymphocyte ratio, and hemoglobin-to-red blood cell distribution width ratio in the progression of non-small cell lung cancer. *PLoS ONE* **2020**, *15*, e0237947. [CrossRef]
44. Bayramoglu, A.; Gunes, H.V.; Metintas, M.; Degirmenci, I.; Mutlu, F.; Alatas, F. The Association of MMP-9 Enzyme Activity, MMP-9 C1562T Polymorphism, and MMP-2 and-9 and TIMP-1,-2,-3, and-4 Gene Expression in Lung Cancer. *Genet. Test. Mol. Biomark.* **2009**, *13*, 671–678. [CrossRef]
45. Hu, Z.; Huo, X.; Lu, D.; Qian, J.; Zhou, J.; Chen, Y.; Xu, L.; Ma, H.; Zhu, J.; Wei, Q.; et al. Functional Polymorphisms of Matrix Metalloproteinase-9 Are Associated with Risk of Occurrence and Metastasis of Lung Cancer. *Clin. Cancer Res.* **2005**, *11*, 5433–5439. [CrossRef]
46. Heist, R.S.; Marshall, A.L.; Liu, G.; Zhou, W.; Su, L.; Neuberg, D.; Lynch, T.J.; Wain, J.; Christiani, D.C. Matrix metalloproteinase polymorphisms and survival in stage I non-small cell lung cancer. *Clin. Cancer Res.* **2006**, *12*, 5448–5453. [CrossRef]
47. Schveingert, D.; Cicenas, S.; Bruzas, S.; Samalavicius, N.E.; Gudleviciene, Z.; Didziapetriene, J. The value of MMP-9 for breast and non-small cell lung cancer patients' survival. *Adv. Med. Sci.* **2013**, *58*, 73–82. [CrossRef] [PubMed]

48. Campa, D.; Zienolddiny, S.; Maggini, V.; Skaug, V.; Haugen, A.; Canzian, F. Association of a common polymorphism in the cyclooxygenase 2 gene with risk of non-small cell lung cancer. *Carcinogenesis* **2004**, *25*, 229–235. [CrossRef] [PubMed]
49. Butkiewicz, D.; Krześniak, M.; Drosik, A.; Giglok, M.; Gdowicz-Kłosok, A.; Kosarewicz, A.; Rusin, M.; Masłyk, B.; Gawkowska-Suwińska, M.; Suwiński, R. The VEGFR2, COX-2 and MMP-2 polymorphisms are associated with clinical outcome of patients with inoperable non-small cell lung cancer. *Int. J. Cancer* **2015**, *137*, 2332–2342. [CrossRef] [PubMed]
50. Horn, L.; Lovly, C.M. Neoplasms of the lung. In *Harrison's Principles of Internal Medicine*, 20th ed.; Jameson, J., Fauci, A.S., Kasper, D.L., Hauser, S.L., Longo, D.L., Loscalzo, J., Eds.; McGraw-Hill: New York, NY, USA, 2018.
51. Mets, O.; Smthuis, R. Lung—Cancer TNM 8th Edition. Available online: https://radiologyassistant.nl/chest/lung-cancer/tnm-classification-8th-edition (accessed on 29 June 2020).

13.3. Załącznik 3 – II artykuł oryginalny

Katarzyna Wadowska, Piotr Błasiak, Adam Rzechonek, Iwona Bil-Lula, Mariola Śliwińska-Mossoń: Hepcidin as a diagnostic biomarker in anaemic lung cancer patients. *Cancers*, **2023**, vol. 15, nr 1, art. 224 [24 s.], DOI:10.3390/cancers15010224

MDPI

Article

Hepcidin as a Diagnostic Biomarker in Anaemic Lung Cancer Patients

Katarzyna Wadowska [1,*], Piotr Błasiak [2,3], Adam Rzechonek [2,3], Iwona Bil-Lula [1] and Mariola Śliwińska-Mossoń [1]

[1] Department of Medical Laboratory Diagnostics, Division of Clinical Chemistry and Laboratory Haematology, Faculty of Pharmacy, Wroclaw Medical University, Borowska 211A, 50-556 Wroclaw, Poland
[2] Department and Clinic of Thoracic Surgery, Faculty of Medicine, Wroclaw Medical University, Grabiszyńska 105, 53-439 Wroclaw, Poland
[3] Lower Silesian Centre of Oncology, Pulmonology and Haematology, Lower Silesian Thoracic Surgery Centre, Grabiszyńska 105, 53-439 Wroclaw, Poland
* Correspondence: katarzyna.wadowska@student.umw.edu.pl; Tel.: +48-71-784-0626

Simple Summary: Lung cancer is the leading cause of cancer-related deaths, with a low overall survival rate. Anaemia is one of the most common cancer side effects, reducing the patient's survival time among other things. Lung cancer patients have the highest incidence of anaemia, with 50–70% of patients experiencing anaemia during the course of their disease. In clinical practice, however, anaemia is not a problem in cancer patients until it becomes severe or life-threatening, at which point therapeutic actions are taken. We choose to change this perception by first investigating the characteristics of proteins involved in the pathogenesis of anaemia, as well as their diagnostic capabilities. We assume that predicting anaemia using diagnostic biomarkers, and thus preventing and treating anaemia in lung cancer patients, is beneficial to both the patient and the economy.

Abstract: We aim to describe the characteristics of hepcidin, IL-6, and TNF-α levels in anaemia of lung cancer patients with operative tumour as well as to investigate the potential diagnostic capabilities of hepcidin in combination with IL-6, TNF-α, and acute phase proteins. We present a retrospective study of 112 lung cancer patients (41 women and 71 men) who were surgically treated at the Lower Silesian Centre for Lung Diseases in Wroclaw, Poland. Serum blood samples were collected from all these patients prior to any surgical treatment and used to determine hepcidin, IL-6, TNF-α, SAA_1, and CRP concentrations. Patients were also examined with a complete blood count several times during their hospitalization. The female and male groups were divided based on the occurrence of anaemia during their hospitalization. Patients who developed anaemia post-operatively had significantly lower hepcidin concentrations than non-anaemic patients ($p = 0.000694$ in females with $\geq$3 complete blood count examinations and $p = 0.007905$ in males with 2 complete blood count examinations), whereas patients with anaemia since hospital admission had higher hepcidin concentrations. We observed two hepcidin roles related to two cancer anaemia pathogeneses: (1) higher hepcidin concentrations in patients with anaemia since hospital admission (anaemia of inflammation) and (2) lower hepcidin concentrations in patients who developed anaemia after surgery (anaemia of iron deficiency). Our data support the role of hepcidin, IL-6, and TNF-α in cancer-related anaemia and provide diagnostic values for predicting post-operative anaemia in lung cancer patients.

Keywords: lung cancer; non-small cell lung carcinoma; anaemia of chronic disease; anaemia of inflammation; iron deficiency anaemia; biomarkers; hepcidin; overall survival; quality of life

Citation: Wadowska, K.; Błasiak, P.; Rzechonek, A.; Bil-Lula, I.; Śliwińska-Mossoń, M. Hepcidin as a Diagnostic Biomarker in Anaemic Lung Cancer Patients. *Cancers* **2023**, *15*, 224. https://doi.org/10.3390/cancers15010224

Academic Editor: Tatsuya Nagano

Received: 17 November 2022
Revised: 22 December 2022
Accepted: 27 December 2022
Published: 30 December 2022

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Lung cancer is the most common cause of cancer death, accounting for nearly one-quarter of all cancer deaths. It is estimated that lung cancer will cause more deaths than breast, prostate, and colon cancer together [1,2].

The vast majority of patients with lung cancer (75%) are diagnosed at an advanced stage of the disease, when treatment options are limited, contributing to the high mortality rate. Even as lung cancer treatments and outcomes improve, survival still remains a challenge. Overall five-year relative survival rates increased only slightly over the last 50 years, from 12.4% for 1974–1976 diagnoses to 15.0% for 1996–2002 diagnoses and to 22% in 2020 for all people with all types of lung cancer. Survival rates differ depending on factors such as the subtype of lung cancer, the stage of the disease, and even the gender of the patient [3–5].

Anaemia is one of the most common side effects of both blood cancers and solid tumours, and it has far-reaching consequences for the entire body and cancer treatment, including reduced survival time and sensitivity to chemotherapy and radiotherapy. According to the findings of The European Cancer Anaemia Survey (ECAS), approximately 40% of patients with solid tumours involve anaemia. Among solid tumour patients, patients with lung cancer have the highest incidence of anaemia: approximately 50–70% of patients with lung cancer experience anaemia during the course of their disease [6–12].

Haemoglobin levels are used in clinical practice to define anaemia and its severity, according to World Health Organisation (WHO) and National Cancer Institute (NCI) classification/grading, among others (Table 1). However, the assessment of anaemia's severity is of limited value because it does not allow for an evaluation of anaemia's nature, morphological type, or mechanisms underlying its development [6,7,13,14]. Anaemia of cancer is also known as anaemia of chronic disease (ACD) or anaemia of inflammation (AI), and it has a multifactorial aetiology that includes cancer progression, coexisting inflammation, renal or bone marrow involvement, malnutrition, and oncologic treatment such as chemotherapy and radiotherapy [13–15].

Table 1. Definition of anaemia and its severity according to WHO/NCI classification/grading.

Severity of Anaemia	NCI CTC/WHO Grading	Haemoglobin Levels [g/dL]	
		NCI References	WHO References
No anaemia	0	Normal	≥11
Mild	1	10 to upper limit of normal	9.5–10.9
Moderate	2	8.0–9.9	8.0–9.4
Severe	3	6.5–7.9	6.5–7.9
Life-threatening	4	<6.5	<6.5

NCI CTC—National Cancer Institute Common Toxicity Criteria; WHO—World Health Organisation.

The pathophysiology of ACD/AI involves immune activation in response to tumour antigens with release of several pro-inflammatory cytokines by immune cells, including interleukin (IL)-1, IL-6, IL-22, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, and transforming growth factor (TGF)-β which stimulate hepatic synthesis of hepcidin. Hepcidin is a circulating hormone of inflammation that is the primary regulator of iron availability to developing red blood cells (RBCs) by inhibiting iron release from macrophages and interfering with intestinal iron absorption. Moreover, the pathophysiology of ACD/AI involves immune-mediated effects on the proliferation of erythroid progenitor cells, red blood cell turnover and half-life, and biological activity of erythropoietin. [6,10,12,16–18].

The objective of this study was to describe the characteristics of hepcidin, IL-6, and TNF-α levels in anaemia of lung cancer patients with operative tumours. We also wanted to investigate the potential diagnostic capabilities of hepcidin in combination with IL-6, TNF-α, and acute phase proteins such as C-reactive protein (CRP) and serum amyloid A_1 (SAA_1), via laboratory assessment of the anaemia's basis and prediction of the occurrence of post-operative anaemia.

2. Materials and Methods

2.1. Patients

A total of 112 lung cancer patients were included in the study. All participants were recruited by the Department of Thoracic Surgery, Lower Silesian Centre for Lung Diseases in Wroclaw, Poland, and signed a written informed consent following an explanation of the study protocols. The study protocol conformed to the World Medical Association's Declaration of Helsinki (2000) and was approved by the Bioethics Committee at the Wroclaw Medical University (NR KB: 106/2020).

All patients were surgically treated and underwent lobectomy (61 out of 112, 54.46%), wedge resection (28 out of 112, 25.00%), biopsy (9 out of 112, 8.04%), segmentectomy (7 out of 112, 6.25%), bilobectomy (5 out of 112, 4.46%), or pulmonectomy (2 out of 112, 1.79%). Diagnosis of lung cancer was confirmed by histopathological examination, performed on the tumour tissue obtained. The lung cancer diagnosis was established in accordance with the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology and was staged in accordance with the American Joint Committee on Cancer's (AJCC) 8th TNM Staging System. Clinical, laboratory, and pathological data for these patients were acquired from hospital medical records using the AMMS IT system (Asseco Medical Management Solutions). The essential characteristics of patients for this study, divided by gender, are presented in Table 2.

Table 2. Lung cancer patients' characteristics divided by gender.

	Women	Men	Men with Anaemia upon Admission to the Hospital	Overall
N (%)	41 (36.61%)	71 (63.39%)	32 (28.57%)	112 (100%)
Age				
Mean ± SD	66.2 ± 8.3	67.8 ± 8.2	71.1 ± 6.5	67.2 ± 8.2
Range	40–81	39–84	58–84	39–84
Median	67	69	73	68
Surgery [n, (%)]				
Lobectomy	30 (73.17%)	31 (43.66%)	16 (50.00%)	61 (54.46%)
Wedge Resection	8 (19.51%)	19 (26.76%)	9 (28.13%)	27 (24.11%)
Biopsy	-	10 (14.08%)	5 (15.63%)	10 (8.93%)
Segmentectomy	2 (4.88%)	5 (7.04%)	1 (3.13%)	7 (6.25%)
Bilobectomy	1 (2.44%)	4 (5.63%)	1 (3.13%)	5 (4.46%)
Pulmonectomy	-	2 (2.82%)	-	2 (1.79%)
Lung cancer subtype [n, (%)]				
Adenocarcinoma	21 (51.22%)	29 (40.85%)	13 (40.63%)	50 (44.64%)
Squamous cell carcinoma	8 (19.51%)	27 (38.03%)	12 (37.50%)	35 (31.25%)
Other NSCLC	5 (12.20%)	8 (11.27%)	4 (12.50%)	13 (11.61%)
Not NSCLC	7 (17.07%)	7 (9.86%)	3 (9.38%)	14 (12.50%)
Stage [n, (%)]				
IA	14 (34.15%)	11 (15.49%)	4 (12.50%)	25 (22.32%)
IB	9 (21.95%)	9 (12.68%)	5 (15.63%)	18 (16.07%)
IIA	-	5 (7.04%)	1 (3.13)	5 (4.46%)
IIB	9 (21.95%)	12 (16.90%)	7 (21.88%)	21 (18.75%)
IIIA	2 (4.88%)	10 (14.08%)	4 (12.50%)	12 (10.71%)
IIIB	1 (2.44%)	10 (14.08%)	5 (15.63%)	11 (9.82%)
IVA	4 (9.76%)	11 (15.49%)	4 (12.50%)	15 (13.39%)
IVB	-	-	-	-
Grading [n, (%)]				
G1	1 (2.44%)	-	-	1 (0.89%)
G2	17 (41.46%)	28 (39.44%)	13 (40.63%)	45 (40.18%)
G3	8 (19.51%)	12 (16.90%)	5 (15.63%)	20 (17.86%)
NA	15 (36.59%)	31 (43.66%)	14 (43.75%)	46 (41.07%)

Table 2. *Cont.*

	Women	Men	Men with Anaemia upon Admission to the Hospital	Overall
Inflammation in the tumour environment [n, (%)]				
YES	19 (46.34%)	28 (39.44%)	13 (40.63%)	47 (41.96%)
NO	22 (53.66%)	43 (60.56%)	19 (59.38%)	65 (58.04%)
Smoking history [n, (%)]				
Current	11 (26.83%)	20 (28.17%)	7 (21.88%)	31 (27.68%)
Former	18 (43.90%)	36 (50.70%)	18 (56.25%)	54 (48.21%)
Passive	2 (4.88%)	-	-	2 (1.79%)
Never	1 (2.44%)	1 (1.41%)	1 (3.13%)	2 (1.79%)
NA	9 (21.95%)	14 (19.72%)	6 (18.75%)	23 (20.54%)
Comorbidities [n, (%)]				
YES	26 (63.41%)	47 (66.20%)	22 (68.75%)	73 (65.18%)
NO	15 (36.59%)	24 (33.80%)	10 (31.25%)	39 (34.82%)
Type of comorbidity [n, (%)]				
NO	15 (36.59%)	24 (33.80%)	10 (31.25%)	39 (34.82%)
DM	1 (2.44%)	2 (2.82%)	1 (3.13%)	3 (2.68%)
COPD	2 (4.88%)	4 (5.63%)	2 (6.25%)	6 (5.36%)
Hypertension	16 (39.02%)	22 (30.99%)	9 (28.13%)	38 (33.93%)
DM + Hypertension	5 (12.20%)	9 (12.68%)	5 (15.63%)	14 (12.50%)
COPD + Hypertension	1 (2.44%)	7 (9.86%)	3 (9.38%)	8 (7.14%)
DM + COPD + Hypertension	1 (2.44%)	3 (4.23%)	2 (6.25%)	4 (3.57%)

SD—standard deviation; IA-IVB—stages of lung cancer based on the AJCC 8th TNM Staging System; NA—not available; G1—grade 1, well-differentiated; G2—grade 2, moderately differentiated; G3—grade 3, poorly differentiated; DM—diabetes mellitus; COPD—chronic obstructive pulmonary disease.

2.2. Methods

2.2.1. Study Design

Prior to any surgical treatment, venous blood samples were collected into tubes with clot activator from all patients. Blood samples were centrifuged at 2000× *g* for 8–10 min at room temperature to separate sera, which were then stored at −80 °C until use. Serum blood samples were used to determine the concentrations of IL-6, TNF-α, SAA_1, hepcidin, and CRP. Commercial enzyme-linked immunosorbent assays (ELISA) test kits: Human IL-6 DuoSet ELISA (Catalog # DY206), Human TNF-alpha DuoSet ELISA (Catalog # DY210), Human Serum Amyloid A1 DuoSet ELISA (Catalog # DY3019-05), and Human Hepcidin DuoSet ELISA (Catalog # DY8307-05), R&D Systems, Inc., Minnesota, MN, USA, were used in accordance with manufacturer's protocols. The detection limits of the used ELISA kits correspond to the standard curves, i.e., Human IL-6 DuoSet ELISA: 9.38–600 pg/mL, Human TNF-alpha DuoSet ELISA: 15.63–1000 pg/mL, Human Serum Amyloid A1 DuoSet ELISA: 1.56–100 ng/mL (we performed 1000-, 2000-, and 3000-fold reconstitution using serial dilutions), Human Hepcidin DuoSet ELISA: 3.13–200 pg/mL (we performed 1000-, 2000-, and 3000-fold reconstitution using serial dilutions). The sensitivity of our ELISA kits, also known as the Lowest Limit of Detection (LLOD), is the lowest calculated/statistical point that is different from zero that the kit can statistically detect. We performed the analysis in the double-check mode, which means we measured each sample twice to reduce the likelihood of random errors. CRP levels were determined using a commercial kit based on turbidimetric method: C-REACTIVE PROTEIN (Catalog # 31321), BioSystems S.A., Barcelona, Spain, in accordance with the manufacturer's instructions. The detection limits for CRP determination are 1.0–150 mg/L, the repeatability CVs (in series) are 3.6–4.5%, and the reproducibility CVs (between series) are 3.7–4.6%.

Moreover, venous blood samples were collected several times during patients' hospitalization into tubes containing ethylenediaminetetraacetic acid (EDTA): before surgery, on the day of surgery (after surgery), and at longer intervals following surgery to determine

peripheral blood count. Complete blood counts (CBCs) were analysed in the hospital's clinical laboratory using automated haematology analysers XN-550 and XN-1000 (Sysmex Corporation, Kobe, Japan).

2.2.2. Grouping Methods

The research group was divided based on gender due to gender-related differences in haemoglobin concentration. Then, the women (*n* = 41) and men (*n* = 71) were both divided into three groups based on the number of CBCs performed during operable patients' hospitalization. A total of 14 women and 26 men were examined $\geq$3 times during follow-up: before surgery, on the day of surgery after surgery, and in the days following surgery. During their hospitalization, 23 women were examined twice, once before surgery and once just after surgery, whereas 4 women were examined only once, upon admission to the hospital. In the case of men, 38 were examined twice during their hospitalization, while 7 were examined only once, including 6 patients who were examined only upon admission to the hospital and 1 patient who was examined only shortly after surgery.

Then, we analysed peripheral blood count parameters from CBC results obtained from patients upon admission to the hospital, post-operatively, and in the days following surgery. We chose the result with the lowest haemoglobin concentration from patients who had $\geq$3 CBC examinations and applied parameters from these results to the analyses as the third examination. We also considered the occurrence of anaemia at all stages of the patient's hospitalization. Using haemoglobin levels less than 11 g/dL as the diagnostic standard for anaemia in women and 13.5 g/dL for men, we grouped patients into anaemia groups (including anaemia since the day of hospital admission and anaemia developed during the course of the hospitalization) and non-anaemia groups. We reduced the risk of incorrectly classifying patients who might have developed anaemia during their hospitalization as non-anaemia patients by dividing the study group based on the number of CBCs performed. Patients with anaemia since the day of hospital admission, as well as those with CBC examined three or more times throughout their hospitalization, i.e., with an evident and specified endpoint, provided us with the most accurate and valuable information. Figures S1 and S2 in the Supplement Materials show how the study group was divided and how the incidence of anaemia was distributed.

2.3. Statistical Analysis

The obtained data were statistically analysed using TIBCO Software Inc. (Palo Alto, CA, USA) (2017), Statistica, version 13 (http://statistica.io, accessed on 16 December 2021 and 27 June 2022) with the additional Plus Package (version 5.0.96), and a significance level of $p < 0.05$. The Shapiro–Wilk test was used to determine whether the data for each parameter was normally distributed across all analysed groups. We used chi-square test to compare discrete variables. The parametric Student's *t*-test and one-way analysis of variance (ANOVA) were used to compare independent, continuous variables between two and more groups, respectively. Repeated measures ANOVA was used to compare parameters from consecutive CBCs examinations. Post hoc analyses using the Student–Newman–Keuls method and its modification, Duncan's new multiple range test, supplemented ANOVA. We used the Pearson correlation in variates method to compute the pairwise correlations between CBC parameters from consecutive examinations and analysed protein concentrations in women and men based on the number of CBC examinations and the presence of anaemia.

3. Results

3.1. The Incidence of Anaemia in the Consecutive Stages of Hospitalization

The study included 41 women (36.61%) and 71 men (63.39%). Using haemoglobin levels less than 11 g/dL as the diagnostic standard for anaemia in women, we found only 1 (2.44%) patient with anaemia and 40 (97.66%) patients without anaemia upon admission to the hospital. Among these 40 patients, 12 (29.27%) patients developed anaemia during their

hospitalization and 28 (68.29%) did not. Using haemoglobin levels less than 13.5 g/dL as the diagnostic standard for anaemia in men, we discovered that nearly half of the male lung cancer group (32 out of 71, 45.07%) had anaemia upon admission to the hospital. Moreover, another 16 (22.54%) patients developed anaemia post-operatively, while 23 (32.39%) had no anaemia during their hospitalization. Figure 1 shows the prevalence of anaemia in women and men in the consecutive stages of hospitalization, i.e., upon admission to the hospital, on the day of surgery after surgery, and in the days following surgery, as well as a comparison of its prevalence by gender.

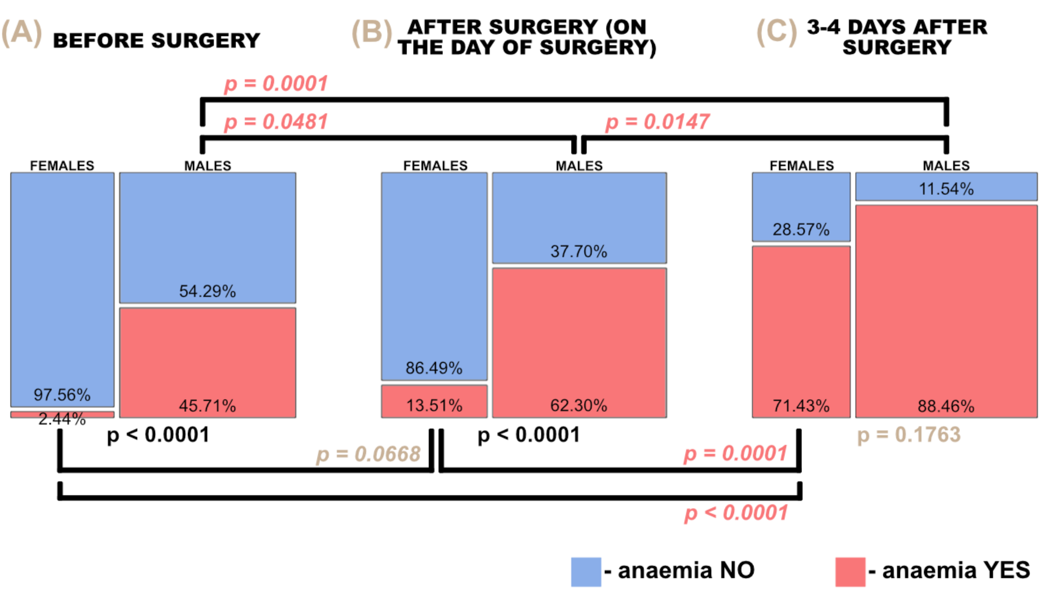


Figure 1. Comparison of anaemia prevalence in women and men (**A**) before surgery, (**B**) after on the day of surgery, and (**C**) 3–4 days after surgery.

We used contingency tables to summarize the relationships between the gender of the patients and the prevalence of anaemia during the course of their hospitalization. Using the chi-square test, we found statistically significant differences in the prevalence of anaemia between females and males before surgery ($p < 0.0001$) and after surgery (on the day of surgery, $p < 0.0001$), with males having a higher frequency of anaemia. We also noticed a statistically significant increase in the frequency of anaemia in both females and males over the course of their hospitalization. In the case of women, we found a significant increase in anaemia prevalence between hospital admission and 3–4 days after surgery ($p < 0.0001$), as well as between the day of surgery (after surgery) and 3–4 days after surgery ($p = 0.0001$). We observed a significant increase in anaemia prevalence in men between the day of admission to hospital and the day of surgery (after surgery) ($p = 0.0481$), as well as between the day of admission to hospital and 3–4 days after surgery ($p = 0.0001$), and between the day of surgery (after surgery) and 3–4 days after surgery ($p = 0.0147$) (Figure 1).

3.2. Complete Blood Count Parameters

The incidence of anaemia in women and men was reflected in the levels of CBC parameters. In women, we found haemoglobin concentrations and red blood cell counts within the laboratory's reference ranges in the first two stages of hospitalization. Normal haemoglobin concentrations (13.15 g/dL before, and 12.44 g/dL after) and red blood cell counts ($4.37 \times 10^6/\mu L$ before, and $4.13 \times 10^6/\mu L$ after) before and after surgery (on the day of surgery) are related to a low prevalence of anaemia in women, with 2.44% and 13.51%, respectively. Only in a CBC performed 3–4 days after surgery we found a decrease

in haemoglobin concentration (10.13 g/dL), as well as haematocrit (30.71%) and red blood cell counts ($3.42 \times 10^6/\mu L$) below the laboratory's reference values, indicating anaemia in women.

Since the first stage of the patient's hospitalization, the mean haemoglobin concentrations and red blood cell count values in men have been lower than the laboratory's reference values, decreasing from 13.39 g/dL to 10.99 g/dL in haemoglobin concentration and from $4.44 \times 10^6/\mu L$ to $3.69 \times 10^6/\mu L$ in red blood cell count values. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) of red blood cells did not deviate from the laboratory's references values in either women or men, indicating normocytic normochromic anaemia. Table S1 in the supplement materials shows the values of CBC parameters at each stage of the patient's hospitalization in relation to the laboratory's reference values.

3.3. The Effect of Independent Variables (Subtype and Stage of Lung Cancer etc.) on the Values of Complete Blood Count Parameters

Prior to examining the influence of hepcidin, IL-6, and TNF-α on CBC parameters, we investigated the potential impact of subtype and stage of lung cancer, type and extent of surgery that patient underwent, presence of inflammation in the tumour environment (observed in the tumour histopathological examination as inflammatory infiltration next to dust deposition and necrosis), patients' smoking status, and underlying lung diseases and other comorbidities on the values of CBC parameters in both women and men. We found statistically significant differences in haemoglobin concentrations and haematocrit values before surgery between lung cancer subtypes in women. A post hoc analysis did not reveal which lung cancer subtypes had statistically significant differences in haematocrit values. However, we found that haemoglobin concentrations differ between lung adenocarcinoma ($\bar{x} = 13.8$ g/dL $\pm$ 0.3) and lung squamous cell carcinoma ($\bar{x} = 12.3$ g/dL $\pm$ 0.3; $p = 0.039425$). In women with adenocarcinoma, the haematocrit value was $\bar{x} = 40.0\%$, and in squamous cell carcinoma, it was $\bar{x} = 37.5\%$. The mean haemoglobin concentrations and haematocrit values in both cases of adenocarcinoma and squamous cell carcinoma patients were fitted in the laboratory's reference values and did not indicate anaemia in these patients.

In the case of the remaining analysed variables, there were no statistically significant differences in the values of CBC parameters in both women and men. We concluded that the stage of lung cancer, the type and extent of surgery that patients underwent, the presence of inflammation in the tumour environment, and the patient's smoking status had no effect on the values of CBC parameters.

3.4. Analysis of Hepcidin, IL-6, and TNF-α Concentrations in Females

We began the analysis by examining the effect of independent variables (subtype and stage of lung cancer, etc.), on the levels of hepcidin, IL-6, and TNF-α. There were no statistically significant differences in hepcidin, IL-6, and TNF-α concentrations between lung cancer subtypes or stages, as well as between patients with and without underlying lung diseases and other comorbidities such as type 2 diabetes mellitus and hypertension in female patients.

Then, we compared the group of females divided based on the number of CBCs performed, and we observed statistically significant differences in the number of hospitalization days between patients who had one ($\bar{x} = 5.5$ days) or ≥ 3 ($\bar{x} = 9.7$ days) ($p = 0.002569$) CBC examinations, as well as between patients who had two ($\bar{x} = 6.7$ days) or ≥ 3 ($\bar{x} = 9.7$ days) ($p = 0.000361$) CBC examinations. Women who had three or more CBC verifications spent 3 to 4 days longer in the hospital than patients who were only examined once or twice during their hospitalization.

3.4.1. A Group of Women with One Complete Blood Count

A CBC was only performed once on four women, upon admission to the hospital. One of the patients was anaemic (haemoglobin concentration = 10.5 g/dL), while the other

three were not. The anaemia patient in this group was the only one of the 41 lung cancer women in the study group who had anaemia prior to surgery, which could be attributed to the patient's advanced lung cancer at stage IVA, because of oesophageal squamous cell carcinoma metastases in the respiratory tract. Anaemia patients had higher cytokine concentrations (126.81 pg/mL vs. 8.74 pg/mL for IL-6 and 302.38 pg/mL vs. 2.09 pg/mL for TNF-α) and more than double the increase in the hepcidin concentration than non-anaemia patients in this group (194.56 ng/mL and $\bar{x} = 80.02$ ng/mL, respectively), but lower CRP and SAA_1 concentrations (2.78 mg/L vs. 9.57 mg/L for CRP and 81.11 μg/mL vs. 131.07 μg/mL for SAA_1). Descriptive statistics for all studied proteins are also included in Table S2 in the supplemental materials.

Moreover, we found a positive, strong, statistically significant correlation between IL-6 and hepcidin (r = 0.99, p = 0.045) and IL-6 and TNF-α concentrations (r = 0.99, p = 0.026) in a group of women with one CBC. All correlations are listed in Table 3.

Table 3. List of statistically important correlations between analysed parameters in women and men with one complete blood count examination, as well as ≥3 complete blood count examinations.

	Hepcidin [ng/mL]	**IL-6 [pg/mL]**	**TNF-α [pg/mL]**	**CRP [mg/L]**	**SAA_1 [μg/mL]**
Patients with one complete blood count examination					
Women					
Hepcidin		r = 0.99, p = 0.045			
IL-6	r = 0.99, p = 0.045		r = 0.99, p = 0.026		
TNF-α		r = 0.99, p = 0.026			
Men					
Hb from 1st CBC				r = −0.96, p = 0.035	
RBC from 1st CBC			r = −0.99, p = 0.014		
Patients with two complete blood count examinations					
Women					
TNF-α					r = 0.55, p = 0.013
SAA_1			r = 0.55, p = 0.013		
Hospital stay				r = 0.57, p = 0.008	
Men					
CRP					r = 0.51, p = 0.018
SAA_1				r = 0.51, p = 0.018	
Patients with ≥3 complete blood count examinations					
Women					
CRP					r = 0.92, p = 0.000
SAA_1				r = 0.92, p = 0.000	
RBC from 1st CBC	r = −0.75, p = 0.019				
Hb from 3rd CBC	r = 0.73, p = 0.026				
Ht from 3rd CBC	r = 0.72, p = 0.029				
RBC from 3rd CBC	r = 0.70, p = 0.036				
Men					
Hb from 1st CBC			r = −0.51, p = 0.016		
Ht from 1st CBC			r = −0.53, p = 0.011		
RBC from 1st CBC			r = −0.61, p = 0.002		
Hb from 2nd CBC				r = −0.51, p = 0.021	
Hb from 3rd CBC	r = 0.46, p = 0.033				
Ht from 3rd CBC	r = 0.44, p = 0.040				
RBC from 3rd CBC	r = 0.43, p = 0.044		r = −0.44, p = 0.040		

IL-6—interleukin 6; TNF-α—tumour necrosis factor α; CRP—C-reactive protein; SAA_1—serum amyloid A_1; CBC—complete blood count; RBC—red blood count; Hb—haemoglobin; Ht—haematocrit; r—correlation coefficient measuring the degree of correlation.

3.4.2. A Group of Women with Two Complete Blood Counts

A CBC was performed on 23 women twice during their hospitalization, once upon admission and once on the day of surgery (after surgery). Only two women in this group had post-operative anaemia. Statistical analyses revealed no significant differences in the concentrations of analysed proteins in this group of patients. However, patients with post-operative anaemia had lower mean hepcidin concentration ($\overline{x}$ = 49.75 ng/mL) and higher mean IL-6 concentration ($\overline{x}$ = 31.54 pg/mL) than non-anaemia patients ($\overline{x}$ = 106.33 ng/mL and $\overline{x}$ = 26.42 pg/mL, respectively). Table S3 in the supplementary materials provides descriptive statistics for all studied proteins.

However, we found a positive, statistically significant correlation between TNF-α and SAA_1 concentrations (r = 0.55, p = 0.013), as well as between CRP concentration and the number of days patients were hospitalized (r = 0.57, p = 0.008). Table 3 contains a list of all correlations.

3.4.3. A Group of Women with ≥3 Complete Blood Counts

On admission to the hospital, no patient in the group with ≥3 CBCs had anaemia, whereas only four women were non-anaemic in the third CBC examination.

When comparing haemoglobin concentrations upon admission to the hospital between women that developed anaemia in the course of hospitalization and non-anaemia women, there were practically no differences in the means, with values of 12.64 g/dL and 12.63 g/dL, respectively. However, we observed a significant decrease in haemoglobin concentration in anaemia patients (from 12.64 g/dL to 9.27 g/dL, p = 0.000019) and a slight decrease in non-anaemia patients (from 12.63 g/dL to 12.28 g/dL, p = 0.188120) during hospitalization. Furthermore, when we compared haemoglobin concentrations in anaemia and non-anaemia women in consecutive CBC examinations, we found statistically significant differences in the second ($\overline{x}$ = 11.36 g/dL and $\overline{x}$ = 12.70 g/dL, p = 0.028790), and third ($\overline{x}$ = 9.27 g/dL and $\overline{x}$ = 12.28 g/dL, p = 0.000312) CBC examination. The dynamics of haemoglobin level changes in anaemia and non-anaemia women in consecutive CBC examinations are depicted in Figure 2. Descriptive statistics for CBC parameters are also included in Table 4.

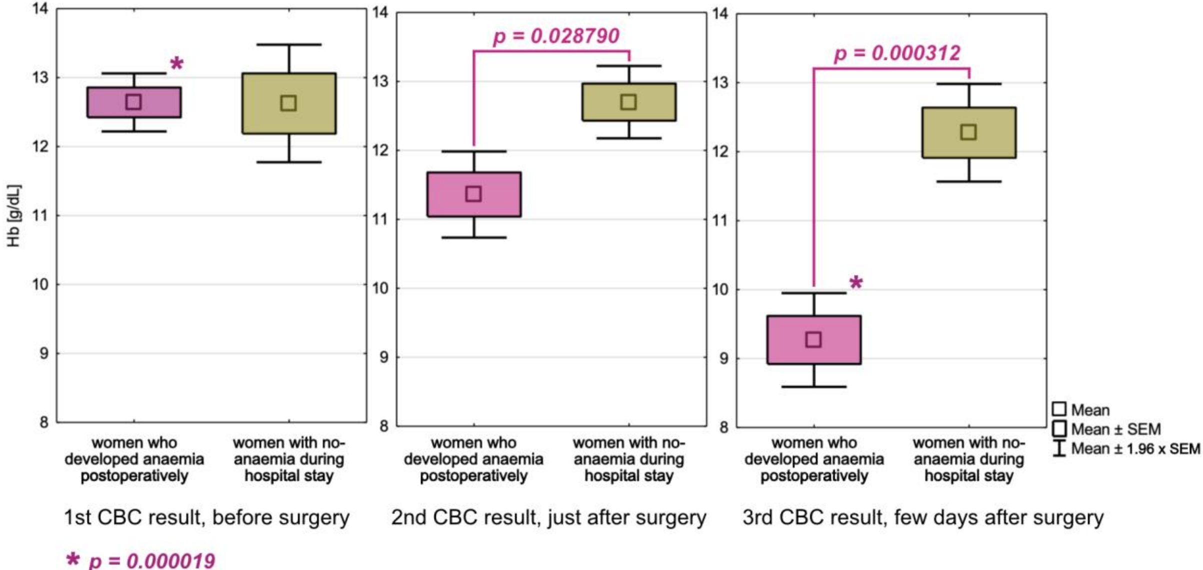


Figure 2. Dynamics of haemoglobin level changes in three consecutive complete blood count examinations in anaemia and non-anaemia women from a group of women with ≥3 complete blood counts.

Table 4. Descriptive statistics of complete blood count parameters as well as hepcidin, IL-6, TNF-α, CRP, and SAA_1 levels in women with and without post-operative anaemia in the third complete blood count examination.

	The Prevalence of Anaemia 3–4 Days after Surgery			
	No Anaemia (N = 4)		**Developed Anaemia (N = 10)**	
Parameter	**1st CBC**	**3rd CBC**	**1st CBC**	**3rd CBC**
NCI grading [n (%)]				
Grade 0	4 (100%)	4 (100%)	10 (100%)	-
Grade 1	-	-	-	3 (30%)
Grade 2	-	-	-	6 (60%)
Grade 3	-	-	-	1 (10%) *
Grade 4	-	-	-	-
Hb [g/dL]				
Mean ± SEM	12.63 ± 0.43	12.28 ± 0.36 [a]	12.64 ± 0.21	9.27 ± 0.35 [a]
Median	12.55	12.3	12.5	9.3
Min–Max	11.70–13.70	11.60–12.90	11.30–13.50	7.20–10.70
RBC [mln/μL]				
Mean ± SEM	4.09 ± 0.08	4.02 ± 0.08 [b]	4.31 ± 0.09	3.18 ± 0.13 [b]
Median	4.11	3.98	4.25	3.24
Min–Max	3.89–4.24	3.89–4.24	3.94–4.72	2.51–3.82
Hepcidin [ng/mL]				
Mean ± SEM	331.43 ± 54.41 [c]		99.34 ± 17.56 [c]	
Median	351.96		116.35	
Min–Max	181.01–440.77		16.18–143.47	
IL-6 [pg/mL]				
Mean ± SEM	24.64 ± 4.72 [d]		53.72 ± 7.68 [d]	
Median	21.15		50.36	
Min–Max	17.81–38.45		34.37–99.69	
TNF-α [pg/mL]				
Mean ± SEM	4.22 ± 2.87		3.17 ± 0.65	
Median	1.54		2.84	
Min–Max	0.99–12.84		1.85–6.87	
CRP [mg/L]				
Mean ± SEM	33.86 ± 15.89		4.76 ± 1.64	
Median	29.74 [e]		1.85 [e]	
Min–Max	1.96–74.01		0.73–16.42	
SAA_1 [μg/mL]				
Mean ± SEM	256.28 ± 155.64		68.03 ± 30.14	
Median	194.47		18.64	
Min–Max	0.32–635.87		0.50–282.63	

CBC—complete blood count; NCI—National Cancer Institute; Hb—haemoglobin; RBC—red blood count; IL-6—interleukin 6; TNF-α—tumour necrosis factor α; CRP—C-reactive protein; SAA_1—serum amyloid A_1; SEM—standard error of the mean. Statistical significances, Student's *t*-test: [a] $p = 0.000312$ in 3rd CBC examination haemoglobin concentrations between women with and without anaemia after surgery; [b] $p = 0.000217$ in 3rd CBC examination RBC values between women with and without anaemia after surgery; [c] $p = 0.000694$ between women with and without anaemia after surgery in hepcidin concentrations; [d] $p = 0.030727$ between women with and without anaemia after surgery in IL-6 concentrations; Mann–Whitney U test: [e] $p = 0.040306$ between women with and without anaemia after surgery in CRP concentrations; chi-square test: * $p = 0.030522$ between females and males who developed anaemia during their hospitalization, in the frequency of developing grade 1 and 2 anaemia.

Furthermore, when we compared hepcidin and cytokines levels in anaemia and non-anaemia women, we discovered statistically significant differences in hepcidin and IL-6 concentrations. Anaemia patients had lower hepcidin concentration ($\overline{x} = 99.34$ ng/mL) than non-anaemia patients ($\overline{x} = 331.43$ ng/mL, $p = 0.000694$), with simultaneously higher IL-6 levels ($\overline{x} = 53.72$ pg/mL) than non-anaemia patients ($\overline{x} = 24.64$ pg/mL, $p = 0.030727$).

The power analysis of the obtained results revealed that the hepcidin analysis had a high power of 0.86 and the IL-6 analysis had a power of 0.51. Moreover, we discovered statistically significant differences in CRP concentrations, with non-anaemia women having significantly higher CRP levels (me = 29.74 mg/L) than women who developed anaemia post-operatively (me = 1.85 mg/L, p = 0.040306). Figure 3 depicts the differences in hepcidin, IL-6, and CRP concentrations in women with and without post-operative anaemia. Table 4 also includes descriptive statistics for all analysed proteins with marked statistical significance.

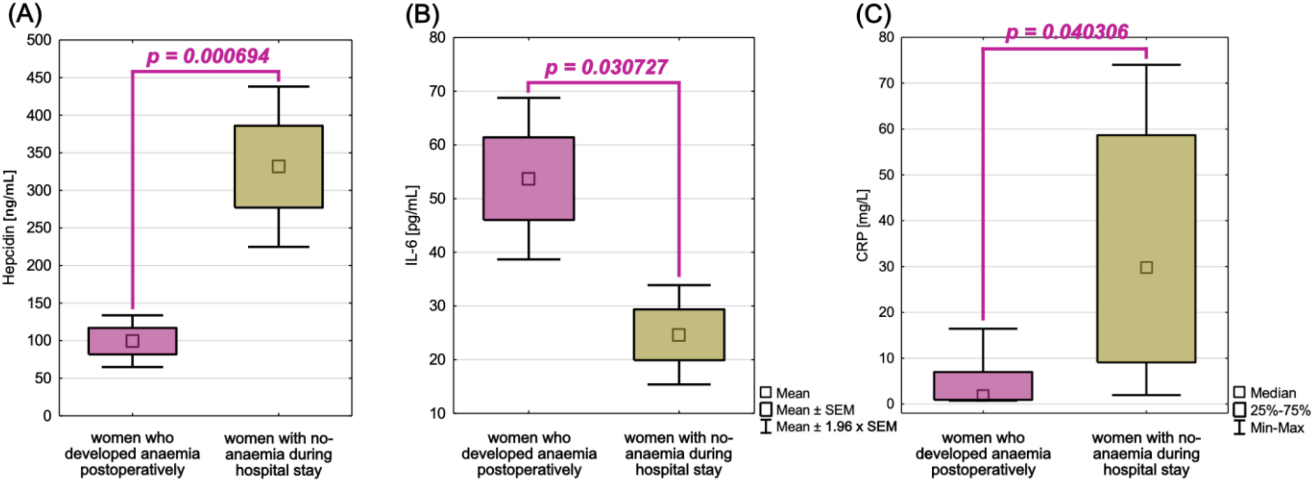


Figure 3. Hepcidin (**A**), IL-6 (**B**), and CRP (**C**) levels in women with and without post-operative anaemia.

We also performed correlation analyses. In a group of women who had ≥3 CBCs, we found a strong, statistically significant, positive correlation between CRP and SAA_1 concentrations (r = 0.92, p = 0.000). Furthermore, we observed a statistically significant negative correlation between hepcidin concentration and RBC value (r = −0.7531, p = 0.019) from the first CBC, as well as statistically significant positive correlations between hepcidin concentration and haemoglobin concentration (r = 0.7282, p = 0.026), haematocrit (r = 0.7189, p = 0.029), and RBC values (r = 0.6986, p = 0.036) from the third CBC. All correlations are listed in Table 3.

3.5. Analysis of Hepcidin, IL-6, and TNF-α Concentrations in Males

Almost half of the men in our study group had anaemia when they were admitted to the hospital. We first looked to see if there were any differences in the essential characteristics of anaemia patients and no anaemia patients, such as subtype and stage of lung cancer, presence of inflammation in the tumour environment, patients' smoking status, or age. The characteristics of males admitted to the hospital with anaemia did not differ from those of the overall group of male patients in our studied lung cancer population (Table 2). There were, however, age differences between males with anaemia and the rest of our study group. We discovered statistically significant age differences between anaemia and non-anaemia males (p = 0.004810), as well as anaemia males and non-anaemia females (p = 0.007810), with anaemia males being 5 years older on average than non-anaemia males and non-anaemia females. Because of the small number of patients (N = 1), we did not include the group of females with pre-operative anaemia in the analysis. Figure 4 depicts the age disparities between patients with and without pre-operative anaemia, demonstrating that females and males without pre-operative anaemia are of comparable age, in contrast to anaemia males.

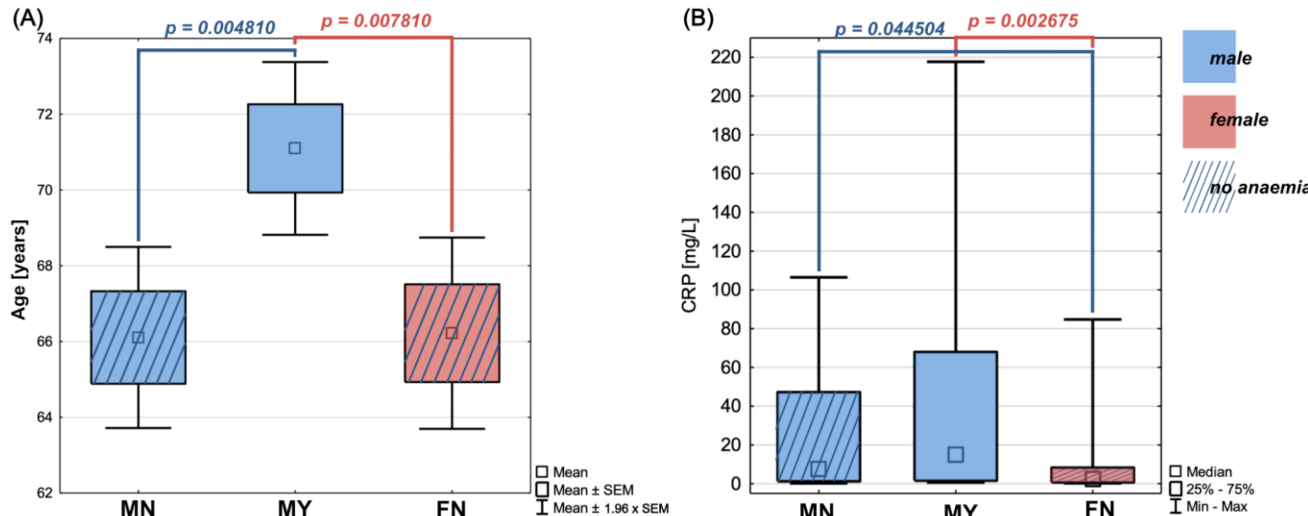


Figure 4. Age (**A**) and CRP levels (**B**) disparities between patients with and without pre-operative anaemia; MN—males without anaemia; MY—males with anaemia; FN—females without anaemia.

Then, we looked to see if there were any statistically significant differences in protein concentrations between groups based on the presence of anaemia at the time of admission to the hospital. We found that non-anaemia women had significantly lower hepcidin concentrations ($\overline{x}$ = 125.27 ng/mL) than non-anaemia men ($\overline{x}$ = 174.64 ng/mL, p = 0.041324). Simultaneously, we discovered that non-anaemia women had significantly lower CRP concentrations (me = 2.27 mg/L) than non-anaemia men (me = 7.56 mg/L, p = 0.044504) and anaemia men (me = 15.06 mg/L, p = 0.002675). Table 5 provides descriptive statistics for age and analysed proteins in women and men, subdivided by the presence of anaemia at the time of admission to the hospital.

Table 5. Descriptive statistics of age and hepcidin, IL-6, TNF-α, CRP, and SAA_1 levels in lung cancer patients, subdivided by the presence of anaemia at the time of admission to the hospital.

	Anaemia upon Admission to the Hospital		
Parameter	**Non-Anaemia Women**	**Non-Anaemia Men**	**Anaemia Men**
Age (years)			
Mean ± SD	66.1 ± 8.3 [a]	66.1 ± 7.5 [b]	71.1 ± 6.5 [a,b]
Median	66.5	67	73
Min–Max	40.0–81.0	47.0–82.0	58.0–84.0
Hepcidin (ng/mL)			
Mean ± SEM	125.27 ± 16.78 [c]	174.64 ± 16.86 [c]	169.02 ± 24.05
Median	109.16	161.5	147.36
Min–Max	11.94–440.77	31.44–429.52	0.08–525.15
IL-6 (pg/mL)			
Mean ± SEM	30.85 ± 3.46	33.49 ± 4.99	36.23 ± 4.50
Median	27.74	26.78	34.95
Min–Max	0.32–99.69	0.21–113.83	0.36–112.70
TNF-α (pg/mL)			
Mean ± SEM	2.64 ± 0.36	6.15 ± 2.96	3.18 ± 0.66
Median	2.1	2.18	2.29
Min–Max	0.99–12.84	1.29–111.55	0.57–20.57

Table 5. *Cont.*

	Anaemia upon Admission to the Hospital		
Parameter	**Non-Anaemia Women**	**Non-Anaemia Men**	**Anaemia Men**
CRP (mg/L)			
Mean ± SEM	10.06 ± 2.95	24.40 ± 5.12	41.53 ± 9.60
Median	2.27 [d,e]	7.56 [d]	15.06 [e]
Min–Max	0.25–84.72	0.22–106.44	0.60–217.67
SAA_1 (µg/mL)			
Mean ± SEM	101.30 ± 22.26	107.99 ± 23.88	132.95 ± 26.39
Median	45.63	7.58	110
Min–Max	0.21–635.87	0.03–548.31	0.63–636.77

SD—standard deviation; SEM—standard error of the mean; IL-6—interleukin 6; TNF-α—tumour necrosis factor α; CRP—C-reactive protein; SAA_1—serum amyloid A_1. Statistical significances, One-way ANOVA: [a] $p = 0.007810$ between non-anaemia women and anaemia men in patients' age; [b] $p = 0.004810$ between non-anaemia men and anaemia men in patients' age; [c] $p = 0.041324$ between non-anaemia women and non-anaemia men in hepcidin concentrations. One-way ANOVA on ranks (Kruskal–Wallis test by ranks): [d] $p = 0.044504$ between non-anaemia women and non-anaemia men in CRP concentrations; [e] $p = 0.002675$ between non-anaemia women and anaemia men in CRP concentrations.

We also examined the effect of independent variables (subtype and stage of lung cancer etc.) on hepcidin, IL-6, and TNF-α concentrations in male lung cancer patients. There were no statistically significant differences in hepcidin, IL-6, and TNF-α concentrations between lung cancer subtypes or stages, or between patients with and without underlying lung diseases. However, we found statistically significant differences in IL-6 concentrations between males with anaemia since hospital admission and comorbidities and those without comorbidities. Anaemic patients with comorbidities (type 2 diabetes mellitus, hypertension, chronic obstructive pulmonary disease) had significantly higher IL-6 concentrations ($\bar{x} = 46.33$ pg/mL) than anaemic patients without comorbidities ($\bar{x} = 21.92$ pg/mL) ($p = 0.005271$). There were no such differences in men who developed anaemia during hospitalization, and in men who did not develop anaemia during hospitalization. Table S4 of the Supplementary Materials contains all descriptive statistics for the proteins studied.

Finally, we compared a group of males divided by the number of CBCs performed and found statistically significant differences in the number of hospitalization days between patients who had two ($\bar{x} = 7.0$ days) or ≥3 ($\bar{x} = 9.0$ days) ($p = 0.001500$) CBC examinations. Men who had three or more CBC verifications spent two to three days longer in the hospital than patients who were only examined once or twice during their hospitalization.

3.5.1. A Group of Men with One Complete Blood Count

A CBC was performed only once on seven men, five of whom were non-anaemic and two of whom had anaemia upon admission to the hospital. In this group, there were no statistically significant differences in hepcidin, IL-6, or TNF-α levels between anaemia and non-anaemia men. However, compared to non-anaemic men, men with anaemia had hepcidin concentrations that were twice as low ($\bar{x} = 155.97$ ng/mL vs. $\bar{x} = 82.09$ ng/mL, respectively), and IL-6 concentrations that were marginally higher ($\bar{x} = 12.91$ pg/mL vs. $\bar{x} = 15.59$ pg/mL, respectively). All descriptive statistics of analysed proteins are included in Table S5 of the Supplementary Materials.

We also performed correlation analyses and discovered a strong negative, statistically significant relationship between TNF-α concentration and the number of red blood cells (r = −0.99, $p = 0.014$), as well as between CRP and haemoglobin concentrations (r = −0.96, $p = 0.035$). We also found a strong negative correlation (r = −0.90) between IL-6 and TNF-α concentrations, but it was not statistically significant ($p = 0.098$). Table 3 contains a list of all statistically important correlations.

3.5.2. A Group of Men with Two Complete Blood Counts

A CBC was performed twice on 38 men, 15 of whom had no anaemia during their hospitalization, 16 had anaemia since their admission, and 7 developed anaemia while in the hospital. When we compared haemoglobin concentrations upon admission to the hospital between men who developed anaemia during their hospitalization, non-anaemia patients, and anaemia patients, we discovered statistically significant differences in each group, i.e., between men who developed anaemia and non-anaemia patients (p = 0.023919), anaemia and non-anaemia patients (p = 0.000123), and men who developed anaemia and anaemia patients (p = 0.000123). The highest haemoglobin concentrations were found in non-anaemia men ($\overline{x}$ = 14.82 g/dL), slightly lower in men who developed anaemia ($\overline{x}$ = 13.96 g/dL), and the lowest in anaemia patients ($\overline{x}$ = 11.83 g/dL). Figure 5 depicts differences in haemoglobin concentrations from the first CBC examination between groups.

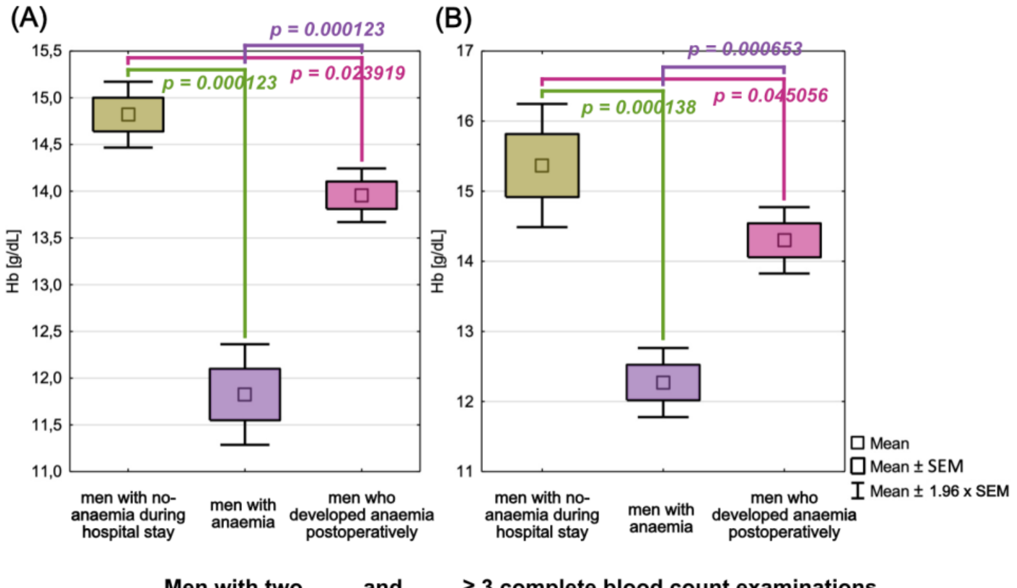


Figure 5. Differences in haemoglobin concentrations in the first complete blood count examination between men with and without anaemia, as well as men who developed anaemia post-operatively, who had two (**A**) and ≥ 3 complete blood count examinations (**B**).

We found statistically significant differences in hepcidin concentrations between non-anaemia men and men who developed anaemia when we compared hepcidin, IL-6, and TNF-α concentrations between non-anaemia and anaemia patients, as well as men who developed anaemia during their hospitalization. Men with developed anaemia had significantly lower hepcidin concentrations (x = 90.84 ng/mL) than non-anaemia patients (x =161.50 ng/mL, p = 0.007905). The obtained results' power analysis revealed that the hepcidin analysis in this group had a satisfactory (as for medical research) power of 0.66. Figure 6 depicts differences in hepcidin concentrations between non-anaemia men and men who developed anaemia during their hospitalization. Table S6 of the Supplementary Materials also includes descriptive statistics for all analysed proteins.

We also performed correlation analyses and discovered a positive, statistically significant relationship between CRP and SAA_1 concentrations (r = 0.51, p = 0.018). Table 3 contains a list of all statistically important correlations.

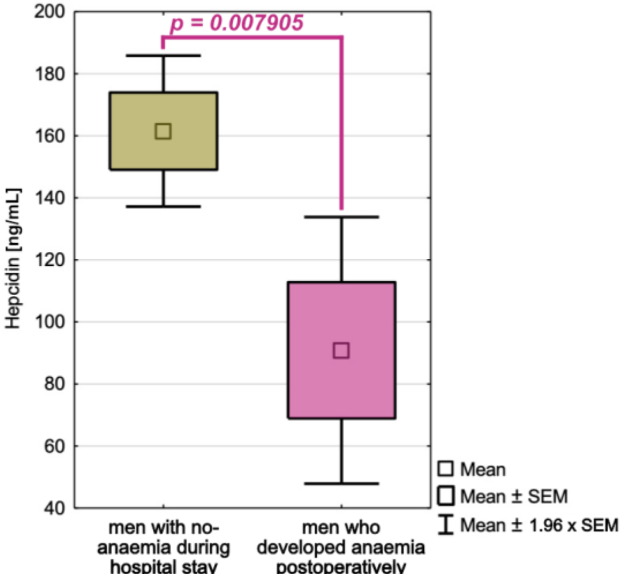


Figure 6. Hepcidin concentration differences between men without anaemia and men who developed anaemia during hospitalization and had two complete blood count examinations.

3.5.3. A Group of Men with ≥3 Complete Blood Counts

A CBC was performed thrice on 26 men, 3 of whom had no anaemia during their hospitalization, 14 had anaemia since their admission, and 9 developed anaemia while in the hospital. When we compared haemoglobin concentrations upon admission to the hospital between men who developed anaemia during their hospitalization, non-anaemia patients, and anaemia patients, we discovered statistically significant differences in each group, i.e., between men who developed anaemia and non-anaemia patients (p = 0.045056), anaemia and non-anaemia patients (p = 0.000138), and men who developed anaemia and anaemia patients (p = 0.000653). The highest haemoglobin concentrations were found in non-anaemia men ($\overline{x}$ = 15.37 g/dL), slightly lower in men who developed anaemia ($\overline{x}$ = 14.30 g/dL), and the lowest in anaemia patients ($\overline{x}$ = 12.27 g/dL). Figure 6 depicts differences in haemoglobin concentrations from the first CBC examination between groups. Table 6 includes descriptive statistics for CBC parameters (haemoglobin concentration and RBC) from both the first and third examinations.

We found no statistically significant differences in hepcidin, IL-6, TNF-α, CRP, and SAA_1 concentrations between non-anaemia and anaemia patients, as well as men who developed anaemia during their hospitalization. Table 6 compiles descriptive statistics for hepcidin, IL-6, TNF-α, CRP, and SAA_1 concentrations in men who developed anaemia, as well as non-anaemia and anaemia men who were tested with CBC three and more times.

We also conducted correlation analyses and discovered a negative, statistically significant relationship between TNF-α concentration and haemoglobin concentration (r = −0.51, p = 0.016), haematocrit value (r = −0.53, p = 0.011), and red blood cell count (r = −0.61, p = 0.002) from the first CBC examination, as well as between TNF-α concentration and the number of red blood cells (r = −0.44, p = 0.040) in the third CBC examination. There was also a negative, statistically significant relationship between CRP and haemoglobin concentrations (r = −0.51, p = 0.021) from the second CBC examination. Additionally, we found a positive, statistically significant relationship between hepcidin concentration and haemoglobin concentration (r = 0.46, p = 0.033), haematocrit value (r = 0.44, p = 0.040), and the number of red blood cells (r = 0.43, p = 0.044). All correlations are listed in Table 3.

Table 6. Descriptive statistics of complete blood count parameters from the first and third complete blood count examinations, as well as hepcidin, IL-6, TNF-α, CRP, and SAA_1 levels in men with and without anaemia, and in men who developed anaemia after surgery.

	The Prevalence of Anaemia 3–4 Days after Surgery					
	No Anaemia (N = 3)		Developed Anaemia (N = 9)		Anaemia (N = 14)	
Parameter	**1st CBC**	**3rd CBC**	**1st CBC**	**3rd CBC**	**1st CBC**	**3rd CBC**
NCI grading (n (%))						
Grade 0	3 (100.0%)	3 (100.0%)	9 (100.0%)	-	-	-
Grade 1	-	-	-	6 (66.7%)	14 (100.0%)	10 (71.4%)
Grade 2	-	-	-	2 (22.2%)	-	4 (28.6%)
Grade 3	-	-	-	1 (11.1%) *	-	-
Grade 4	-	-	-	-	-	-
Hb (g/dL)						
Mean ± SEM	15.37 ± 0.45 [a,b]	13.80 ± 0.12 [d,e]	14.30 ± 0.24 [a,c]	10.72 ± 0.62 [d]	12.27 ± 0.25 [b,c]	10.56 ± 0.32 [e]
Median	15.6	13.8	14	10.7	12.55	11.1
Min–Max	14.50–16.00	13.60–14.00	13.70–15.90	7.60–13.30	10.20–13.30	8.50–12.00
RBC (mln/μL)						
Mean ± SEM	4.94 ± 0.17 [f]	4.41 ± 0.07 [h,i]	4.89 ± 0.10 [g]	3.71 ± 0.27 [h]	4.06 ± 0.08 [f,g]	3.52 ± 0.10 [i]
Median	5.03	4.39	4.79	3.75	4.07	3.53
Min–Max	4.61–5.19	4.31–4.54	4.55–5.33	2.69–5.04	3.51–4.53	2.92–3.99
Hepcidin (ng/mL)						
Mean ± SEM	251.19 ± 58.30		240.74 ± 41.34		214.84 ± 34.74	
Median	247.96		259.62		188.37	
Min–Max	151.86–353.74		78.61–429.52		60.85–525.15	
IL-6 (pg/mL)						
Mean ± SEM	67.45 ± 18.45		52.93 ± 12.34		45.82 ± 6.95	
Median	64.62		46.14		45.32	
Min–Max	37.00–100.72		0.21–113.83		0.36–112.70	
TNF-α (pg/mL)						
Mean ± SEM	1.99 ± 0.27		1.98 ± 0.15		2.71 ± 0.30	
Median	2.2		1.91		2.54	
Min–Max	1.45–2.33		1.44–2.71		1.20–4.53	
CRP (mg/L)						
Mean ± SEM	54.24 ± 21.44		29.49 ± 11.10		48.05 ± 16.90	
Median	65.52		17.33		13.7	
Min–Max	12.78–84.43		1.44–81.32		0.81–217.67	
SAA_1 (μg/mL)						
Mean ± SEM	59.44 ± 55.68		162.43 ± 61.76		146.22 ± 50.08	
Median	7.58		101.35		57.12	
Min–Max	0.03–170.72		0.08–515.75		0.63–636.77	

CBC—complete blood count; NCI—National Cancer Institute; Hb—haemoglobin; RBC—red blood count; IL-6—interleukin 6; TNF-α—tumour necrosis factor α; CRP—C-reactive protein; SAA_1—serum amyloid A_1; SEM—standard error of the mean. Statistical significances, one-way ANOVA: [a] p = 0.045056 in haemoglobin concentration upon hospital admission between men who did not have anaemia and men who developed anaemia post-operatively; [b] p = 0.000138 in haemoglobin concentration upon hospital admission between men who did not have anaemia and men who had anaemia; [c] p = 0.000653 in haemoglobin concentration upon hospital admission between men who developed anaemia post-operatively and men who had anaemia; [d] p = 0.002236 in haemoglobin concentration from the 3rd CBC examination between men who did not have anaemia and men who developed anaemia post-operatively; [e] p = 0.003834 in haemoglobin concentration from the 3rd CBC examination between men who did not have anaemia and men who had anaemia; [f] p = 0.000199 in RBC values upon hospital admission between men who did not have anaemia and men who had anaemia; [g] p = 0.000212 in RBC values upon hospital admission between men who developed anaemia post-operatively and men who had anaemia; [h] p = 0.040572 in RBC values from the 3rd CBC examination between men who did not have anaemia and men who developed anaemia post-operatively; [i] p = 0.028428 in RBC values from the 3rd CBC examination between men who did not have anaemia and men who had anaemia; chi-square test: * p = 0.030522 between females and males who developed anaemia during their hospitalization, in the frequency of developing grade 1 and 2 anaemia.

3.6. Diagnostic Value of Hepcidin, IL-6, TNF-α, and CRP

We supplemented our findings with a statistical analysis of the diagnostic value of hepcidin, IL-6, TNF-a, CRP, and SAA_1 in the examination and differentiation of anaemia in lung cancer patients. Among the examined models, we discovered a statistically significant diagnostic model consisting of hepcidin and IL-6 in the differential diagnosis of females without anaemia from females who will develop anaemia post-operatively during the hospitalization, with the highest sensitivity (87.5%) and specificity (71.4%), as well as an area under the curve (AUC) value considered excellent (0.8809).

We also found statistically significant diagnostic models when distinguishing (1) males without anaemia from males with anaemia since hospital admission, (2) females who will develop anaemia post-operatively from males with anaemia since hospital admission, and (3) females without anaemia from males with anaemia since hospital admission. Table 7 shows the diagnostic efficiency of the best biomarker models for differential diagnosis in each and every group of lung cancer patients studied, whereas Figure 7b shows receiver operating characteristic (ROC) curves of the three statistically significant models with the highest sensitivity, specificity, and AUC values.

Table 7. The diagnostic efficiency of biomarker models to differentiate lung cancer patients without anaemia, patients with anaemia since the day of hospitalization, and patients who developed anaemia during their hospitalization based on patient gender.

Model	*p* Value	AIC	AUC	Sensitivity	Specificity	Cut-Off Values
Females without anaemia vs. Females with developed anaemia						
Hepcidin + IL-6	0.00574	28.8	0.8809	87.50%	71.40%	Hepcidin: 44.29 ng/mL IL-6: 24.39 pg/mL
Males without anaemia vs. Males with developed anaemia						
IL-6	0.171698	40.37	0.6458	75.00%	50.00%	IL-6: 18.77 pg/mL
Males without anaemia vs. Males with anaemia						
TNF-α	0.032369	54.27	0.6687	72.00%	56.30%	TNF-α: 2.59 pg/mL
Males with anaemia vs. Males with developed anaemia						
IL-6	0.448175	50.05	0.5533	60.00%	50.00%	IL-6: 39.58 pg/mL
Females with developed anaemia vs. Males with anaemia						
Hepcidin + IL-6 + CRP	0.00927	30.11	0.8285	(1) 72.0%	(1) 85.7%	(1) Hepcidin: 66.16 ng/mL IL-6: 57.61 pg/mL CRP: 21.23 mg/L
				(2) 92.0%	(2) 42.9%	(2) Hepcidin: 0.08 ng/mL IL-6: 2.63 pg/mL CRP: 0.60 mg/L
Females without anaemia vs. Males with anaemia						
IL-6 + CRP	0.02763	66.73	0.6966	(1) 68.0%	(1) 70.8%	(1) IL-6: 44.14 pg/mL CRP: 0.88 mg/L
				(2) 80.0%	(2) 62.5%	(2) IL-6: 34.95 pg/mL CRP: 0.60 mg/L

AIC—Akaike information criterion; AUC—area under the curve; IL-6—interleukin 6; TNF-α—tumour necrosis factor α; CRP—C-reactive protein; bold black text indicates statistically significant diagnostic model.

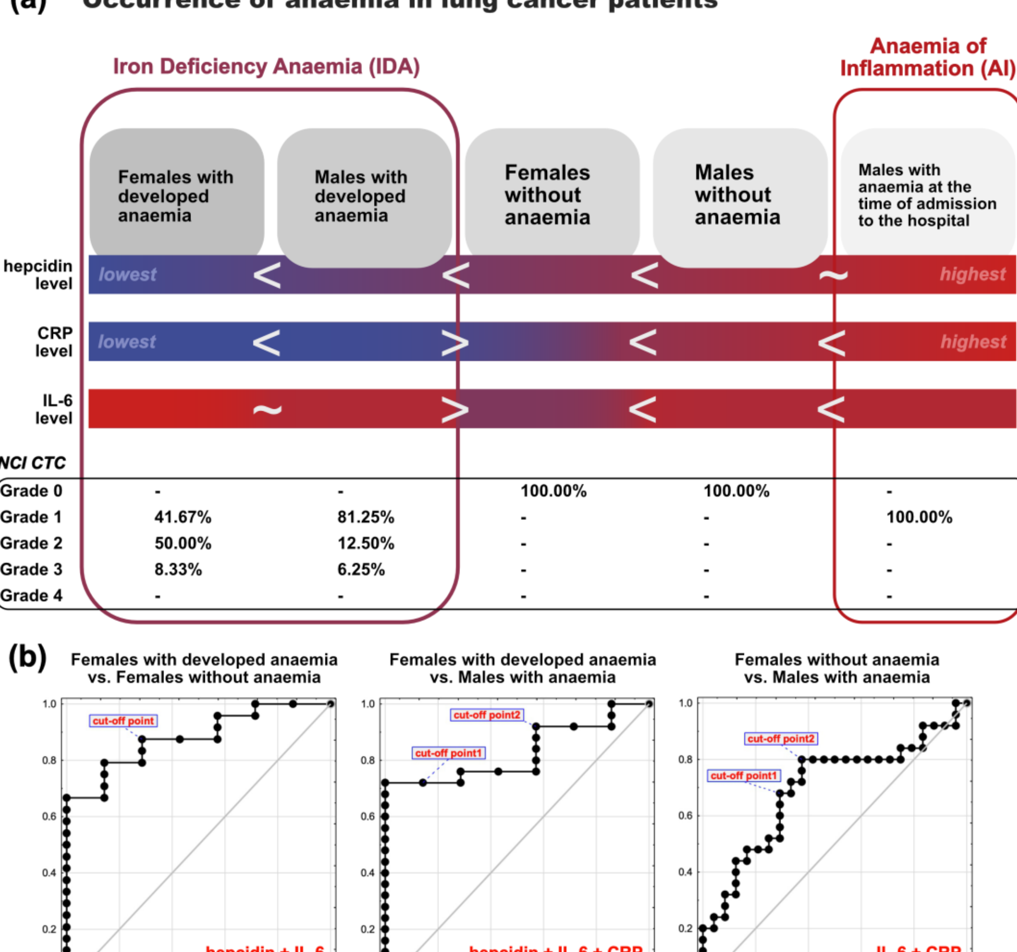


Figure 7. The potential diagnostic model for (**a**) determining the foundation in the pathogenesis of anaemia in lung cancer patients, as well as predicting anaemia severity, and (**b**) distinguishing patients with anaemia since hospital admission from patients with anaemia developed during hospitalization from patients without anaemia during hospitalization.

4. Discussion

In clinical practice, anaemia is not a problem in cancer patients until it is severe or life-threatening anaemia, at which point therapeutic actions are taken. Researchers are also focusing on severe or life-threatening anaemia, particularly in terms of the side effects of used chemotherapy or radiotherapy [8,19–21]. To expose the lack of interest/negligibility of the anaemia problem in lung cancer patients, we obtained the following numbers after searching the PubMed database. After conducting a keyword search on 23 September 2022: (1) (lung cancer OR lung neoplasms OR lung tumour) AND (anaemia OR anemia), we found 3547 results; (2) (lung cancer OR lung neoplasms OR lung tumour) AND (anaemia OR anemia) AND chemotherapy, we found 2282 results; (3) (lung cancer OR lung neoplasms OR lung tumour) AND (anaemia OR anemia) AND radiotherapy, we found 316 results; (4) (lung cancer OR lung neoplasms OR lung tumour) AND hepcidin, we found 22 results, compared to 432,949 results after searching for lung cancer OR lung tumour OR lung neoplasm.

Lung cancer patients not only have the highest incidence of anaemia, but they also have the highest mortality rate, with low overall five-year relative survival rates [2,5,13]. Given that anaemia is independently associated with shorter cancer patient survival times, even mild or moderate anaemia should be a significant issue in lung cancer patients [9,12,13].

In the present study, we focused on anaemia associated with the condition of lung cancer, as well as anaemia that developed in lung cancer patients post-operatively. We discovered that males outnumbered females in the group of patients admitted to the hospital with anaemia. Anaemia was found in 29.46% of 112 cancer patients at the time of admission to the hospital, including 45.07% of 71 male cancer patients and 2.44% of 41 female cancer patients. We also found that the anaemia group had a higher mean age. All of these patients with anaemia since hospital admission had mild (grade 1) anaemia, according to the NCI grading. Furthermore, 35.44% of the 79 remaining lung cancer patients who did not have anaemia at the time of admission to the hospital developed anaemia in the course of their hospitalization, including 41.03% of 39 male cancer patients and 30.00% of 40 female cancer patients. In contrast to the group with anaemia since hospital admission, we observed a variety of anaemia severity in the "developed anaemia group", with patients having mild, moderate, or severe anaemia. We found that females with developed anaemia had a higher proportion of moderate and severe anaemia than males with developed anaemia.

Statistical analyses revealed that males with anaemia on admission to the hospital were not only older than non-anaemia patients in our study group, but they also had the highest CRP concentrations, above reference values (>5 mg/L). The current state of the art indicates that comparing hepcidin to CRP may be a quick and simple way to determine whether anaemia is the result of an iron deficiency, an inflammation, or a mixture of both [15,22]. Hence, increased hepcidin concentrations in combination with high CRP concentrations enable us to determine the presence of inflammation in the pathogenesis of anaemia in lung cancer males admitted to the hospital with anaemia.

Anaemia of inflammation is also known as anaemia of chronic disease because it occurs in people who have chronic conditions that may be associated with inflammation. Cancer is one of these chronic conditions. [15] Anaemia of inflammation is more common in the elderly and may be associated with the term inflammageing coined by Claudio Franceschi in ~2000 [16,17,23]. Our finding confirmed the concept of inflammaeging and anaemia of inflammation, as people with anaemia on hospital admission were on average 5 years older than non-anaemia patients.

Franceschi and colleagues [23] reported an elevated basal level of pro-inflammatory mediators in the blood even in healthy elderly people. High circulating levels of IL-1, IL-1 receptor antagonist protein (IL-1RN), IL-6, IL-8, IL-13, IL-18, CRP, IFN-α, IFN-β, TGF-β, TNF-α and its soluble receptors, and SAA_1 characterise this mild pro-inflammatory state. Furthermore, cancer itself also stimulates the production of pro-inflammatory cytokines. [12,14,16,17,24,25] TNF-α and IL-6 cytokines are secreted by non-small cell lung cancer (NSCLC) cells and immune cells infiltrating the tumour microenvironment, and they regulate liver cell production of the non-specific acute phase proteins CRP and SAA_1 [26]. In addition, IL-6 regulates hepcidin expression through the direct binding of STAT3 (signal transducer and activator of transcription 3) to the promoter. As a result, chronic IL-6 elevation is involved in the development of anaemia, among other things, by increasing hepcidin production and thus serum hepcidin levels. Both cytokines and hepcidin have been shown to inhibit erythropoiesis by (1) storing iron in reticuloendothelial system cells, (2) decreasing iron absorption in the digestive system, (3) inhibiting erythropoietin production in the kidneys, and (4) directly inhibiting proliferation and differentiation of erythroid progenitor cells [10,12,15,22,25,27].

Furthermore, other studies show that healthy women have higher IL-6 serum levels later in life than healthy men, implying that women experience the effects of inflammageing later than men [23,28]. Because lung cancer males have additive effects on pro-inflammatory cytokine levels from inflammageing and cancer, and lung cancer females have higher concentrations of pro-inflammatory cytokines from cancer itself, we may conclude that anaemia of inflammation is more common in older male lung cancer patients. This conclusion would explain why men outnumber women in the group of patients with anaemia upon admission to the hospital (45.07% of all males vs. 2.44% of all females).

We also observed that patients who developed anaemia post-operatively were characterized by significantly lower hepcidin concentrations than non-anaemic patients and patients who had anaemia since their hospital admission. A low hepcidin concentration combined with a low CRP concentration (within reference values, <5 mg/L) indicates the presence of iron deficiency in the pathogenesis of anaemia [15], in this case, anaemia that develops during the patient's hospitalization. Moreover, we found that female patients who developed anaemia during hospitalization had a higher frequency of moderate or severe anaemia than male patients. Additionally, females who developed anaemia had statistically lower hepcidin concentrations than males. We concluded that the severity of developed anaemia, which is characterized by iron deficiency in its pathogenesis, is related to the patient's hepcidin level, i.e., lower hepcidin concentrations are associated with more severe anaemia.

While hepcidin and CRP examination may point to the pathogenesis of anaemia, we discovered that hepcidin and IL-6 examination may predict the development of anaemia during hospitalization in female lung cancer patients. The model with (area under the curve) AUC = 0.8809 provides good discrimination and allows differentiation of women who will not develop anaemia from women who will develop anaemia during the hospitalization, with a high sensitivity and specificity. Based on our observations, section (a) of Figure 7 depicts a scheme of a possible diagnostic procedure in differentiation and determination of the foundation in the pathogenesis of anaemia in lung cancer patients, as well as the prediction of anaemia severity. While section (b) of Figure 7 depicts receiver operating characteristic (ROC) curves assessing the ability of multi-biomarker models to distinguish (1) women who will not develop anaemia from women who will develop anaemia during the hospitalization, (2) men with anaemia from women who will develop anaemia during the hospitalization, and (3) men with anaemia from women who will not develop anaemia during the hospitalization.

An algorithm for diagnosing and predicting anaemia in lung cancer patients is required. The ECAS was one of the first to raise awareness of the significance of mild-to-moderate anaemia on patients who may not be treated according to Anaemia Society of Haematology/American Society of Clinical Oncology (ASH/ASCO) guidelines. Even mild-to-moderate anaemia, according to ECAS, has a significant impact on a patient's QoL and performance status, as measured by the physician-reported WHO Performance Score [9]. Anaemia in cancer patients causes micro changes such as tumour hypoxia and the production of angiogenic factors, both of which control further tumour growth and progression, and have been linked to the development of treatment resistance [12,20]. In addition to resistance to chemo- and radiotherapy, anaemia of cancer is responsible for increased post-operative mortality, impaired organ function, decreased QoL, and poor prognoses [11,13]. Cancer patients with haemoglobin levels less than 12 g/dL have significantly more fatigue, more non-fatigue anaemia symptoms, poorer physical, functional, and mental well-being, and lower general QoL [12]. Furthermore, a Southwest Oncology Group study that enrolled patients with advanced-stage NSCLC between 1974 and 1988 discovered that patients with haemoglobin levels greater than 11 g/dL had a better prognosis [13,29]. There is also evidence that higher haemoglobin levels, even when increased by epoetin, are associated with better outcome measures such as survival [12,13].

When mild or moderate anaemia appears, there is a lack of awareness about the potential consequences for patients' quality of life and overall survival. Clinicians must become more aware of the clinical impact of not only severe and life-threatening anaemia, but also mild and moderate anaemia, and offer adequate treatment to their patients [11]. At this point, anaemia treatment should be a critical component of cancer treatment. Especially since we can predict which operative lung cancer patients will develop anaemia during hospitalization using hepcidin, IL-6, and CRP testing and include these patients in anaemia prevention treatment. In the short term, preventing and treating anaemia in lung cancer patients may improve post-operative recovery as well as shorten the duration of the patient's hospitalization, as patients with developed anaemia spent 2 to 4 days longer in

the hospital on average. In the long term, preventing and treating anaemia in lung cancer patients may be another key point in improving their survival rates, resulting in decreased lung cancer patient mortality. Overall, prevention and treatment of anaemia in lung cancer patients appear to be beneficial for the patient themself and from the economical point of view.

Our main findings demonstrate and confirm the role of hepcidin and IL-6 in the pathogenesis of lung cancer-related anaemia, as well as present diagnostic values for hepcidin, IL-6, and CRP. TNF-α, however, cannot be ruled out as a factor in the anaemia of cancer. Numerous studies described the central role of TNF-α in the pathogenesis of anaemia [10,12,24]. TNF-α's inhibitory effect on erythroid differentiation was first described over 30 years ago. Blick and colleagues [30] discovered in 1987 a decrease in haemoglobin synthesis in cancer patients treated with TNF-α, and an in vitro study revealed that TNF-α inhibited the formation of burst-forming unit-erythrocyte (BFU-E) cells.

In the present study, we discovered statistical significance in the model consisting of TNF-α alone in distinguishing men without anaemia during hospitalization from men with anaemia since hospital admission, with men with anaemia since hospital admission, recognized by us as anaemia of inflammation, having higher TNF-α concentrations. Correlation analysis confirmed these findings. TNF-α concentration was found to be negatively correlated with CBC parameters examined upon hospital admission: haemoglobin concentration, haematocrit value, and red blood cell count, implying that the values of CBC parameters were decreased in patients with higher TNF-α concentration. The significance of TNF-α in our study group's males, with a focus on males with anaemia since hospital admission, may point to TNF-α's role in the pathogenesis of anaemia of inflammation. The activation of STAT3 by IL-6 regulates hepcidin expression. However, because STAT3 can be activated by a variety of other cytokines and growth factors, hepcidin expression may be influenced by a variety of other factors, including TNF-α [22,25,27].

Serum hepcidin concentrations and local hepcidin expression have been reported to increase in a variety of neoplasms, including lung cancer, breast cancer, renal carcinoma, prostate cancer, colorectal cancer, and acute leukaemia. Elevated serum hepcidin levels and local expression have been linked to disease progression, cancer metastatic potential, and shorter overall survival in oncological patients [31–33]. Despite knowledge and awareness of the role and potential consequences of increased hepcidin levels in oncological patients, there is a lack of studies focusing on both the potential diagnostic or therapeutic use of hepcidin and its role in the development of cancer-related anaemia, as well as how to incorporate this ready-to-use marker into the clinical field.

Our study is the first to investigate hepcidin's diagnostic capabilities in lung cancer anaemia in a comprehensive and detailed manner. We discovered that hepcidin reacts differently in lung cancer patients with anaemia. We linked two hepcidin roles with two cancer anaemia pathogeneses. We found higher hepcidin concentrations in patients with anaemia since hospital admission, which we labelled as anaemia of inflammation in its pathogenesis, not related to lung cancer treatment (in our case, surgery), but to the cancer itself. On the other hand, we found lower levels of hepcidin in patients who developed anaemia after surgery, which we labelled anaemia of iron deficiency in its pathogenesis. We proposed a diagnostic strategy that would enable clinicians to predict which lung cancer patients are at risk of developing anaemia after surgery. This type of strategy would aid in the reduction of post-operative side effects while also increasing the overall survival of lung cancer patients.

Our study had a few limitations, including the lack of iron distribution analysis in our lung cancer patients. We also struggled with a scarcity of comparable studies that could confirm or refute our hypotheses and findings. Despite meeting the high power of the power analysis test in the key examinations that supported our hypotheses, some analyses had insufficient power analysis. The number of patients in our study group should be increased for this purpose. Moreover, not every patient had a clear and defined endpoint. We reduced the risk of incorrectly classifying patients who might have developed anaemia

during their hospitalization as non-anaemia patients but having a full study group with a minimum of three CBCs examinations: upon admission to the hospital, on the day of surgery after surgery, and 3–4 days later would be the perfectly designed study for our analyses. Given these comments, our study should be regarded as a pilot study.

5. Conclusions

There is a need to incorporate hepcidin as a diagnostic marker into clinical practice, which necessitates the development of a quantitative, sensitive, quick, easy, and low-cost diagnostic method for routine diagnosis in diagnostic laboratories. In future studies, the cut-off values, at which hepcidin, IL-6, and CRP concentrations may differentiate future anaemia patients from patients who are not expected to develop anaemia, must be determined.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers15010224/s1, Figure S1: The prevalence of anaemia in women with operable lung cancer before and after surgery; Figure S2: The prevalence of anaemia in men with operable lung cancer before and after surgery; Table S1: Complete blood count parameters determined during hospitalization of women and men with operable lung cancer (dynamics of changes); Table S2: Hepcidin, IL-6, TNF-α, CRP, and SAA_1 concentrations in non-anaemia and anaemia women who had one complete blood count examination (upon hospital admission); Table S3: Hepcidin, IL-6, TNF-α, CRP, and SAA_1 concentrations in non-anaemia women and women with post-operative anaemia who had two complete blood counts; Table S4: Hepcidin, IL-6, and TNF-α concentrations in (a) males with anaemia since hospital admission without and with comorbidities, (b) males who developed anaemia during hospitalization without and with comorbidities, and (c) males with no anaemia during hospitalization without and with comorbidities; Table S5: Hepcidin, IL-6, TNF-α, CRP, and SAA_1 concentrations in non-anaemia and anaemia men who had one complete blood count examination (upon hospital admission); Table S6: Descriptive statistics of complete blood count parameters from the first and second complete blood count examinations, as well as hepcidin, IL-6, TNF-α, CRP, and SAA_1 levels in men with and without anaemia and men with post-operative anaemia.

Author Contributions: Conceptualization, K.W. and M.Ś.-M.; methodology, K.W. and M.Ś.-M.; formal analysis, K.W., P.B., A.R. and M.Ś.-M.; investigation, K.W., P.B., A.R. and M.Ś.-M.; resources, K.W., P.B. and A.R.; data curation, K.W.; writing—original draft preparation, K.W.; writing—review and editing, K.W., I.B.-L. and M.Ś.-M.; visualization, K.W.; supervision, M.Ś.-M.; project administration, K.W. and M.Ś.-M.; funding acquisition, I.B.-L. and M.Ś.-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the Ministry of Health subvention according to the number of SUBZ.D010.22.039 from the IT Simple system of Wroclaw Medical University.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Wroclaw Medical University (protocol code 106/2020, date of approval 25 February 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy and ethical restriction.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Horn, L.; Lovly, C.M. Chapter 74: Neoplasms of the Lung. In *Harrison's Principles of Internal Medicine*, 20th ed.; Jameson, J., Fauci, A.S., Kasper, D.L., Hauser, S.L., Longo, D.L., Loscalzo, J., Eds.; McGraw-Hill: New York, NY, USA, 2018.
2. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* **2021**, *71*, 209–249. [CrossRef] [PubMed]
3. Schwartz, A.G.; Prysak, G.M.; Bock, C.H.; Cote, M.L. The molecular epidemiology of lung cancer. *Carcinogenesis* **2007**, *28*, 507–518. [CrossRef] [PubMed]

4. Wadowska, K.; Bil-Lula, I.; Trembecki, Ł.; Śliwińska-Mossoń, M. Genetic Markers in Lung Cancer Diagnosis: A Review. *Int. J. Mol. Sci.* **2020**, *21*, 4569. [CrossRef] [PubMed]
5. Lu, T.; Yang, X.; Huang, Y.; Zhao, M.; Li, M.; Ma, K.; Yin, J.; Zhan, C.; Wang, Q. Trends in the incidence, treatment, and survival of patients with lung cancer in the last four decades. *Cancer Manag. Res.* **2019**, *11*, 943–953. [CrossRef]
6. Blohmer, J.-U.; Dunst, J.; Harrison, L.; Johnston, P.; Khayat, D.; Ludwig, H.; O'Brien, M.; van Belle, S.; Vaupel, P. Cancer-Related Anemia: Biological Findings, Clinical Implications and Impact on Quality of Life. *Oncology* **2005**, *68* (Suppl. S1), 12–21. [CrossRef]
7. Birgegård, G.; Aapro, M.S.; Bokemeyer, C.; Dicato, M.; Drings, P.; Hornedo, J.; Krzakowski, M.; Ludwig, H.; Pecorelli, S.; Schmoll, H.-J.; et al. Cancer-Related Anemia: Pathogenesis, Prevalence and Treatment. *Oncology* **2005**, *68* (Suppl. S1), 3–11. [CrossRef]
8. Groopman, J.E.; Itri, L.M. Chemotherapy-Induced Anemia in Adults: Incidence and Treatment. *J. Natl. Cancer Inst.* **1999**, *91*, 1616–1634. [CrossRef]
9. Ludwig, H.; Van Belle, S.; Barrett-Lee, P.; Birgegård, G.; Bokemeyer, C.; Gascón, P.; Kosmidis, P.; Krzakowski, M.; Nortier, J.; Olmi, P.; et al. The European Cancer Anaemia Survey (ECAS): A large, multinational, prospective survey defining the prevalence, incidence, and treatment of anaemia in cancer patients. *Eur. J. Cancer* **2004**, *40*, 2293–2306. [CrossRef]
10. Dicato, M.; Plawny, L.; Diederich, M. Anemia in cancer. *Ann. Oncol.* **2010**, *21* (Suppl. S7), 167–172. [CrossRef]
11. Knight, K.; Wade, S.; Balducci, L. Prevalence and outcomes of anemia in cancer: A systematic review of the literature. *Am. J. Med.* **2004**, *116* (Suppl. S7A), 11S–26S. [CrossRef]
12. Spivak, J.L.; Gascón, P.; Ludwig, H. Anemia Management in Oncology and Hematology. *Oncol.* **2009**, *14* (Suppl. S1), 43–56. [CrossRef]
13. Pirker, R.; Wiesenberger, K.; Pohl, G.; Minar, W. Anemia in Lung Cancer: Clinical Impact and Management. *Clin. Lung Cancer* **2003**, *5*, 90–97. [CrossRef] [PubMed]
14. Kang, H.S.; Shin, A.Y.; Yeo, C.D.; Park, C.K.; Kim, J.S.; Kim, J.W.; Kim, S.J.; Lee, S.H.; Kim, S.K. Clinical significance of anemia as a prognostic factor in non-small cell lung cancer carcinoma with activating epidermal growth factor receptor mutations. *J. Thorac. Dis.* **2020**, *12*, 1895–1902. [CrossRef]
15. Sasu, B.J.; Li, H.; Rose, M.J.; Arvedson, T.L.; Doellgast, G.; Molineux, G. Serum hepcidin but not prohepcidin may be an effective marker for anemia of inflammation (AI). *Blood Cells Mol. Dis.* **2010**, *45*, 238–245. [CrossRef]
16. Ferrucci, L.; Fabbri, E. Inflammageing: Chronic inflammation in ageing, cardiovascular disease, and frailty. *Nat. Rev. Cardiol.* **2018**, *15*, 505–522. [CrossRef] [PubMed]
17. Kovacs, E.J.; Boe, D.M.; Boule, L.A.; Curtis, B.J. Inflammaging and the Lung. *Clin. Geriatr. Med.* **2017**, *33*, 459–471. [CrossRef]
18. Morceau, F.; Dicato, M.; Diederich, M. Pro-Inflammatory Cytokine-Mediated Anemia: Regarding Molecular Mechanisms of Erythropoiesis. *Mediat. Inflamm.* **2009**, *2009*, 405016. [CrossRef] [PubMed]
19. Bryer, E.; Henry, D. Chemotherapy-induced anemia: Etiology, pathophysiology, and implications for contemporary practice. *Int. J. Clin. Transfus. Med.* **2018**, *6*, 21–31. [CrossRef]
20. Harrison, L.B.; Chadha, M.; Hill, R.J.; Hu, K.; Shasha, D. Impact of Tumor Hypoxia and Anemia on Radiation Therapy Outcomes. *Oncologist* **2002**, *7*, 492–508. [CrossRef]
21. Aapro, M.; Beguin, Y.; Bokemeyer, C.; Dicato, M.; Gascón, P.; Glaspy, J.; Hofmann, A.; Link, H.; Littlewood, T.; Ludwig, H.; et al. Management of anaemia and iron deficiency in patients with cancer: ESMO Clinical Practice Guidelines. *Ann. Oncol.* **2018**, *29* (Suppl. S4), iv96–iv110. [CrossRef]
22. Kim, A.; Rivera, S.; Shprung, D.; Limbrick, D.; Gabayan, V.; Nemeth, E.; Ganz, T. Mouse Models of Anemia of Cancer. *PLoS ONE* **2014**, *9*, e93283. [CrossRef]
23. Franceschi, C.; Bonafe, M.; Valensin, S.; Olivieri, F.; De Luca, M.; Ottaviani, E.; De Benedictis, G. Inflamm-aging: An evolutionary perspective on immunosenescence. *Ann. N. Y. Acad. Sci.* **2000**, *908*, 244–254. [CrossRef]
24. Macciò, A.; Madeddu, C.; Massa, D.; Mudu, M.C.; Lusso, M.R.; Gramignano, G.; Serpe, R.; Melis, G.B.; Mantovani, G. Hemoglobin levels correlate with interleukin-6 levels in patients with advanced untreated epithelial ovarian cancer: Role of inflammation in cancer-related anemia. *Blood* **2005**, *106*, 362–367. [CrossRef]
25. Tang, H.; Bai, Y.; Shen, W.; Wei, Y.; Xu, M.; Zhou, X.; Zhao, J. Clinical significance of combined detection of interleukin-6 and tumour markers in lung cancer. *Autoimmunity* **2018**, *51*, 191–198. [CrossRef]
26. Wadowska, K.; Błasiak, P.; Rzechonek, A.; Bil-Lula, I.; Śliwińska-Mossoń, M. New Insights on Old Biomarkers Involved in Tumor Microenvironment Changes and Their Diagnostic Relevance in Non-Small Cell Lung Carcinoma. *Biomolecules* **2021**, *11*, 1208. [CrossRef]
27. Wrighting, D.M.; Andrews, N.C. Interleukin-6 induces hepcidin expression through STAT3. *Blood* **2006**, *108*, 3204–3209. [CrossRef]
28. Milan-Mattos, J.; Anibal, F.; Perseguini, N.; Minatel, V.; Rehder-Santos, P.; Castro, C.; Vasilceac, F.; Mattiello, S.; Faccioli, L.; Catai, A. Effects of natural aging and gender on pro-inflammatory markers. *Braz. J. Med. Biol. Res.* **2019**, *52*, e8392. [CrossRef]
29. Albain, K.S.; Crowley, J.J.; Leblanc, M.; Livingston, R.B. Survival determinants in extensive-stage non-small-cell lung cancer: The Southwest Oncology Group experience. *J. Clin. Oncol.* **1991**, *9*, 1618–1626. [CrossRef]
30. Blick, M.; Sherwin, S.A.; Rosenblum, M.; Gutterman, J. Phase I study of recombinant tumor necrosis factor in cancer patients. *Cancer Res.* **1987**, *47*, 2986–2989.
31. Vela, D.; Vela-Gaxha, Z. Differential regulation of hepcidin in cancer and non-cancer tissues and its clinical implications. *Exp. Mol. Med.* **2018**, *50*, e436. [CrossRef]

32. Słomka, A.; Łęcka, M.; Styczyński, J. Hepcidin in Children and Adults with Acute Leukemia or Undergoing Hematopoietic Cell Transplantation: A Systematic Review. *Cancers* **2022**, *14*, 4936. [CrossRef]
33. Di Grazia, A.; Di Fusco, D.; Franzè, E.; Colella, M.; Strimpakos, G.; Salvatori, S.; Formica, V.; Laudisi, F.; Maresca, C.; Colantoni, A.; et al. Hepcidin Upregulation in Colorectal Cancer Associates with Accumulation of Regulatory Macrophages and Epithelial–Mesenchymal Transition and Correlates with Progression of the Disease. *Cancers* **2022**, *14*, 5294. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Supplementary Materials

Hepcidin as a Diagnostic Biomarker in Anaemic Lung Cancer Patients

Katarzyna Wadowska [1,*], Piotr Błasiak [2,3], Adam Rzechonek [2,3], Iwona Bil-Lula [1] and Mariola Śliwińska-Mossoń [1]

1. Department of Medical Laboratory Diagnostics, Division of Clinical Chemistry and Laboratory Haematology, Faculty of Pharmacy, Wroclaw Medical University, Borowska 211A, 50-556 Wroclaw, Poland
2. Department and Clinic of Thoracic Surgery, Faculty of Medicine, Wroclaw Medical University, Grabiszyńska 105, 53-439 Wroclaw, Poland
3. Lower Silesian Centre of Oncology, Pulmonology and Haematology, Lower Silesian Thoracic Surgery Centre, Grabiszyńska 105, 53-439 Wroclaw, Poland

* Correspondence: katarzyna.wadowska@student.umw.edu.pl; Tel.: +48-71-784-0626

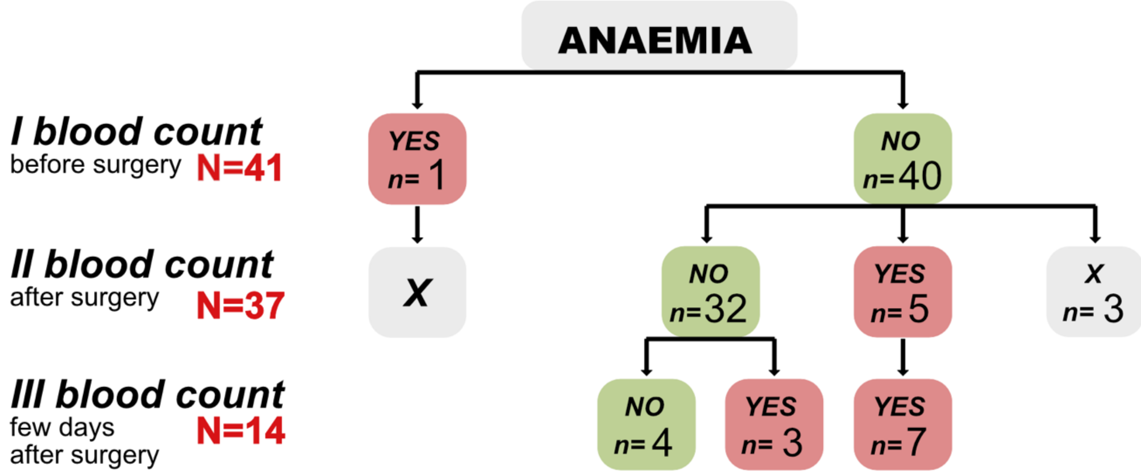


Figure S1. The prevalence of anaemia in women with operable lung cancer before and after surgery.

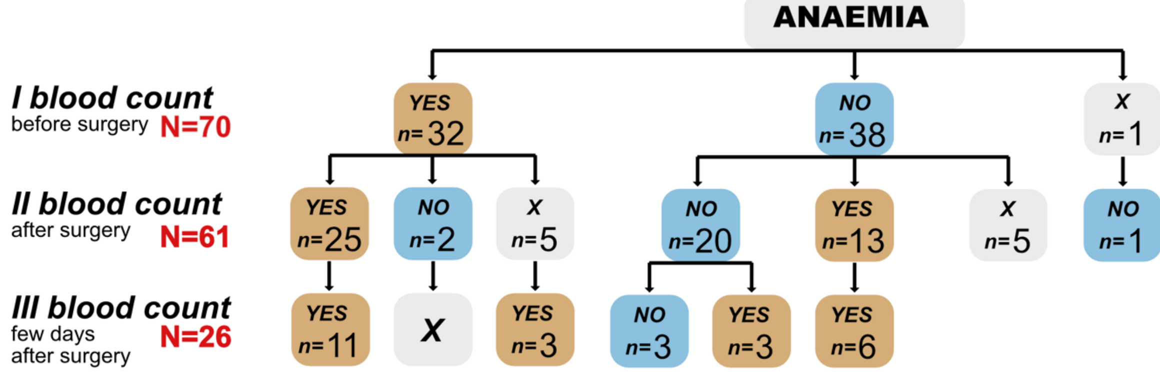


Figure S2. The prevalence of anaemia in men with operable lung cancer before and after surgery.

Table S1. Complete blood count parameters determined during hospitalization of women and men with operable lung cancer (dynamics of changes).

	Before surgery	After surgery (on the day of surgery)	3-4 days after surgery	Laboratory's reference values
Women	N=41	N=37	N=14	
Hb [g/dL] ± SEM	13.15 ± 0.20	12.44 ± 0.23	**10.13 ± 0.46**	11.2 – 15.7
Ht [%] ± SEM	39.39 ± 0.51	37.16 ± 0.64	**30.71 ± 1.32**	34 – 45
RBC [10^6/µL] ± SEM	4.37 ± 0.06	4.13 ± 0.08	**3.42 ± 0.14**	3.93 – 5.22
MCV [fL] ± SEM	90.44 ± 0.80	90.16 ± 0.88	90.21 ± 1.33	79 – 95
MCH [pg] ± SEM	30.15 ± 0.31	30.19 ± 0.35	29.64 ± 0.46	26 – 32
MCHC [g/dL] ± SEM	33.33 ± 0.15	33.44 ± 0.17	32.77 ± 0.22	32.2 – 35.5
RDW-SD [fL] ± SEM	44.93 ± 0.66	44.31 ± 0.69	43.46 ± 0.93	36.4 – 46.3
RDW-CV [%] ± SEM	13.96 ± 0.20	13.79 ± 0.21	14.10 ± 0.27	11.7 – 14.4
Men	N=71	N=60	N=26	
Hb [g/dL] ± SEM	**13.39 ± 0.18**	**12.71 ± 0.22**	**10.99 ± 0.35**	13.7 – 17.5
Ht [%] ± SEM	**40.19 ± 0.47**	**38.15 ± 0.59**	**33.46 ± 0.99**	40 – 51
RBC [10^6/µL] ± SEM	**4.44 ± 0.06**	**4.20 ± 0.08**	**3.69 ± 0.12**	4.63 – 6.08
MCV [fL] ± SEM	90.90 ± 0.79	91.52 ± 0.82	91.04 ± 0.87	79 – 92
MCH [pg] ± SEM	30.33 ± 0.32	30.51 ± 0.32	29.85 ± 0.35	26 – 32
MCHC [g/dL] ± SEM	33.28 ± 0.15	33.28 ± 0.15	32.75 ± 0.20	32.3 – 36.5
RDW-SD [fL] ± SEM	**46.32 ± 0.52**	**46.16 ± 0.59**	**46.08 ± 0.89**	35.1 – 43.9
RDW-CV [%] ± SEM	14.33 ± 0.16	14.18 ± 0.18	14.31 ± 0.24	11.6 – 14.4

Hb – haemoglobin; Ht – haematocrit; RBC – red blood count; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; RDW-SD – red cell distribution width-standard deviation; RDW-CV – red cell distribution width-coefficient of variation; SEM – standard error of the mean; bold black text indicates mean values above laboratory's references, whereas bold blue text indicates mean values below laboratory's references

Table S2. Hepcidin, IL-6, TNF-α, CRP, and SAA_1 concentrations in non-anaemia and anaemia women who had one complete blood count examination (upon hospital admission).

	Anaemia upon admission to the hospital	
Parameter	**No-anaemia (N=3)**	**Anaemia (N=1)**
Hepcidin [ng/mL]		
Mean ± SEM	80.02 ± 34.16	194.56
Median	109.16	
Min – Max	11.94 – 118.97	
IL-6 [pg/mL]		
Mean ± SEM	8.74 ± 1.56	126.81
Median	7.26	
Min – Max	7.11 – 11.86	
TNF-α [pg/mL]		
Mean ± SEM	2.09 ± 0.73	302.38
Median	1.38	
Min – Max	1.35 – 3.55	
CRP [mg/L]		
Mean ± SEM	9.57 ± 6.77	2.78
Median	5.28	
Min – Max	0.60 – 22.84	
SAA_1 [µg/mL]		
Mean ± SEM	131.07 ± 71.62	81.11
Median	131.07	
Min – Max	59.45 – 202.68	

IL-6 – interleukin 6; TNF-α - tumour necrosis factor α; CRP – C-reactive protein; SAA_1 – serum amyloid A_1; SEM – standard error of the mean

Table S3. Hepcidin, IL-6, TNF-α, CRP, and SAA_1 concentrations in non-anaemia women and women with post-operative anaemia who had two complete blood counts.

	Post-operative anaemia in the day of surgery	
Parameter	**No-anaemia (N=21)**	**Developed anaemia (N=2)**
Hepcidin [ng/mL]		
Mean ± SEM	106.33 ± 15.30	49.75 ± 16.41
Median	91.33	49.75
Min – Max	15.20 – 239.36	33.34 – 66.16
IL-6 [pg/mL]		
Mean ± SEM	26.42 ± 3.69	31.54 ± 26.08
Median	24.39	31.54
Min – Max	0.32 – 57.28	5.46 – 57.61
TNF-α [pg/mL]		
Mean ± SEM	2.27 ± 0.24	2.14 ± 0.43
Median	2.06	2.14
Min – Max	1.21 – 5.54	1.71 – 2.56
CRP [mg/L]		
Mean ± SEM	8.04 ± 4.12	10.92 ± 10.32
Median	2.13	10.92
Min – Max	0.25 – 84.72	0.60 – 21.23
SAA_1 [μg/mL]		
Mean ± SEM	81.59 ± 23.51	134.83 ± 3.53
Median	7.33	134.83
Min – Max	0.21 – 330.01	131.30 – 138.35

IL-6 – interleukin 6; TNF-α - tumour necrosis factor α; CRP – C-reactive protein; SAA_1 – serum amyloid A_1; SEM – standard error of the mean

Table S4. Hepcidin, IL-6, and TNF-α concentrations in (a) males with anaemia since hospital admission without and with comorbidities, (b) males who developed anaemia during hospitalization without and with comorbidities, and (c) males with no anaemia during hospitalization without and with comorbidities

(a)	Males with anaemia since hospital admission	
Parameter	**Without comorbidities**	**With comorbidities**
Hepcidin [ng/mL]		
Mean ± SEM	156.77 ± 33.35	177.44 ± 34.21
Median	180.89	144.02
Min – Max	0.08 – 380.71	1.58 – 525.15
IL-6 [pg/mL]		
Mean ± SEM	21.92 ± 5.32[a]	46.33 ± 5.61[a]
Median	22.24	45.87
Min – Max	0.36 – 59.84	3.97 – 112.70
TNF-α [pg/mL]		
Mean ± SEM	4.05 ± 1.69	2.68 ± 0.41
Median	2.38	2.21
Min – Max	0.67 – 20.57	0.57 – 8.92
(b)	Males who developed anaemia during their hospitalization	
	Without comorbidities	**With comorbidities**
Hepcidin [ng/mL]		
Mean ± SEM	234.75 ± 82.20	161.41 ± 33.78
Median	259.62	115.17

Min – Max	81.59 – 363.05	39.16 – 429.52
IL-6 [pg/mL]		
Mean ± SEM	28.31 ± 5.78	46.81 ± 11.42
Median	27.42	46.14
Min – Max	18.77 – 38.74	0.21 – 113.83
TNF-α [pg/mL]		
Mean ± SEM	2.43 ± 0.54	11.19 ± 8.40
Median	2.43	1.92
Min – Max	1.89 – 2.96	1.29 – 111.55
(c)	Males with no anaemia during their hospitalization	
	Without comorbidities	**With comorbidities**
Hepcidin [ng/mL]		
Mean ± SEM	203.60 ± 22.96	141.55 ± 23.83
Median	169.25	143.16
Min – Max	128.42 – 353.74	31.44 – 247.96
IL-6 [pg/mL]		
Mean ± SEM	32.50 ± 9.73	21.50 ± 3.78
Median	21.60	26.13
Min – Max	5.26 – 100.72	5.80 – 37.00
TNF-α [pg/mL]		
Mean ± SEM	2.86 ± 0.59	4.42 ± 1.07
Median	1.93	3.01
Min – Max	1.30 – 7.61	1.46 – 10.95

IL-6 – interleukin 6; TNF-α - tumour necrosis factor α; SEM – standard error of the mean; Statistical significances, Student's t-test: [a]p=0.005271 in IL-6 concentrations between males with anaemia since hospital admission and without comorbidities, and males with anaemia since hospital admission and with comorbidities

Table S5. Hepcidin, IL-6, TNF-α, CRP, and SAA_1 concentrations in non-anaemia and anaemia men who had one complete blood count examination (upon hospital admission).

	Anaemia upon admission to the hospital	
Parameter	**No-anaemia (N=5)**	**Anaemia (N=2)**
Hepcidin [ng/mL]		
Mean ± SEM	155.97 ± 49.01	82.09 ± 3.84
Median	154.87	82.09
Min – Max	31.44 – 295.55	78.25 – 85.92
IL-6 [pg/mL]		
Mean ± SEM	12.91 ± 2.72	15.59 ± 7.56
Median	10.60	15.59
Min – Max	9.80 – 18.33	8.03 – 23.15
TNF-α [pg/mL]		
Mean ± SEM	2.69 ± 0.41	3.19 ± 1.25
Median	2.78	3.19
Min – Max	1.71 – 4.01	1.94 – 4.44
CRP [mg/L]		
Mean ± SEM	12.01 ± 8.70	65.81 ± 15.60
Median	4.40	65.81
Min – Max	0.60 – 46.46	50.21 – 81.40
SAA_1 [μg/mL]		
Mean ± SEM	61.54 ± 57.38	150.90 ± 47.32
Median	5.19	150.90
Min – Max	1.26 – 291.01	103.58 – 198.21

IL-6 – interleukin 6; TNF-α - tumour necrosis factor α; CRP – C-reactive protein; SAA_1 – serum amyloid A_1; SEM – standard error of the mean

Table S6. Descriptive statistics of complete blood count parameters from the first and second complete blood count examinations, as well as hepcidin, IL-6, TNF-α, CRP, and SAA_1 levels in men with and without anaemia and men with post-operative anaemia.

	The prevalence of anaemia in the day of surgery (after surgery)					
	No-anaemia (N=15)		Developed anaemia (N=7)		Anaemia (N=16)	
Parameter	**1st CBC**	**2nd CBC**	**1st CBC**	**2nd CBC**	**1st CBC**	**2nd CBC**
NCI grading [n (%)]						
Grade 0	15 (100%)	15 (100%)	7 (100%)	-	-	-
Grade 1	-	-	-	7 (100%)	16 (100%)	12 (75%)
Grade 2	-	-	-	-	-	4 (25%)
Grade 3	-	-	-	-	-	-
Grade 4	-	-	-	-	-	-
Hb [g/dL]						
Mean ± SEM	14.82 ± 0.18[a,b]	14.30 ± 0.16	13.96 ± 0.15[a,c]	12.70 ± 0.24	11.83 ± 0.27[b,c]	11.28 ± 0.34
Median	14.80	14.20	13.80	12.80	11.80	11.05
Min – Max	13.80 – 16.60	13.50 – 15.40	13.50 – 14.60	11.40 – 13.30	10.00 – 13.40	9.40 – 13.80
RBC [mln/μL]						
Mean ± SEM	4.75 ± 0.08	4.58 ± 0.09	4.57 ± 0.10	4.26 ± 0.15	3.99 ± 0.15	3.78 ± 0.14
Median	4.79	4.66	4.61	4.27	4.06	3.69
Min – Max	4.23 – 5.24	4.00 – 5.17	4.25 – 4.91	3.70 – 4.92	2.83 – 5.27	2.94 – 4.63
Hepcidin [ng/mL]						
Mean ± SEM	161.50 ± 12.41[d]		90.84 ± 21.93[d]		126.50 ± 33.29	
Median	165.90		81.59		140.67	
Min – Max	85.39 – 243.65		39.16 – 205.37		0.08 – 344.26	
IL-6 [pg/mL]						
Mean ± SEM	21.34 ± 3.69		25.93 ± 9.93		29.08 ± 5.52	
Median	24.86		18.77		34.37	
Min – Max	5.26 – 46.68		10.56 – 64.75		2.63 – 58.34	
TNF-α [pg/mL]						
Mean ± SEM	4.12 ± 0.85		19.23 ± 15.45		3.60 ± 1.31	
Median	2.41		2.96		1.91	
Min – Max	1.30 – 10.95		1.29 – 111.55		0.57 – 20.57	
CRP [mg/L]						
Mean ± SEM	21.58 ± 8.52		19.98 ± 12.53		32.79 ± 12.25	
Median	1.22		7.87		12.35	
Min – Max	0.22 – 106.44		1.08 – 93.34		0.60 – 153.93	
SAA_1 [μg/mL]						
Mean ± SEM	101.86 ± 38.96		105.15 ± 53.91		119.10 ± 30.52	
Median	10.96		19.83		127.67	
Min – Max	0.62 – 548.31		2.63 – 336.87		0.75 – 395.53	

CBC – complete blood count; NCI – National Cancer Institute; Hb – haemoglobin; RBC – red blood count; IL-6 – interleukin 6; TNF-α - tumour necrosis factor α; CRP – C-reactive protein; SAA_1 – serum amyloid A_1; SEM – standard error of the mean; Statistical significances, One-way ANOVA: [a]p=0.023919 in haemoglobin concentration upon hospital admission between men who did not have anaemia and men who developed anaemia post-operatively; [b]p=0.000123 in haemoglobin concentration upon hospital admission between men who did not have anaemia and men who had anaemia; [c]p=0.000123 in haemoglobin concentration upon hospital admission between men who developed anaemia post-operatively and men who had anaemia; [d]p=0.007905 in hepcidin concentration between men without anaemia and men that developed anaemia post-operatively

13.4. Załącznik 4 – III artykuł oryginalny

Katarzyna Wadowska, Piotr Błasiak, Adam Rzeczonek, Mariola Śliwińska-Mossoń: Analysis of MMP-2 -735C/T (rs2285053) and MMP-9 -1562C/T (rs3918242) polymorphisms in the risk assessment of developing lung cancer. *International Journal of Molecular Sciences*, **2023**, vol. 24, nr 13, art. 10576 [20 s.], DOI:10.3390/ijms241310576

International Journal of
Molecular Sciences

MDPI

Article

Analysis of *MMP-2*-735C/T (rs2285053) and *MMP-9*-1562C/T (rs3918242) Polymorphisms in the Risk Assessment of Developing Lung Cancer

Katarzyna Wadowska [1], Piotr Błasiak [2,3], Adam Rzechonek [3] and Mariola Śliwińska-Mossoń [1,*]

1 Department of Medical Laboratory Diagnostics, Division of Clinical Chemistry and Laboratory Haematology, Wroclaw Medical University, Borowska 211A, 50-556 Wroclaw, Poland; katarzyna.wadowska@student.umw.edu.pl
2 Department and Clinic of Thoracic Surgery, Wroclaw Medical University, Grabiszyńska 105, 53-439 Wroclaw, Poland; blasiakpiotr@gmail.com
3 Department of Thoracic Surgery, Lower Silesian Centre of Oncology, Lung Diseases and Haematology, Grabiszyńska 105, 53-439 Wroclaw, Poland; adam.rzechonek@gmail.com
* Correspondence: mariola.sliwinska-mosson@umw.edu.pl

Abstract: Matrix metalloproteinase (MMP)-2 and -9 are gelatinases which are capable of degrading type IV collagen and have been linked to cancer invasion and metastatic development. MMP-2 and MMP-9 gene polymorphisms may affect their biological function, and thus their role in cancer development and progression. We analyzed the association of the polymorphism frequencies of *MMP-2*-735C/T and *MMP-9*-1562C/T with MMP-2 and MMP-9 serum concentrations, as well as their potential effects in lung cancer patients. We conducted a retrospective, case-control study consisting of 112 lung cancer patients and 100 healthy individuals from a Caucasian population in Poland. Polymerase chain reaction with restriction fragment length polymorphism (PCR/RFLP) and electrophoresis was used to genotype genomic DNA from whole blood samples. MMP-2 and MMP-9 serum concentrations were then determined using ELISA. For statistical analysis, Statistica version 13 from TIBCO Software Inc. was utilized with a significance level <0.05. Logistic regression analysis revealed that *MMP-2*-735CC (OR = 5.39; 95% CI = 0.62–47.17; $p = 0.238504$) and -735CT genotype (OR = 7.22; 95% CI = 0.78–67.14; $p = 0.072836$), as well as *MMP-9*-1562CC (OR = 1.45; 95% CI = 0.31–6.70; $p = 0.757914$) and -1562CT genotype (OR = 1.60; 95% CI = 0.33–7.83; $p = 0.548801$) were associated with a higher risk of lung cancer. There were statistically significant differences observed in the MMP-2 concentration between individuals with the -735CC genotype and the -735CT genotype (non-smoking control: 204.04 ng/mL vs. 237.00 ng/mL, respectively, $p = 0.041479$; adenocarcinoma patients: 157.69 ng/mL vs. 126.37 ng/mL, respectively, $p = 0.013222$), as well as differences in the MMP-9 concentration between individuals with the -1562CC genotype and the -1562CT genotype (smoking control: 385.67 ng/mL vs. 562.80 ng/mL, respectively, $p = 0.000936$; patients with other lung neoplasms: 821.64 ng/mL vs. 928.88 ng/mL, respectively $p = 0.023315$). The role of *MMP-2*-735C/T and *MMP-9* -1562C/T polymorphisms in an increased risk of lung cancer cannot be dismissed. Specific genotypes affect MMP-2 and MMP-9 concentrations in both lung cancer patients and healthy controls, which may thereby increase lung cancer risk, disease aggressiveness, and patient survival outcomes.

Keywords: lung cancer; polymorphism; matrix metalloproteinases; *MMP-2*-735C/T; rs2285053; *MMP-9*-1562C/T; rs3918242; ethnicity

Citation: Wadowska, K.; Błasiak, P.; Rzechonek, A.; Śliwińska-Mossoń, M. Analysis of *MMP-2*-735C/T (rs2285053) and *MMP-9*-1562C/T (rs3918242) Polymorphisms in the Risk Assessment of Developing Lung Cancer. *Int. J. Mol. Sci.* **2023**, *24*, 10576. https://doi.org/10.3390/ijms241310576

Academic Editors: Cristina Peña and Robert Arthur Kratzke

Received: 9 May 2023
Revised: 12 June 2023
Accepted: 20 June 2023
Published: 24 June 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes which are capable of cleaving extracellular matrix (ECM) proteins. The first identified MMP was MMP-1, which was discovered in 1962 by Gross and Lapierre [1] as a result of collagen

remodeling during tadpole tail metamorphosis. Since then, more than 28 MMPs have been discovered [2]. MMPs play a role in a variety of biological processes that occur during embryonic development, organogenesis, and wound healing, such as cell proliferation, migration, differentiation, tissue invasion, and vascularization. On the other hand, MMPs have piqued the interest of researchers due to their overexpression in numerous human disorders, including cardiovascular diseases, inflammatory diseases, lung and liver diseases, and malignancies, with lung cancer taking the lead [2–5].

Lung cancer accounts for nearly 25% of all cancer deaths worldwide. In 2020, an estimated 1,796,144 people died from lung cancer worldwide, which is not much fewer than the combined deaths from colorectal (935,173), breast (684,996), and prostate (375,304) cancer [6,7]. Such a high mortality rate of lung cancer patients is the effect, among others, of the fact that the vast majority of patients (75%) are diagnosed at an advanced stage of the disease, when treatment options are limited [8–12].

The leading cause of lung cancer development is tobacco use. Approximately 90% of lung cancer patients have a smoking history. However, only 10–20% (depending on the source of data) of people who smoke throughout their lives develop lung cancer [13–15]. Differences in these numbers imply that other factors, such as genetic susceptibility in the form of genotypic and phenotypic variables, including genetic polymorphism, contribute to lung carcinogenesis and influence individual differences in response to environmental factors, with carcinogens implicated in tobacco smoke exposure [9,13,16–18]. Over the last two decades, researchers have focused on low-penetrance genes involved in carcinogen metabolism and the DNA repair of damage caused by tobacco smoke, as well as changes in genes encoding proteins implicated in tumor formation, growth, and dissemination [19,20].

In the present study, we focused on low-penetrance variations in two genes of two MMPs, MMP-2 and MMP-9, which, along with MMP-1 and MMP-7, are the primary MMPs responsible for ECM remodeling in lung tissue [21,22]. MMP-2 and MMP-9 belong to gelatinases that, by degrading type IV collagen in the basement membrane, can contribute to carcinogenesis processes, such as cell proliferation, angiogenesis, and tumor metastasis when their activity is dysregulated [23–25]. MMP activity is regulated at several levels, including gene expression, compartmentalization, proenzyme activation, and enzyme inactivation [26–28].

The main studied polymorphisms of *MMP-2* are rs243865 and rs2285053, which are located in the *MMP-2* promoter at positions -1306 and -735, respectively, while the *MMP-9* polymorphism rs3918242 is located in the *MMP-9* promoter at position -1562, all of which induce the transition of the allele C to T [25]. *MMP-2* and *MMP-9* promoter polymorphisms can affect mRNA and protein expression levels by modifying transcriptional activity, eventually leading to the development of several types of cancer, including breast, lung, esophageal, and colorectal cancer [29–32]. According to growing research, *MMP-2* appears to also have a key role in the metastasis of a number of malignancies, including glioma and colorectal cancer [33,34].

In recent years, numerous genetic studies on *MMP-2* and *MMP-9*, along with their roles in cancer risk have been published. The majority of these studies, however, have been focused on breast, colorectal, or prostate cancer. In the previous decade, just five studies on the role of *MMP-2* and *MMP-9* polymorphism in lung cancer patients have been published and indexed in the MEDLINE (PubMed) database, which appears to be an understatement given the seriousness of the lung cancer problem. The objective of this study was to re-examine polymorphisms in the genes encoding the gelatinases MMP-2 and MMP-9 at positions -735C/T (rs2285053) and -1562C/T (rs3918242), respectively, and their potential effects on lung cancer, with our rationale for conducting this research being that our understanding of lung cancer has improved over the last decade. We sought answers to the following questions: (1) Can we identify populations at an increased risk of developing lung cancer by analyzing *MMP-2*-735C/T and *MMP-9*-1562C/T polymorphisms? (2) Do the polymorphisms *MMP-2*-735C/T and *MMP-9*-1562C/T affect the MMP-2 and MMP-9

concentrations? (3) Does the prevalence of certain polymorphic variants of *MMP-2* (735C/T) and *MMP-9* (1562C/T) vary amongst lung cancer subtypes?

2. Results

2.1. Characteristics of Cases and Controls

The study included 112 lung cancer patients and 100 healthy controls, including 47 non-smokers and 51 smokers, respectively. The selected characteristics of lung cancer patients and healthy individuals are summarized in Table 1. There were significant differences observed in the distribution of basic characteristics, such as age, gender, and smoking status between the cases and the controls. Lung cancer patients were mostly over the age of 60 (80.4% of patients), with an advantage of males (63.4%), and moderate (29.5%) to heavy (25.9%) smokers, whereas healthy individuals were mostly under the age of 60 (85.0% of controls), more often females (54.0%), and if smokers—light smokers (37.0%).

Table 1. Selected characteristics of the lung cancer patients and controls.

Variable	Cases [*n*, (%)] *n* = 112	Controls [*n*, (%)] *n* = 100	*p*-Value (Pearson's Chi-Square Test)
Age [years]			
≤60	22 (19.6%)	85 (85.0%)	$p < 0.00001$
>60	90 (80.4%)	15 (15.0%)	
Gender			
Male	71 (63.4%)	46 (46.0%)	$p = 0.01102$
Female	41 (36.6%)	54 (54.0%)	
Smoking status			
Never smoker	3 (2.7%)	47 (47.0%)	$p < 0.00001$
Light smoker	7 (6.3%)	37 (37.0%)	
Moderate smoker	33 (29.5%)	11 (11.0%)	
Heavy smoker	29 (25.9%)	2 (2.0%)	
NA	40 (35.7%)	3 (3.0%)	
MMP-2-735 C/T			
CC	83 (74.1%)	77 (77.0%)	$p = 0.14379$
CT	26 (23.2%)	18 (18.0%)	
TT	1 (0.9%)	5 (5.0%)	
NA	2 (1.8%)	0 (0.0%)	
MMP-9-1562 C/T			
CC	76 (67.9%)	70 (70.0%)	$p = 0.83358$
CT	30 (26.8%)	25 (25.0%)	
TT	3 (2.7%)	4 (4.0%)	
NA	3 (2.7%)	1 (1.0%)	

Notes: Light smokers are defined as people who smoke from >0 to <20 pack-years; moderate smokers are defined as people who smoke from ≥20 to <40 pack-years; and heavy smokers are defined as people who smoke ≥40 pack-years. NA—not available.

Table 2 presents basic information regarding the picked and analyzed SNPs, as well as the allele frequency distributions among the cases and controls. The Hardy–Weinberg equilibrium (HWE) was used to verify the observed *MMP-2*-735C/T and *MMP-9* -1562C/T genotype frequencies in the cases and controls (Table S1 of the Supplementary Materials). In the control group, we found a minor discrepancy in the frequency of the *MMP-2*-735C/T genotypes ($p = 0.041271$). When we subdivided the control group into non-smokers and smokers, we found a statistically significant difference between the observed and expected frequencies of the *MMP-2*-735C/T genotypes among non-smokers ($p = 0.012686$), but not among smokers ($p = 0.600091$).

Table 2. Characteristics of the analyzed single nucleotide variations.

SNP	Gene	Band	Position	Alleles	Molecular Consequences	MAF—Cases	MAF—Controls	*p*-Value
rs2285053	*MMP-2*	16q12.2	55478465	C>T	2KB upstream variant	0.1273	0.1400	0.7376
rs3918242	*MMP-9*	20q13.2	46007337	C>T	2KB upstream variant	0.1651	0.1667	0.9717

In the analyses of both the *MMP-2*-735C/T and *MMP-9*-1562C/T polymorphisms, there were no statistically significant variations observed in the minor allele frequency (MAF) values between the patients and controls. Furthermore, when we investigated the relationship between the *MMP-2*-735C/T and *MMP-9*-1562C/T polymorphisms and ethnicity, we discovered that the MAFs received in our study's population (0.1333 and 0.1659, respectively) correspond to MAFs that were obtained in other studies conducted on the European population (0.1101 and 0.1666, respectively).

We also conducted an analysis using the available data on the *MMP-2*-735C/T and *MMP-9*-1562C/T MAFs in various populations to examine whether there is any association between the ethnic distribution and the MMP mutation risk (Table 3). When we compared five major populations, i.e., European, East Asian, South Asian, American, and African, we found that the East Asian population (0.2591) had a statistically significantly higher T allele frequency of *MMP-2*-735C/T than the European (0.1101, $p = 0.000134$), South Asian (0.1454, $p = 0.000132$), African (0.1164, $p = 0.000169$), and American (0.1761, $p = 0.000254$) populations. Moreover, we observed statistically significantly higher MAF values in the American population compared to the European ($p = 0.006904$) and African ($p = 0.008623$) populations. There were no similar trends observed in the *MMP-9*-1562C/T T allele frequency. We observed the highest MAF values in the South Asian (0.2343) and African (0.2199) populations, and the lowest in the American (0.0809) population, with their *p*-values being at the limit of statistical significance of 0.053710 and 0.064237, respectively.

Table 3. Descriptive statistics on the minor allele frequencies of rs2285053 and rs3918242 across various populations.

Population	N	Mean ± SEM	Median	Min–Max
MMP-2-735C/T (rs2285053)				
European	9	0.1101 ± 0.0087 [a,b]	0.1133	0.0500–0.1346
East Asian	9	0.2591 ± 0.0047 [a,c,d,e]	0.2604	0.2368–0.2784
South Asian	5	0.1454 ± 0.0280 [c]	0.1186	0.1110–0.2570
African	5	0.1164 ± 0.0020 [d,f]	0.1153	0.1105–0.1221
American	3	0.1761 ± 0.0048 [b,e,f]	0.1801	0.1666–0.1816
Semitic	2	0.1411 ± 0.0024	0.1411	0.1387–0.1435
Latin 1	2	0.1227 ± 0.0132	0.1227	0.1096–0.1358
Latin 2	2	0.1872 ± 0.0068	0.1872	0.1803–0.1940
Other	2	0.1393 ± 0.0102	0.1393	0.1291–0.1494
MMP-9-1562C/T (rs3918242)				
European	8	0.1666 ± 0.0051	0.1671	0.1463–0.1873
East Asian	8	0.1583 ± 0.0110	0.1562	0.1294–0.2260
South Asian	4	0.2343 ± 0.0415 *	0.2427	0.1250–0.3269
African	5	0.2199 ± 0.0788 **	0.1214	0.1103–0.5231
American	3	0.0809 ± 0.0039 *,**	0.0796	0.0749–0.0883
Semitic	2	0.1610 ± 0.0057	0.1610	0.1553–0.1667

Table 3. *Cont.*

Population	N	Mean ± SEM	Median	Min–Max
Latin 1	1	0.1096	0.1096	
Latin 2	1	0.0836	0.0836	
Other	2	0.1741	0.1741	0.1257–0.2225

Notes: N—number of studies included; SEM—standard error of the mean; statistical significances, one-way ANOVA: [a] $p = 0.000134$ between Europeans and East Asians, [b] $p = 0.006904$ between Europeans and Americans, [c] $p = 0.000132$ between East Asians and South Asians, [d] $p = 0.000169$ between East Asians and Africans, [e] $p = 0.000254$ between East Asians and Americans, and [f] $p = 0.008623$ between Africans and Americans in the rs2285053 MAF; * $p = 0.053710$ between South Asians and Americans, and ** $p = 0.064237$ between Africans and Americans in the rs3918242 MAF.

Next we investigated whether the *MMP-2*-735C/T and *MMP-9*-1562C/T genotypes were associated with environmental factors in lung cancer patients and healthy controls, as well as clinicopathological characteristics in lung cancer patients (Table 4). The only significant association we observed was between age and the *MMP-9*-1562C/T genotype in lung cancer patients ($p = 0.03854$), with lung cancer patients with the CT and TT genotypes being found to be more often younger than those with the CC genotype.

Of the total 112 lung cancer cases, 50 (44.6%) were adenocarcinoma, 35 (31.3%) were squamous cell carcinoma, and 27 (24.1%) were other lung neoplasms, respectively, including large-cell carcinoma, pleomorphic carcinoma, undifferentiated carcinomas, and metastases of other neoplasms to the lungs. Table 5 shows the selected characteristics of lung cancer patients divided by their lung tumor subtype. Lung cancer patients did not significantly differ between their subtypes in terms of their age, gender, and smoking status. There were also no associations found between the genotypes of *MMP-2*-735C/T and *MMP-9*-1562C/T and the lung cancer subtype (Table 4). However, there were significant differences observed in the frequency of metastases between adenocarcinoma patients and patients with other lung neoplasms ($p = 0.01420$), as well as between squamous cell carcinoma patients and patients with other lung neoplasms ($p = 0.00701$), with squamous cell carcinoma patients having the highest frequency of metastases to the lymph nodes and patients with other lung neoplasms having the highest frequency of distant metastases. Despite the metastasis factor, the characteristics of the lung cancer patient group by lung tumor subtype were found to be homogeneous, which was important information for further analyses.

2.2. The Effect of Dependent Variables on the Risk of Developing Lung Cancer

Prior to examining the impact of these specific *MMP-2*-735C/T and *MMP-9*-1562C/T genotypes on the MMP-2 and MMP-9 concentrations, we performed logistic regression analyses to estimate the impact of each examined factor on the risk of lung cancer. Table 6 summarizes the results of logistic regression analyses with comparisons between the controls and lung cancer patients. We observed that male gender increased the risk of lung cancer by two-fold. Aging and an increase in the number of smoked pack-years were also found to have statistically significantly increased the risk of lung cancer development. With every year, the risk of lung cancer incidence increased by 23%, whereas each packyear smoked increased the risk of lung cancer by 15%, respectively. Logistic regression analysis confirmed the Pearson's chi-square test results in terms of the significance of the *MMP-2*-735C/T and *MMP-9*-1562C/T genotype frequencies between the lung cancer patients and the controls. Despite the lack of statistical significance, we observed that the *MMP-2*-735CC genotype increases the lung cancer risk by 5-fold, while the CT genotype increases the risk by 7-fold, respectively. In case of the *MMP-9*-1562C/T genotypes the increase was not as significant, but it was still 45% and 60% for the CC and the CT genotypes, respectively. Importantly, we observed that both the decrease in the MMP-2 concentration and the increase in the MMP-9 concentration further enhance the risk of lung cancer development.

Table 4. Association of the (**a**) rs2285053 and (**b**) rs3918242 genotypes with selected qualitative variables in lung cancer patients and in healthy controls.

(a) ***MMP-2*-735C/T (rs2285053)**

Variable	Cases			Controls			*p*-Value (Pearson's Chi-Square Test)
	CC (*n* = 83)	CT (*n* = 26)	TT (*n* = 1)	CC (*n* = 77)	CT (*n* = 18)	TT (*n* = 5)	
Age [years]							
≤60	18 (21.7%)	3 (11.5%)	1 (100.0%)	65 (84.4%)	16 (88.9%)	4 (80.0%)	[a] $p = 0.07026$
>60	65 (78.3%)	23 (88.5%)	0 (0.0%)	12 (15.6%)	2 (11.1%)	1 (20.0%)	[b] $p = 0.84697$
Gender							
Male	54 (65.1%)	15 (57.7%)	1 (100.0%)	35 (45.5%)	9 (50.0%)	2 (40.0%)	[a] $p = 0.59419$
Female	29 (34.9%)	11 (42.3%)	0 (0.0%)	42 (54.5%)	9 (50.0%)	3 (60.0%)	[b] $p = 0.90591$
Smoking status							
Never smoker	2 (2.4%)	1 (3.8%)	0 (0.0%)	39 (50.6%)	5 (27.8%)	3 (60.0%)	[a] $p = 0.90223$
Light smoker	7 (8.4%)	0 (0.0%)	0 (0.0%)	27 (35.1%)	8 (44.4%)	2 (40.0%)	[b] $p = 0.45752$
Moderate smoker	25 (30.1%)	7 (26.9%)	0 (0.0%)	7 (9.1%)	4 (22.2%)	0 (0.0%)	
Heavy smoker	23 (27.7%)	6 (23.1%)	0 (0.0%)	2 (2.6%)	0 (0.0%)	0 (0.0%)	
NA	26 (31.3%)	12 (46.2%)	1 (100.0%)	2 (2.6%)	1 (5.6%)	0 (0.0%)	
Lung cancer subtype							
Adenocarcinoma	39 (47.0%)	10 (38.5%)	0 (0.0%)				[a] $p = 0.21120$
Squamous cell carcinoma	28 (33.7%)	7 (26.9%)	0 (0.0%)				
Other lung neoplasms	16 (19.3%)	9 (34.6%)	1 (100.0%)				
Metastases							
No metastases	40 (48.2%)	11 (42.3%)	1 (100.0%)				[a] $p = 0.80839$
To lymph nodes	32 (38.6%)	12 (46.2%)	0 (0.0%)				
Distant metastases	11 (13.3%)	3 (11.5%)	0 (0.0%)				

(b) ***MMP-9*-1562C/T (rs3918242)**

Variable	Cases			Controls			*p*-Value (Pearson's Chi-Square Test)
	CC (*n* = 76)	CT (*n* = 30)	TT (*n* = 3)	CC (*n* = 70)	CT (*n* = 25)	TT (*n* = 4)	
Age [years]							
≤60	11 (14.5%)	8 (26.7%)	2 (66.7%)	61 (87.1%)	20 (80.0%)	3 (75.0%)	[a] $p = 0.03854$
>60	65 (85.5%)	22 (73.3%)	1 (33.3%)	9 (12.9%)	5 (20.0%)	1 (25.0%)	[b] $p = 0.59287$
Gender							
Male	47 (61.8%)	20 (66.7%)	3 (100.0%)	30 (42.9%)	13 (52.0%)	3 (75.0%)	[a] $p = 0.37972$
Female	29 (38.2%)	10 (33.3%)	0 (0.0%)	40 (57.1%)	12 (48.0%)	1 (25.0%)	[b] $p = 0.37092$
Smoking status							
Never smoker	2 (2.6%)	1 (3.3%)	0 (0.0%)	35 (50.0%)	10 (40.0%)	2 (50.0%)	[a] $p = 0.84560$
Light smoker	6 (7.9%)	1 (3.3%)	0 (0.0%)	25 (35.7%)	10 (40.0%)	1 (25.0%)	[b] $p = 0.91162$
Moderate smoker	25 (32.9%)	7 (23.3%)	0 (0.0%)	7 (10.0%)	3 (12.0%)	1 (25.0%)	
Heavy smoker	18 (23.7%)	11 (36.7%)	0 (0.0%)	1 (1.4%)	1 (4.0%)	0 (0.0%)	
NA	25 (32.9%)	10 (33.3%)	3 (100.0%)	2 (2.9%)	1 (4.0%)	0 (0.0%)	
Lung cancer subtype							
Adenocarcinoma	39 (51.3%)	9 (30.0%)	1 (33.3%)				[a] $p = 0.11658$
Squamous cell carcinoma	22 (28.9%)	12 (40.0%)	0 (0.0%)				
Other lung neoplasms	15 (19.7%)	9 (30.0%)	2 (66.7%)				
Metastases							
No metastases	35 (46.1%)	14 (46.7%)	2 (66.7%)				[a] $p = 0.94658$
To lymph nodes	30 (39.5%)	12 (40.0%)	1 (33.3%)				
Distant metastases	11 (14.5%)	4 (13.3%)	0 (0.0%)				

Notes: Light smokers are defined as people who smoke from >0 to <20 pack-years; moderate smokers are defined as people who smoke from ≥20 to <40 pack-years; and heavy smokers are defined as people who smoke ≥40 pack-years; NA—not available; statistical values of comparisons between individuals with CC, CT, and TT genotypes among cases are marked with an "a," while among controls are marked with a "b".

Table 5. Selected characteristics of lung cancer patients divided by their lung cancer subtype.

Variable	Adenocarcinoma [n, (%)] [a,b] n = 50 (44.6%)	Squamous Cell Carcinoma [n, (%)] [a,c] n = 35 (31.3%)	Other Lung Neoplasms [n, (%)] [b,c] n = 27 (24.1%)	p-Value (Pearson's Chi-Square Test)
Age [years]				[a] $p = 0.74016$
≤60	9 (18.0%)	6 (17.1%)	5 (18.5%)	[b] $p = 0.81861$
>60	41 (82.0%)	29 (82.9%)	22 (81.5%)	[c] $p = 0.61571$
Gender				[a] $p = 0.06694$
Male	29 (58.0%)	27 (77.1%)	15 (56.6%)	[b] $p = 0.83614$
Female	21 (42.0%)	8 (22.9%)	12 (44.4%)	[c] $p = 0.07140$
Smoking status				
Never smoker	3 (6.0%)	0 (0.0%)	0 (0.0%)	
Light smoker	4 (8.0%)	3 (8.6%)	0 (0.0%)	[a] $p = 0.35977$
Moderate smoker	17 (34.0%)	11 (31.4%)	5 (18.5%)	[b] $p = 0.33032$
Heavy smoker	11 (22.0%)	12 (34.3%)	6 (22.2%)	[c] $p = 0.49688$
NA	15 (30.0%)	9 (25.7%)	16 (59.3%)	
Metastases				
No metastases	26 (52.0%)	15 (42.9%)	12 (44.4%)	[a] $p = 0.57388$
To the lymph nodes	20 (40.0%)	18 (51.4%)	6 (33.3%)	[b] $p = 0.01420$
Distant	4 (8.0%)	2 (5.7%)	9 (22.2%)	[c] $p = 0.00701$

Notes: Light smokers are defined as people who smoke from >0 to <20 pack-years; moderate smokers are defined as people who smoke from ≥20 to <40 pack-years; and heavy smokers are defined as people who smoke ≥40 pack-years; NA—not available. Comparisons between adenocarcinoma and squamous cell carcinoma patients are denoted by an "a", while comparisons between adenocarcinoma and patients with other lung neoplasms are represented with a "b", whereas comparisons between squamous cell carcinoma and patients with other lung neoplasms are denoted by a "c".

Table 6. The effect of each variable on the risk of lung cancer when compared to healthy individuals.

Variable	Lung Cancer Patient vs. Control	
	OR (95% CI)	p-Value
Gender–Male	2.03 (1.17–3.52)	0.011458
Age	1.23 (1.16–1.30)	<0.000001
Pack-years	1.15 (1.10–1.19)	<0.000001
MMP-2 [ng/mL]	0.96 (0.95–0.97)	<0.000001
MMP-2-735 CC	5.39 (0.62–47.17)	0.238504
MMP-2-735 CT	7.22 (0.78–67.14)	0.072836
MMP-9 [ng/mL]	1.01 (1.00–1.01)	<0.000001
MMP-9-1562 CC	1.45 (0.31–6.70)	0.757914
MMP-9-1562 CT	1.60 (0.33–7.83)	0.548801

Abbreviation: OR—odds ratio; and CI—confidence interval.

2.3. Concentration of MMP-2 Depending on the MMP-2-735C/T Genotypes

MMP-2 concentrations were compared in two groups of controls, non-smokers (NSC) and smokers (SC), with three lung cancer subtypes, including adenocarcinoma (ADC), squamous cell carcinoma (SqCC), and other lung neoplasms (OLN), in relation to the *MMP-2*-735C/T genotype. There were no significant differences observed in the MMP-2 concentrations between the non-smokers and smokers in both the -735CC and -735CT genotypes. However, there were statistically significant differences observed in the MMP-2 concentration among the non-smoking controls with the -735CC genotype ($\bar{x}$ = 204.04 ng/mL) and the -735CT genotype ($\bar{x}$ = 237.00 ng/mL, $p = 0.041479$).

Within both of the CC and CT genotypes of the *MMP-2*-735C/T polymorphism, patients with all lung cancer subtypes (ADC, SqCC, and OLN) had statistically significant differences in their MMP-2 concentrations, with lower levels of MMP-2 observed compared to the non-smoking and smoking controls. Within the -735CC genotype, we observed the highest MMP-2 concentration in the smoking control ($\bar{x}$ = 216.56 ng/mL) and the lowest in patients with other lung neoplasms ($\bar{x}$ = 138.05 ng/mL). In the -735CT genotype, we found the highest MMP-2 level in the non-smoking control ($\bar{x}$ = 237.00 ng/mL), and the lowest in

adenocarcinoma patients ($\bar{x}$ = 126.37 ng/mL). All statistical significances are detailed in the notes section of Table 7, and these results are also presented in Figure 1a.

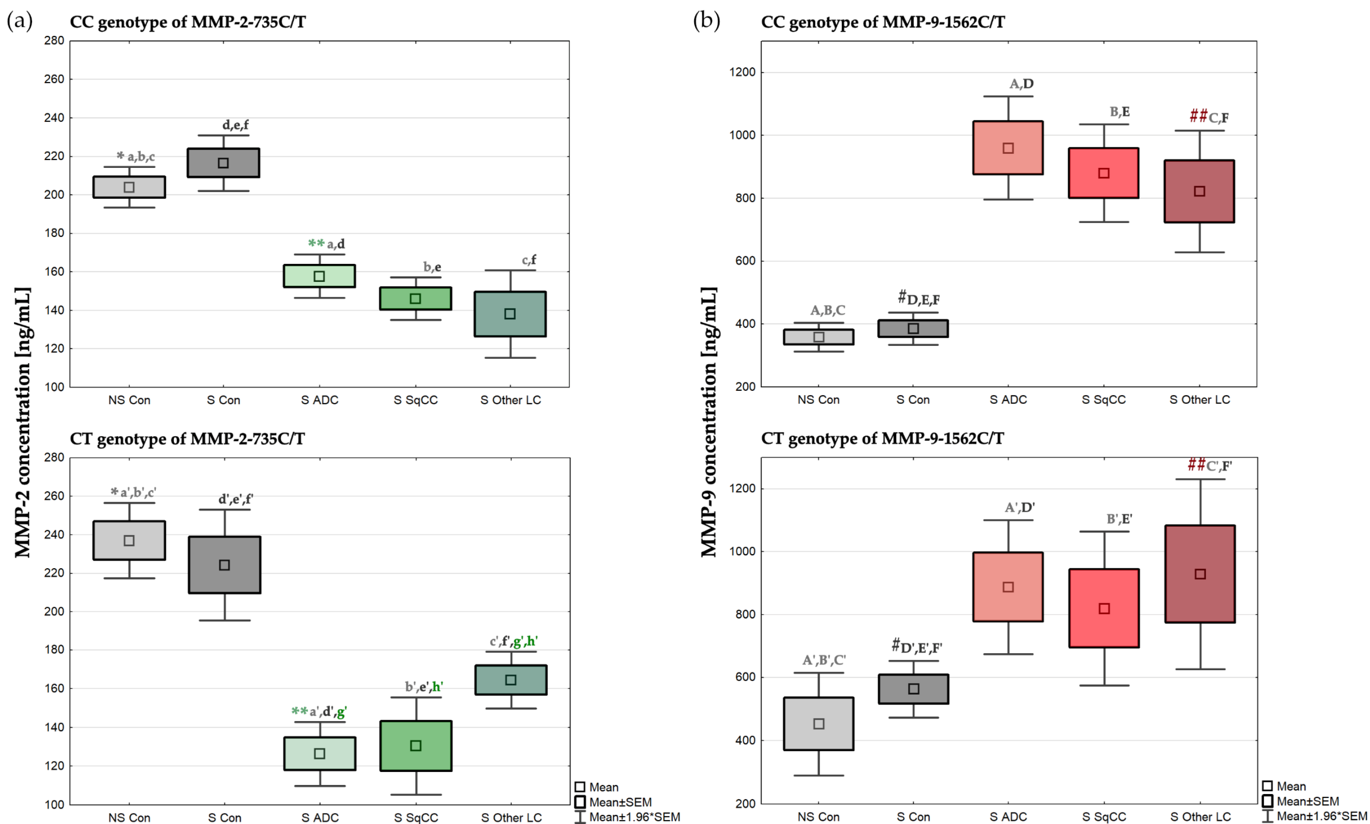


Figure 1. Comparisons of the (**a**) MMP-2 concentrations by the MMP-2-735C/T genotype and (**b**) the MMP-9 concentrations by the MMP-9-1562C/T genotype observed in the lung cancer cases and controls. Controls are depicted in grey in both the MMP-2 and MMP-9 concentration analyses, with non-smoking controls being lighter and smoking controls being darker. MMP-2 concentrations in cases are depicted in green color, with the lightest green in adenocarcinoma patients, medium green in squamous cell carcinoma patients, and darkest green in patients with other lung neoplasms. Similarly, MMP-9 concentrations in cases are depicted in red color, with the lightest red in adenocarcinoma patients, medium red in squamous cell carcinoma, and darkest red in patients with other lung neoplasms. All statistical significances are detailed in the notes section of Table 7.

Furthermore, the MMP-2 concentration was found to be statistically significantly higher in adenocarcinoma patients with the -735CC genotype ($\bar{x}$ = 157.69 ng/mL) than in adenocarcinoma patients with the -735CT genotype ($\bar{x}$ = 126.37 ng/mL, p = 0.013222). In contrast, we observed lower concentrations of MMP-2 in patients with other lung neoplasms and the -735CC genotype ($\bar{x}$ = 138.05 ng/mL), than in patients with other lung neoplasms and the -735CT genotype ($\bar{x}$ = 164.48 ng/mL) at the limit of statistical significance (p = 0.060294). In patients with other lung neoplasms and the -735CT genotype, MMP-2 concentrations were also found to be significantly higher than in patients with the -735CT genotype and adenocarcinoma ($\bar{x}$ = 164.48 ng/mL vs. $\bar{x}$ = 126.37 ng/mL, respectively, p = 0.003789), and squamous cell carcinoma ($\bar{x}$ = 164.48 ng/mL vs. $\bar{x}$ = 130.43 ng/mL, respectively, p = 0.029928). Table 7 summarizes all MMP-2 concentration results by the patient group and genotype of the *MMP-2*-735C/T polymorphism.

Table 7. MMP-2 concentrations by the MMP-2-735C/T genotype, and MMP-9 concentrations by the MMP-9-1562C/T genotype observed in the non-smoking controls, smoking controls, and lung cancer patients divided by lung cancer subtype.

	MMP-2 [ng/mL]			MMP-9 [ng/mL]		
	MMP-2-735C/T Genotype			*MMP-9*-1562C/T Genotype		
	CC	CT	TT	CC	CT	TT
Non-smoking control (NSC)						
	(n = 39)	(n = 5)	(n = 3)	(n = 35)	(n = 10)	(n = 2)
Mean ± SEM	204.04 ^{*,a,b,c} ± 5.43	237.00 $^{*,a',b',c'}$ ± 10.01	207.54 ± 3.97	358.74 ± 23.19	452.62 $^{A',B',C'}$ ± 82.74	358.70 ± 32.02
Median	208.01	239.60	207.41	312.41 ^{A,B,C}	396.93	358.70
Min–Max	145.21–275.71	207.97–269.46	200.74–214.47	192.94–697.64	152.14–941.59	326.68–390.72
Smoking control (SC)						
	(n = 37)	(n = 12)	(n = 2)	(n = 34)	(n = 14)	(n = 2)
Mean ± SEM	216.56 ^{d,e,f} ± 7.37	224.34 $^{d',e',f'}$ ± 14.69	190.92 ± 47.38	385.67 $^{\#,D,E,F}$ ± 26.31	562.80 $^{\#,D',E',F'}$ ± 45.55	648.57 ± 139.44
Median	215.81	217.85	190.92	378.48	519.45	648.57
Min–Max	134.65–317.92	148.72–318.04	143.54–238.30	148.51–633.65	317.65–840.55	509.13–788.02
Adenocarcinoma (ADC)						
	(n = 39)	(n = 10)	(n = 0)	(n = 39)	(n = 9)	(n = 1)
Mean ± SEM	157.69 ^{**,a,d} ± 5.75	126.37 $^{**,a',d',g'}$ ± 8.41		959.95 ^{D} ± 83.76	887.55 $^{A',D'}$ ± 108.93	1307.07
Median	154.72	128.25		936.72A	779.53	1307.07
Min–Max	94.93–237.57	90.70–175.39		73.03–2143.81	654.54–1310.73	
Squamous cell carcinoma (SqCC)						
	(n = 28)	(n = 7)	(n = 0)	(n = 22)	(n = 12)	(n = 0)
Mean ± SEM	146.11 ^{b,e} ± 5.69	130.43 $^{b',e',h'}$ ± 12.81		880.26 ^{E} ± 79.06	819.39 $^{B',E'}$ ± 124.75	
Median	142.00	137.85		778.66B	817.61	
Min–Max	90.70–209.82	81.92–180.09		403.44–1632.66	295.32–1514.18	
Other lung neoplasms (OLN)						
	(n = 16)	(n = 9)	(n = 1)	(n = 15)	(n = 9)	(n = 2)
Mean ± SEM	138.05 ^{c,f} ± 11.62	164.48 $^{c',f',g',h'}$ ± 7.51	231.50	821.64 $^{\#\#,F}$ ± 98.62	928.88 $^{\#\#,C',F'}$ ± 154.40	1527.66 ± 343.10
Median	134.93	164.78	231.50	932.81C	802.18	1527.66
Min–Max	59.16–227.51	133.53–200.94		104.94–1322.75	413.85–1801.21	1184.56–1870.75

Notes: SEM—standard error of the mean; statistical significances, Student's *t*-test: * $p = 0.041479$ between the NSCs with the CC and CT genotypes (*MMP-2*-735C/T) in the MMP-2 concentration, ** $p = 0.013222$ between the ADC patients with the CC and CT genotypes (*MMP-2*-735C/T) in the MMP-2 concentration, ^{a} $p < 0.000001$ between NSCs and ADC patients with *MMP-2*-735CC genotype in MMP-2 concentration, $^{a'}$ $p = 0.000002$ between the NSCs and ADC patients with the *MMP-2*-735CT genotype in the MMP-2 concentration, ^{b} $p < 0.000001$ between the NSCs and SqCC patients with the *MMP-2*-735CC genotype in the MMP-2 concentration, $^{b'}$ $p = 0.000115$ between the NSCs and SqCC patients with the *MMP-2*-735CT genotype in the MMP-2 concentration, ^{c} $p < 0.000001$ between the NSCs and OLN patients with the *MMP-2*-735CC genotype in the MMP-2 concentration, $^{c'}$ $p = 0.000087$ between the NSCs and OLN patients with the *MMP-2*-735CT genotype in the MMP-2 concentration, ^{d} $p < 0.000001$ between the SCs and ADC patients with the *MMP-2*-735CC genotype in the MMP-2 concentration, $^{d'}$ $p = 0.000023$ between the SCs and ADC patients with the *MMP-2*-735CT genotype in the MMP-2 concentration, ^{e} $p < 0.000001$ between the SCs and SqCC patients with the *MMP-2*-735CC genotype in the MMP-2 concentration, $^{e'}$ $p = 0.000456$ between the SCs and SqCC patients with *MMP-2*-735CT genotype in the MMP-2 concentration, ^{f} $p = 0.000001$ between the SCs and OLN patients with the *MMP-2*-735CC genotype in the MMP-2 concentration, $^{f'}$ $p = 0.036566$ between the SCs and OLN patients with the *MMP-2*-735CT genotype in the MMP-2 concentration, $^{g'}$ $p = 0.003790$ between the ADC and OLN patients with the *MMP-2*-735CT genotype in the MMP-2 concentration, $^{h'}$ $p = 0.029928$ between the SqCC and OLN patients with the *MMP-2*-735CT genotype in the MMP-2 concentration, $^{\#}$ $p = 0.000936$ between the SCs with the CC and CT genotypes (*MMP-9*-1562C/T) in the MMP-9 concentration, $^{\#\#}$ $p = 0.023315$ between the OLN patients with the CC and CT genotypes (*MMP-9*-1562C/T) in the MMP-9 concentration, $^{A'}$ $p = 0.006470$ between the NSCs and ADC patients with the *MMP-9*-1562CT genotype in the MMP-9 concentration, $^{B'}$ $p = 0.026914$ between the NSCs and SqCC patients with the *MMP-9*-1562CT genotype in the MMP-9 concentration, $^{C'}$ $p = 0.012350$ between the NSCs and OLN patients with the *MMP-9*-1562CT genotype in the MMP-9 concentration, ^{D} $p < 0.000001$ between the SCs and ADC patients with the *MMP-9*-1562CC genotype in the MMP-9 concentration, $^{D'}$ $p = 0.004315$ between the SCs and ADC patients with the *MMP-9*-1562CT genotype in the MMP-9 concentration, ^{E} $p = 0.000020$ between the SCs and SqCC patients with the *MMP-9*-1562CC genotype in the MMP-9 concentration, $^{E'}$ $p = 0.004493$ between the SCs and SqCC patients with the *MMP-9*-1562CT genotype in the MMP-9 concentration, ^{F} $p = 0.000401$ between the SCs and OLN patients with the *MMP-9*-1562CC genotype in the MMP-9 concentration, $^{F'}$ $p = 0.015655$ between the SCs and OLN patients with the *MMP-9*-1562CT genotype in the MMP-9 concentration, Mann–Whitney U test: ^{A} $p < 0.000001$ between the NSCs and ADC patients with the *MMP-9*-1562CC genotype in the MMP-9 concentration, ^{B} $p < 0.000001$ between the NSCs and SqCC patients with the *MMP-9*-1562CC genotype in the MMP-9 concentration, and ^{C} $p = 0.000043$ between the NSCs and OLN patients with the *MMP-9*-1562CC genotype in the MMP-9 concentration.

2.4. Concentration of MMP-9 Depending on the MMP-9-1562C/T Genotypes

We used the same patient classification and calculation method for MMP-9 concentration analysis as we performed for MMP-2. Similarly, there was no significant difference observed in the MMP-9 concentration between the non-smokers and smokers of the control group in both the -1562CC and -1562CT genotypes. However, a statistically significant

difference in the MMP-9 concentration was found between the smoking controls with the -1562CC genotype ($\bar{x}$ = 385.67 ng/mL) and smoking controls with the -1562CT genotype ($\bar{x}$ = 562.80 ng/mL, p = 0.000936).

Patients with all lung cancer subtypes (ADC, SqCC, and OLN) were found to have statistically significant differences in their MMP-9 concentrations with higher levels of MMP-9 observed compared to the non-smoking and smoking controls within both the CC and CT genotypes of the *MMP-9*-1562C/T polymorphism. Within the -1562CC genotype, we observed the highest MMP-9 concentration in the adenocarcinoma patients ($\bar{x}$ = 959.95 ng/mL) and the lowest in the non-smoking controls ($\bar{x}$ = 358.74 ng/mL). Furthermore, we found the highest MMP-9 concentration in the -1562CT genotype in patients with other lung neoplasms ($\bar{x}$ = 928.88 ng/mL), and the lowest was once again observed in the non-smoking group ($\bar{x}$ = 452.62 ng/mL). All statistical significances are detailed in the notes section of Table 7, and these results are also presented in Figure 1b.

In contrast to the MMP-2 concentration, there were no statistically significant differences observed in the MMP-9 concentration between the lung cancer subtypes within both the -1562CC and -1562CT genotypes. However, we observed that patients with other lung neoplasms with the -1562CT genotype had a statistically higher MMP-9 concentration ($\bar{x}$ = 928.88 ng/mL) than patients with other lung neoplasms with the -1562CC genotype ($\bar{x}$ = 821.64 ng/mL, p = 0.023315). Table 7 summarizes all MMP-9 concentration results by the patient group and *MMP-9*-1562C/T polymorphism genotype.

3. Discussion

Even though tobacco smoke exposure causes lung cancer in 90% of cases, each patient may have a unique molecular pattern that causes this disease. The mechanisms by which smoking promotes lung carcinogenesis are numerous in quantity [35–38]. Individual vulnerability to tobacco smoke, also known as genetic susceptibility, can occur as a result of (1) the inheritance of low-frequency, high-penetrance genes; (2) the inheritance of high-frequency, low-penetrance genes; or (3) acquisition via epigenetic mechanisms. Candidates for lung cancer susceptibility genes have been intensively researched, with an emphasis on the variation in predisposing, low-penetrance genes involved in carcinogen metabolism and the DNA repair of damage caused by tobacco smoke, as well as changes in the genes encoding proteins implicated in tumor formation, growth, and dissemination [19,20]. In this study, we evaluated the effect of two polymorphisms in the promoter regions of two human gelatinases, i.e., MMP-2 and MMP-9, on the risk of lung cancer development.

Among the secreted MMPs, MMP-2 and MMP-9 have long been considered to play an important role in cancer invasion and metastasis due to their ability to degrade the ECM and basement membrane barriers required for each step of tumor progression [39–43]. Recent studies have, however, demonstrated that MMP functions are much more complex, since they are the key mediators of growth factor activation, bioavailability, receptor signaling, cell adhesion and motility, apoptosis and survival mechanisms, angiogenesis, inflammatory responses, and immunological surveillance [44]. As a result, polymorphisms in these MMP genes are being extensively studied in patients suffering from various malignancies. However, the number of published genotypic articles on the role of MMP-2 and MMP-9 in lung cancer has been extremely low in the preceding decade.

We analyzed two SNPs with known functional effects, i.e., rs2285053 of the *MMP-2* gene at position -735 with a C-to-T transition that has been shown to destroy the binding site of specificity protein 1 (Sp1) to MMP-2 mRNA, resulting in the reduction of its transcription level; and rs3918242 of the *MMP-9* gene at position -1562, also with a C-to-T transition resulting in the change in the promoter's activity [29,45,46]. SNPs were analyzed in 112 lung cancer patients and 100 healthy controls in association with the MMP-2 and MMP-9 serum concentrations, revealing that specific genotypes appeared to affect both the MMP-2 and MMP-9 concentrations, which may result in an increased lung cancer risk, a more aggressive course of the disease, and poorer patient survival outcomes.

In our study, the *MMP-2*-735C/T genotype frequencies were found to be CC 74.1%, CT 23.2%, and TT 0.9% in the lung cancer patients group, and CC 77.0%, CT 18.0%, and TT 5.0% in the control group, respectively. There were no statistically significant differences observed in the distribution of the *MMP-2*-735C/T genotypes. As expected, there were no statistically significant differences observed in the MAF of the rs2285053 values between the cases (0.1273) and the controls (0.1400). However, the distribution of the *MMP-2* genotypes in the controls in our study was not in HWE. Interestingly, Gonzalez-Arriaga et al. (2012) [44] observed the same issue in their analysis, which was also based on the European population. Similarly, to Gonzalez-Arriaga et al., the explanation for this problem in our case is unknown, as we used a control group of healthy individuals that were randomly recruited and received the consistency with the HWE in other polymorphic loci—in the *MMP-9* gene located at the -1562 position.

According to logistic regression analysis using the -735TT genotype as the reference level, the prevalence of the -735CC genotype was found to be associated with a 5.4-fold higher risk of developing lung cancer, whereas the -735CT genotype was associated with a 7.2-fold higher risk, respectively. Even though the results were not statistically significant (p-values 0.238504 and 0.072836, respectively), the odds ratios remained quite high, which may be due to the fact that there were no patients with the -735TT genotype among our research group's adenocarcinoma and squamous cell carcinoma patients. On the one hand, the lack of the presence of the -735TT genotype among patients with adenocarcinoma and squamous cell carcinoma may weight our results with an error, but on the other hand, it may be a normal trend among the two most common subtypes of lung cancer. Individuals with the TT genotype of the *MMP-2*-735C/T polymorphism had a lower risk of lung cancer when compared to the CC genotype, according to Wang et al. (2012) [43], and Li et al. (2015) [24], and the TT genotype had a protective effect as a result of a lower promoter activity, and thus lowered the MMP-2 enzyme activity. In our study, a larger sample size would have revealed more about the importance of these findings.

The genotype frequencies for the *MMP-9*-1562C/T polymorphism in lung cancer patients were CC 67.9%, CT 26.8%, and TT 2.7%, and CC 70.0%, CT 25.0%, and TT 4.0% in the control group, respectively, with no statistically significant differences observed between these two groups. In addition, no statistically significant differences in the MAF of the rs3918242 values were found between the cases (0.1651) and controls (0.1667). A few studies have indicated that individuals carrying the T allele had a lower risk of developing lung cancer, whereas those carrying the C allele had an increased risk [24,42,44]. In our study, logistic regression analysis revealed a 1.4-fold increased risk of developing lung cancer in carriers of the -1562CC genotype, and a 1.6-fold increased risk in carriers of the -1562CT genotype, respectively, although these results were not statistically significant. The findings of Bayramoglu et al. (2009) [39] and Rollin et al. (2007) [45] were consistent with the findings obtained in this study, indicating that there is no difference in the distribution of the *MMP-9*-1562C/T genotypes between the lung cancer patients and healthy individuals. Furthermore, Wang et al. (2005) [46] suggested that rs3918242 may not be a good marker for predicting lung cancer susceptibility and the presence of lymphatic metastasis in lung cancer patients.

The reported results of the *MMP-2*-735C/T and *MMP-9*-1562C/T polymorphisms and their role in lung cancer risk are frequently conflicting. The source of the contradictory results could be explained by their ethnicity, producing variation as a result of their genetic backgrounds as well as environmental factors across the different ethnicities. For example, the study by Li et al. (2015) [24] produced significant results of the *MMP-2*-735C/T polymorphism in the overall comparison and among Asians, but not among the Caucasians, whereas the *MMP-9*-1562C/T polymorphism was solely significant among the Asians. To assess the significance of our results in the Polish population, we compared them to the available genomic data on rs2285053 and rs3918242 from projects, such as 1000Genomes, 1000Genomes_30x, Allele Frequency Aggregator, gnomAD-Genomes, and the PAGE Study. We found that the T allele frequencies (minor allele frequencies) of both

the *MMP-2*-735C/T and *MMP-9*-1562C/T polymorphisms in our study population (0.1333, and 1659, respectively) were consistent with the average MAF values in the European population (0.1101 and 0.1666, respectively). When we compared the *MMP-2*-735C/T polymorphism in five major populations, including the European, East Asian, South Asian, American, and African populations, we found statistically significant differences, with the East Asian population having the highest MAF value (0.2591), and the European population having the lowest (0.1101). The *MMP-9*-1562C/T, on the other hand, revealed no statistically significant differences in the MAF values between the ethnicities, with the South Asian population having the highest MAF value (0.2343), and the American population having the lowest (0.0809). The findings corroborate the association between ethnicity and the MMP mutation risk and explain the disparities in the results of studies conducted on different ethnicities. It further demonstrates that ethnicity must be taken into account when assessing the risk of developing lung cancer based on the *MMP* polymorphisms.

We also investigated whether the *MMP-2*-735C/T and *MMP-9*-1562C/T genotypes affected the MMP-2 and MMP-9 concentration levels. We observed significant differences in the MMP-2 and MMP-9 concentrations between the -735CC and -735CT genotypes, as well as the -1562CC and -1562CT genotypes, respectively, not only among the lung cancer subtypes but also among the healthy non-smokers and smokers. We found statistically significant higher MMP-2 concentrations in non-smokers with the -735CT genotype and higher MMP-9 concentrations in smokers with the -1562CT genotype. Moreover, we observed an opposite effect in lung cancer patients with adenocarcinoma, the squamous cell carcinoma subtypes, and the -735CC genotype which all had statistically increased MMP-2 levels, as well as patients with the -1562CC genotype who had higher MMP-9 concentration levels than in patients with the -1562CT genotype. These findings confirm that polymorphisms at the promoter regions of *MMPs* affect the expression levels of these proteins.

Several articles have reported that MMP expression can be induced by smoking, leading changes in the MMP/TIMP ratio [47]. The effect of cigarette smoking on MMP concentrations was also found in our research. We observed the increase in the MMP-9 concentration in the smoking controls when compared to the non-smoking controls. Even though the increase was not statistically significant, we noticed a trend since smoking controls for each *MMP-9*-1562C/T genotype had higher MMP-9 concentrations than the non-smoking controls for each genotype (385.67 ng/mL, 562.80 ng/mL, and 648.57 ng/mL vs. 312.41 ng/mL, 452.62 ng/mL, and 358.70 ng/mL for the CC, CT, and TT genotypes of the smoking and non-smoking control groups, respectively). The increase in the MMP levels in the smoking controls (as compared to the non-smoking controls) suggests that smoking is the factor that induces ECM remodeling. Moreover, connective tissue remodeling may promote tumor development [22,41,43]. As the great majority of lung cancer patients have a smoking history and tobacco-smoke-related increased MMP expression, the subsequent ECM remodeling that arises as a result may be regarded as one of the initial steps of lung carcinogenesis (Figure 2).

Moreover, there have been reports published regarding the additive joint effect of smoking and *MMP* genotypes on lung cancer risk determination. Zhou et al. (2005) [46] investigated the synergistic effect of two polymorphism in the *MMP-2* promoter region at the locations -735 and -1306 with a C-to-T transition. The authors found that the C-735-C-1306 haplotype was associated with an increased risk of lung cancer when compared to the T-735-T-1306 haplotype, and the risk of developing lung cancer being even higher in smokers with the C-735-C-1306 haplotype. At this point, it could be expected that individuals who smoke and carry the *MMP-2*-735CC or -1306CC genotype, or the C-735-C-1306 haplotype, are more susceptible to develop lung cancer than those who smoke and carry either the -735TT or -1306TT genotype, or the T-735-T-1306 haplotype.

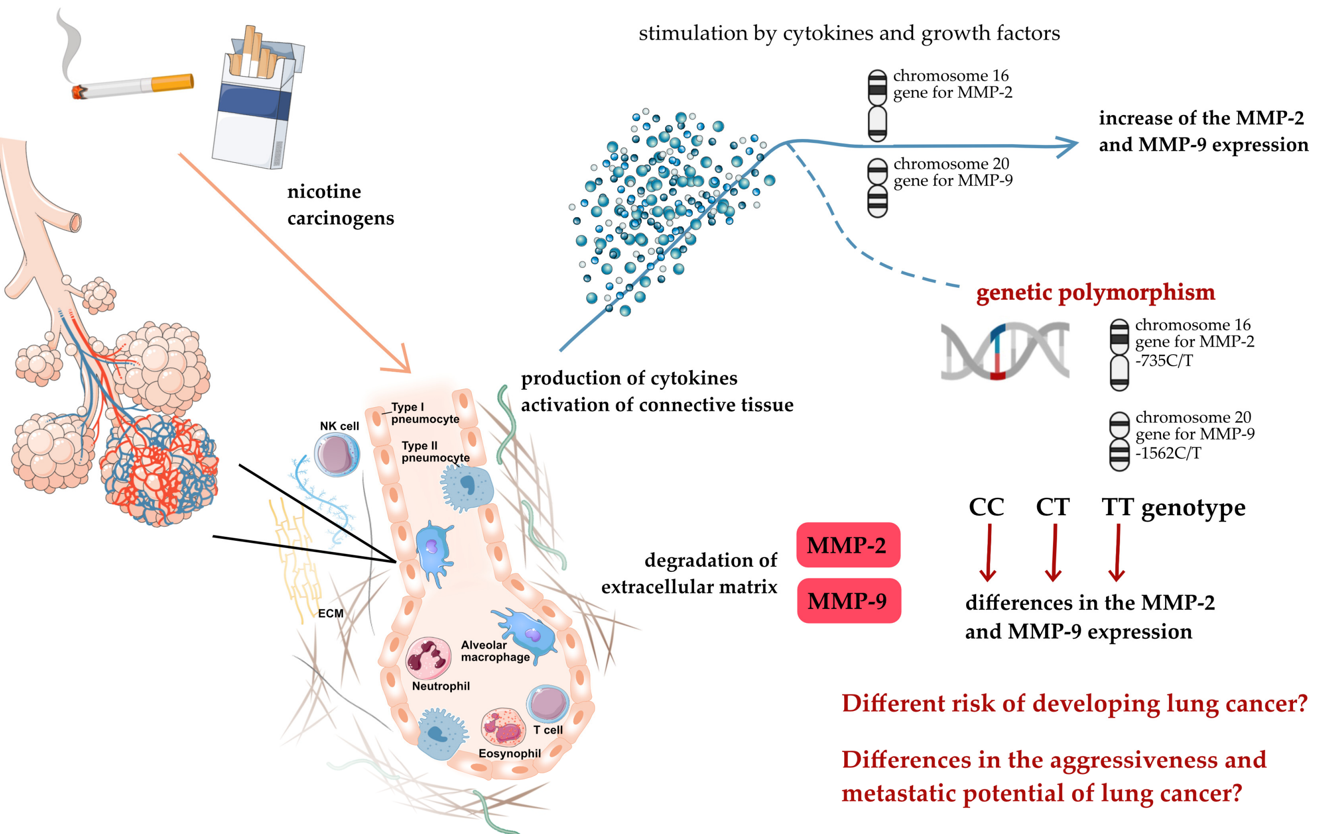


Figure 2. Mechanisms of MMP-2 and MMP-9 stimulation by tobacco smoke exposure. Created with BioRender.com and Affinity Designer. When exposed to tobacco smoke, pulmonary alveoli are stimulated to release cytokines by both epithelial and inflammatory cells; increased cytokine production then causes an inflow of inflammatory cells into the alveoli, exacerbating local inflammation, as well as activation of the connective tissue, i.e., ECM, with the stimulation of the *MMP-2* and *MMP-9* genes in cells producing these MMPs, resulting in an increase in MMP expression; carriers of genotypes predisposed to elevated MMP concentrations are at an increased risk of being transformed or preinvasive lung cells caused by tobacco carcinogens being converted into an invasive tumor under the conditions of a higher lifetime MMP expression.

In our study, we also observed greater levels of MMP-2 in patients with other lung neoplasms than in patients with adenocarcinoma or squamous cell carcinoma that could be attributed to a higher prevalence of distant metastases in this group of patients, which included individuals with metastases to the lungs from other neoplasms. The group of patients with other lung neoplasms was characterized by statistically significant differences in the metastases factor when compared with both adenocarcinoma (22.2% vs. 8.0%, respectively, $p = 0.01420$) and squamous cell carcinoma patients (22.2% vs. 5.7%, respectively, $p = 0.00701$). These observations may confirm a role of MMP-2 in cancer metastasis and its higher serum expression due to metastasis. Since a higher MMP-2 level has been associated with an increased risk of lung cancer, a more aggressive course of the disease, and a higher incidence of distant metastases [23,48,49], we can thereby predict that lung cancer patients with the -735CC genotype, which predisposes to higher MMP concentrations, will have a worsened prognosis and shorter overall survival times than patients with the CT or TT genotype. Gonzalez-Arriaga et al. (2012) [44] partially confirmed this hypothesis by demonstrating that the *MMP-2*-735C allele was related with shorter survival times in carriers compared to those carrying the T allele ($p = 0.02$).

The comparison of lung cancer subtypes in the prevalence of *MMP-2*-735C/T and *MMP-9*-1562C/T genotypes complemented our research. There were no statistically sig-

nificant changes observed in the frequency of the *MMP-2*-735C/T and *MMP-9*-1562C/T genotypes between patients with adenocarcinoma, squamous cell carcinoma, and other lung neoplasms. We also found no significant differences in the frequency of the *MMP-2*-735C/T and *MMP-9*-1562C/T genotypes between lung cancer patients with no metastases, lymph nodes metastases, and distant metastases. There were also no variations observed in the MMP-2 and MMP-9 concentrations based on metastasis incidence. However, we cannot rule out the impact of *MMP* polymorphism on the lung cancer subtype and metastasis presence, since our above-described analyses demonstrate differences in the MMP-2 and MMP-9 concentrations between these lung cancer subtypes based on the *MMP-2*-735C/T and *MMP-9*-1562C/T genotypes, respectively.

Cancer is a multifactorial disease that results from complex interactions between the hereditary and environmental factors. Lung cancer is an aggressive and genomically unstable cancer that progresses with a series of genetic and epigenetic changes [38,50]. It may be naive to expect that a single mutation or nucleotide variation predominates the progression of cancer. Although the differences in allele transcription caused by polymorphisms in the *MMP* promoters are subtle when compared to, for example, oncogene overexpression [46,51], specific genotypes have appeared to affect the concentrations of MMP-2 and MMP-9, which when increased may result over a lifetime in an increased susceptibility to lung cancer, a more aggressive course of the disease, and poorer patient survival outcomes. Candidates for single nucleotide polymorphisms implicated in increased lung cancer risk must be sought for in this regard in order to better understand and link the individual factors involved in lung cancer pathogenesis, and thus improve the diagnostic, screening, and therapeutic options for future lung cancer patients.

4. Materials and Methods

4.1. Patients

We present a non-interventional, retrospective, case-control study. The case group comprised 112 lung cancer patients recruited by the Department of Thoracic Surgery, Lower Silesian Centre for Lung Diseases in Wroclaw, Poland. All participants signed a written informed consent following an explanation of the study protocols. The study protocol conformed to the World Medical Association's Declaration of Helsinki (2000) and was approved by the Bioethics Committee at the Wroclaw Medical University (NR KB: 106/2020 and 433/2022). Clinical, laboratory, and pathological data for these patients were acquired from hospital medical records using the AMMS IT system (Asseco Medical Management Solutions). Lung cancer diagnosis was established in accordance with the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology and was staged in accordance with the American Joint Committee on Cancer's (AJCC) 8th TNM Staging System.

The control group comprises 100 volunteers, 47 of whom were healthy non-smokers and 51 of whom were healthy smokers, respectively. The Biobank Research Group, Łukasiewicz Research Network—PORT Polish Centre for Technology Development provided biological material (sera, whole blood for DNA isolation) and basic data about patients in the control group to the Department of Medical Laboratory Diagnostics, Division of Clinical Chemistry, and Laboratory Hematology. All provided samples were stored at −80 °C until at the point of their utilization.

4.2. Methods

Following admission to the hospital, venous blood samples were collected into tubes with ethylenediaminetetraacetic acid (EDTA) anticoagulant and tubes with the clot activator from all lung cancer patients. Then, 200 µL of whole blood was taken from EDTA blood samples for DNA isolation. Following this, at room temperature, blood samples were centrifuged at 2000× *g* for 8–10 min to separate the plasma and serum, which were then stored at −80 °C until their use.

Patients' exposure to cigarette smoke was assessed based on their smoking history and nicotine metabolite (cotinine) concentrations in their sera. Participants were divided into two groups based on their tobacco consumption: those who had never smoked, defined as subjects who had not smoked at least one cigarette per day regularly for six months or longer in their lifetimes, and those who smoked, including former smokers, who were defined as regular smokers who had quit smoking at least one year before the interview; and current smokers, who were defined as subjects who are active smokers. The concentration of cotinine was measured to confirm active smoking. Cotinine serum levels were determined using a competitive immunoenzymatic assay termed Cotinine direct (Serum/Urine) ELISA (Cat. No: EIA-5496/EIA-5497, DRG International Inc., Springfield, NJ, USA).

Serum blood samples were also used to determine the concentrations of MMP-2 and MMP-9. Commercial enzyme-linked immunosorbent assays (ELISA) test kits: Total MMP-2 Quantikine ELISA (Catalog # MMP200), and Human MMP-9 Quantikine ELISA (Catalog # DMP900), R&D Systems, Inc., Minnesota, MN, USA, were used in accordance with the manufacturer's protocols. ELISA kits characteristics are presented in Table 8.

Table 8. Characteristics of the used ELISA kits.

ELISA Kit	Standard Curve	Intra-Assay Precision	Inter-Assay Precision	Minimum Detectable Dose (MDD)
Cotinine	5–100 ng/mL	4.6–8.6%		1 ng/mL
MMP-2	0.5–32 ng/mL	3.6–7.0%	6.5–7.0%	0.033 ng/mL
MMP-9	0.313–20 ng/mL	1.9–2.9%	6.9–7.9%	<0.156 ng/mL

DNA isolation was performed using the binding column technology of the Syngen Blood/Cell DNA Mini Kit (300) (Cat. No: SY221012, Syngen, Poland). The NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify the purity (the A260/A280 ratio) and concentration (the A260 measurement) of the isolated DNA. All extractions had a high purity of ~1.8, along with an average yield of DNA of 14.55 μg in the case group and 10.77 μg in the control group, respectively (a typical DNA extraction yield from frozen whole blood samples using this kit is between 4 and 12 μg, respectively). Isolated DNAs were stored at −80 °C until their use.

The polymerase chain reaction method with restriction fragment length polymorphism (PCR/RFLP) was used for genotyping. The PCR-RFLP method consists of three analysis steps. The promoter regions containing polymorphisms were amplified using PCR to obtain the amount of DNA needed for RFLP analysis. Then, the amplified DNA sequences were cut with specific restriction endonucleases. Finally, after digestion of the DNA samples, DNA fragments of various lengths were separated using gel electrophoresis in 1.5% agarose gel and analyzed under UV light to reveal differences in the homologous DNA sequences. Table 9 contains a detailed protocol for the PCR-RFLP method, while Table 10 contains the primer sequences. Figure 3 shows a photo of the electrophoretic separation of (a) *MMP-2*-735C/T polymorphism analysis products and (b) *MMP-9*-1562C/T polymorphism analysis products. We performed both random duplications in 20% of the samples to reduce the genotyping error rate.

Table 9. Protocol of the designed and utilized PCR-RFLP method for *MMP-2*-735C/T (rs2285053) and *MMP-9*-1562C/T (rs3918242) polymorphism analyses.

	***MMP-2*-735 C/T**	***MMP-9*-1562 C/T**
1. Amplification of the promoter regions using PCR		
PCR Mix: (given amounts are calculated for one reaction)	Forward primer: 0.6 µL Reverse primer: 0.6 µL Gold Taq polymerase (5 U/µL): 0.2 µL 10× Gold buffer: 2 µL 25 mM $MgCl_2$: 1.6 µL 10 mM dNTP Mix: 0.4 µL DNA: 2 µL PCR water: 12.6 µL	Forward primer: 0.6 µL Reverse primer: 0.6 µL Gold Taq polymerase (5 U/µL): 0.2 µL 10× Gold buffer: 2 µL 25 mM $MgCl_2$: 1.6 µL 10 mM dNTP Mix: 0.4 µL DNA: 2 µL PCR water: 12.6 µL
PCR conditions:	Activation: 15 min at 95 °C 35 cycles of: • Denaturation: 30 s at 95°C; • Annealing: 30 s at 60 °C; • Elongation: 30 s at 72 °C. Final elongation: 15 min at 72 °C Hold: ∞ at 4 °C	Activation: 15 min at 95 °C 35 cycles of: • Denaturation: 30 s at 95 °C; • Annealing: 30 s at 60 °C; • Elongation: 30 s at 72 °C. Final elongation: 15 min at 72 °C Hold: ∞ at 4 °C
2. Digestion of the amplified DNA sequences with restriction enzymes		
Reaction Mix:	PCR product: 10 µL Anza™ 10(×) Buffer: 2 µL HinfI enzyme: 1 µL PCR water: 7 µL	PCR product: 10 µL 10(×) Buffer B: 2 µL PaeI enzyme: 1 µL PCR water: 18 µL
Reaction conditions:	Incubation: 16 h at 37 °C Inactivation: 20 min at 65 °C	Incubation: 16 h at 37 °C Inactivation: 20 min at 65 °C
3. Electrophoresis of the digested DNA fragments		
Agarose gel: Electrophoresis conditions:	Agarose: 1.5 g TBE buffer 1(×): 100 mL Gold DNA gel stain: 5 µL 50 V for 5 min 120 V for 120 min	Agarose: 1.5 g TBE buffer 1(×): 100 mL Gold DNA gel stain: 5 µL 50 V for 5 min 120 V for 120 min
Final products:	CC: 300 bp TT: 254 bp, 46 bp CT: 300 bp, 254 bp, 46 bp	CC: 435 bp TT: 247 bp, 188 bp CT: 435 bp, 247 bp, 188 bp

Notes: PCR-RFLP—polymerase chain reaction—restriction fragment length polymorphism; Mix—mixture; dNTP—deoxynucleoside triphosphate; 10(X)—ten times concentrated; V—voltage; CC—homozygous genotype CC; TT—homozygous genotype TT; CT—heterozygous genotype CT; and bp—base pair.

Table 10. Sequences of the used primers to detect the *MMP-2*-735C/T (rs2285053) and *MMP-9*-1562C/T (rs3918242) polymorphisms.

Genotype	Primer	Sequence
MMP-2-735 C/T	F primer:	5′-ATA GGG TAA ACC TCC CCA CAT T-3′
	R primer:	5′-GGT AAA ATG AGG CTG AGA CCT G-3′
MMP-9-1562 C/T	F primer:	5′-GCC TGG CAC ATA GTA GGC CC-3′
	R primer:	5′-TTC CTA GCC AGC CGG CAT C-3′

Notes: F primer—Forward primer; and R primer—Reverse primer.

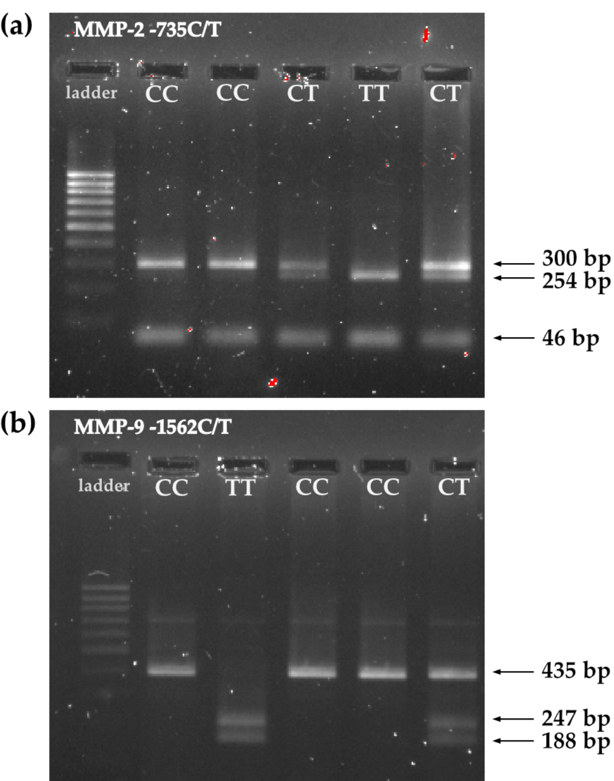


Figure 3. Examples of the electrophoretic separation of the PCR-RFLP products from the (**a**) *MMP-2* promoter -735C/T polymorphism analysis; and the (**b**) *MMP-9* promoter -1562C/T polymorphism analysis.

In our study, we also used results from the *MMP-2*-735C/T and *MMP-9*-1562C/T MAF (in both cases, T allele frequency) available on the website of the Reference SNP (rs) Report of National Library of Medicine, National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/snp/rs2285053, https://www.ncbi.nlm.nih.gov/snp/rs3918242, respectively, both accessed on 3 June 2023).

4.3. Statistical Analysis

The obtained data were statistically analyzed using TIBCO Software Inc. (Palo Alto, CA, USA) (2017), Statistica, version 13 (http://statistica.io, accessed on 17 December 2022) with the additional Plus Package (version 5.0.96), and a significance level of $p < 0.05$. The Shapiro–Wilk test was used to determine whether the data for each parameter was normally distributed across all analyzed groups. Pearson's chi-square test was then applied to analyze sets of categorical data, including deviation from the HWE. Logistic regression analysis was also used to estimate qualitative data. To compare independent, continuous variables between two groups, the parametric Student's *t*-test or the non-parametric Mann–Whitney U test were used, and the one-way analysis of variance (ANOVA) was used if there were more groups. Post-hoc analyses using Tukey's Honest Significant Difference (HSD) corrected for unequal sample sizes supplemented the ANOVA.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms241310576/s1.

Author Contributions: Conceptualization, K.W. and M.Ś.-M.; methodology, K.W. and M.Ś.-M.; formal analysis, K.W., P.B., A.R. and M.Ś.-M.; investigation, K.W., P.B., A.R. and M.Ś.-M.; resources, K.W., P.B. and A.R.; data curation, K.W.; writing—original draft preparation, K.W.; writing—review and editing, K.W. and M.Ś.-M.; visualization, K.W. and M.Ś.-M.; supervision, M.Ś.-M.; project administration, K.W. and M.Ś.-M.; funding acquisition, K.W. and M.Ś.-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the Ministry of Health subvention according to the number of SUBK.D010.22.041 from the IT Simple system of Wroclaw Medical University.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Wroclaw Medical University (protocol code 433/2022, date of approval 26 May 2022).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy and ethical restriction.

Acknowledgments: The control group samples were provided by the Biobank Research Group, Łukasiewicz Research Network—PORT Polish Centre for Technology Development.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gross, J.; Lapiere, C.M. Collagenolytic activity in amphibian tissues: A tissue culture assay. *Proc. Natl. Acad. Sci. USA* **1962**, *48*, 1014–1022. [CrossRef]
2. Liu, G.; Philp, A.M.; Corte, T.; Travis, M.A.; Schilter, H.; Hansbro, N.G.; Burns, C.J.; Eapen, M.S.; Sohal, S.S.; Burgess, J.K.; et al. Therapeutic Targets in Lung Tissue Remodelling and Fibrosis. *Pharm. Ther.* **2021**, *225*, 107839. [CrossRef]
3. Cui, N.; Hu, M.; Khalil, R.A. Biochemical and Biological Attributes of Matrix Metalloproteinases. *Prog. Mol. Biol. Transl. Sci.* **2017**, *147*, 1–73.
4. Page-McCaw, A.; Ewald, A.J.; Werb, Z. Matrix Metalloproteinases and the Regulation of Tissue Remodelling. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 221–233. [CrossRef]
5. Murphy, G. Matrix Metalloproteinases. In *Encyclopedia of Cell Biology*; Elsevier: Amsterdam, The Netherlands, 2016; pp. 621–629.
6. Lung Source: Globocan 2020 Number of New Cases in 2020, Both Sexes, All Ages. 2020. Available online: https://gco.iarc.fr/today/data/factsheets/cancers/15-Lung-fact-sheet.pdf (accessed on 1 June 2023).
7. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* **2021**, *71*, 209–249. [CrossRef]
8. Didkowska, J.; Wojciechowska, U.; Mańczuk, M.; Łobaszewski, J. Lung Cancer Epidemiology: Contemporary and Future Challenges Worldwide. *Ann. Transl. Med.* **2016**, *4*, 150. [CrossRef]
9. Horn, L.; Lovly, C.M. Chapter 74: Neoplasms of the Lung. In *Harrison's Principles of Internal Medicine*; Loscalzo, J., Fauci, A., Kasper, D., Hauser, S., Longo, D., Jameson, J.L., Eds.; McGraw Hill: New York, NY, USA, 2018.
10. Hirsch, F.R.; Franklin, W.A.; Gazdar, A.F.; Bunn, P.A. Early Detection of Lung Cancer: Clinical Perspectives of Recent Advances in Biology and Radiology. *Clin. Cancer Res.* **2001**, *7*, 5–22.
11. Hirsch, F.R.; Scagliotti, G.V.; Mulshine, J.L.; Kwon, R.; Curran, W.J.; Wu, Y.-L.; Paz-Ares, L. Lung Cancer: Current Therapies and New Targeted Treatments. *Lancet* **2017**, *389*, 299–311. [CrossRef] [PubMed]
12. Jakubek, Y.; Lang, W.; Vattathil, S.; Garcia, M.; Xu, L.; Huang, L.; Yoo, S.-Y.; Shen, L.; Lu, W.; Chow, C.-W.; et al. Genomic Landscape Established by Allelic Imbalance in the Cancerization Field of a Normal Appearing Airway. *Cancer Res.* **2016**, *76*, 3676–3683. [CrossRef]
13. Kiyohara, C.; Otsu, A.; Shirakawa, T.; Fukuda, S.; Hopkin, J.M. Genetic Polymorphisms and Lung Cancer Susceptibility: A Review. *Lung Cancer* **2002**, *37*, 241–256. [CrossRef]
14. Li, J.; Lu, X.; Zou, X.; Jiang, Y.; Yao, J.; Liu, H.; Ni, B.; Ma, H. COX-2 Rs5275 and Rs689466 Polymorphism and Risk of Lung Cancer: A PRISMA-Compliant Meta-Analysis. *Medicine* **2018**, *97*, e11859. [CrossRef]
15. Guo, X.T.; Wang, J.F.; Zhang, L.Y.; Xu, G.Q. Quantitative Assessment of the Effects of MMP-2 Polymorphisms on Lung Carcinoma Risk. *Asian Pac. J. Cancer Prev.* **2012**, *13*, 2853–2856. [CrossRef]
16. Hecht, S.S. Cigarette Smoking and Lung Cancer: Chemical Mechanisms and Approaches to Prevention. *Lancet Oncol.* **2002**, *3*, 461–469. [CrossRef] [PubMed]
17. Li, X.; Liu, C.; Ran, R.; Liu, G.; Yang, Y.; Zhao, W.; Xie, X.; Li, J. Matrix Metalloproteinase Family Gene Polymorphisms and Lung Cancer Susceptibility: An Updated Meta-Analysis. *J. Thorac. Dis.* **2020**, *12*, 349–362. [CrossRef] [PubMed]
18. Peng, S.; Chen, M.; Wang, C.; Liu, C.; Luo, K.; Yang, L. Study on the Relationship between MMP-2, MMP-9 Gene Polymorphisms, and the Risk of Colorectal Cancer. *Evid.-Based Complement. Altern. Med.* **2022**, *2022*, 7357160. [CrossRef]

19. Schwartz, A.G.; Prysak, G.M.; Bock, C.H.; Cote, M.L. The Molecular Epidemiology of Lung Cancer. *Carcinogenesis* **2006**, *28*, 507–518. [CrossRef]
20. Shields, P.G.; Harris, C.C. Molecular Epidemiology and the Genetics of Environmental Cancer. *JAMA* **1991**, *266*, 681–687. [CrossRef]
21. Blanco-Prieto, S.; Barcia-Castro, L.; Páez de la Cadena, M.; Rodríguez-Berrocal, F.J.; Vázquez-Iglesias, L.; Botana-Rial, M.I.; Fernández-Villar, A.; De Chiara, L. Relevance of Matrix Metalloproteases in Non-Small Cell Lung Cancer Diagnosis. *BMC Cancer* **2017**, *17*, 823. [CrossRef]
22. Schveigert, D.; Cicenas, S.; Bruzas, S.; Samalavicius, N.; Gudleviciene, Z.; Didziapetriene, J. The Value of MMP-9 for Breast and Non-Small Cell Lung Cancer Patients' Survival. *Adv. Med. Sci.* **2013**, *58*, 73–82. [CrossRef]
23. Butkiewicz, D.; Krzesniak, M.; Drosik, A.; Giglok, M.; Gdowicz-Kłosok, A.; Kosarewicz, A.; Rusin, M.; Masłyk, B.; Gawkowska-Suwińska, M.; Suwiński, R. The VEGFR2, COX-2 and MMP-2 Polymorphisms Are Associated with Clinical Outcome of Patients with Inoperable Non-Small Cell Lung Cancer. *Int. J. Cancer* **2015**, *137*, 2332–2342. [CrossRef]
24. Li, H.; Liang, X.; Qin, X.; Cai, S.; Yu, S. Association of Matrix Metalloproteinase Family Gene Polymorphisms with Lung Cancer Risk: Logistic Regression and Generalized Odds of Published Data. *Sci. Rep.* **2015**, *5*, 10056. [CrossRef]
25. Dofara, S.G.; Chang, S.-L.; Diorio, C. Gene Polymorphisms and Circulating Levels of MMP-2 and MMP-9: A Review of Their Role in Breast Cancer Risk. *Anticancer Res.* **2020**, *40*, 3619–3631. [CrossRef] [PubMed]
26. Parks, W.C. Matrix metalloproteinases. In *Encyclopedia of Respiratory Medicine*; Elsevier: Amsterdam, The Netherlands, 2006; pp. 18–25.
27. Wang, X.; Khalil, R.A. Matrix Metalloproteinases, Vascular Remodeling, and Vascular Disease. *Adv. Pharmacol.* **2018**, *81*, 241–330. [PubMed]
28. Iniesta, P.; Morán, A.; De Juan, C.; Gómez, A.; Hernando, F.; García-Aranda, C.; Frías, C.; Díaz-López, A.; Rodríguez-Jiménez, F.-J.; Balibrea, J.-L.; et al. Biological and Clinical Significance of MMP-2, MMP-9, TIMP-1 and TIMP-2 in Non-Small Cell Lung Cancer. *Oncol. Rep.* **2007**, *17*, 217–223. [CrossRef] [PubMed]
29. Chen, G.-L.; Wang, S.-C.; Shen, T.-C.; Tsai, C.-W.; Chang, W.-S.; Li, H.-T.; Wu, C.-N.; Chao, C.-Y.; Hsia, T.-C.; Bau, D.-T. The Association of Matrix Metalloproteinas-2 Promoter Polymorphisms with Lung Cancer Susceptibility in Taiwan. *Chin. J. Physiol.* **2019**, *62*, 210. [CrossRef]
30. Hsu, S.-W.; Gong, C.-L.; Hsu, H.-M.; Chao, C.-C.; Wang, Y.-C.; Chang, W.-S.; Tsai, Y.-T.; Shih, L.-C.; Tsai, C.-W.; Bau, D.-T. Contribution of Matrix Metalloproteinase-2 Promoter Genotypes to Nasopharyngeal Cancer Susceptibility and Metastasis in Taiwan. *Cancer Genom.-Proteom.* **2019**, *16*, 287–292. [CrossRef] [PubMed]
31. Yueh, T.-C.; Hung, Y.-C.; Lee, H.-T.; Yang, M.-D.; Wang, Z.-H.; Yang, Y.-C.; Ke, T.-W.; Pei, J.-S.; Tsai, C.-W.; Bau, D.-T.; et al. Role of Matrix Metallopeptidase-2 Genotypes in Taiwanese Patients With Colorectal Cancer. *Anticancer Res.* **2022**, *42*, 5335–5342. [CrossRef]
32. Li, P.-H.; Liao, C.-H.; Huang, W.-C.; Chang, W.-S.; Wu, H.-C.; Hsu, S.-W.; Chen, K.-Y.; Wang, Z.-H.; Hsia, T.-C.; Bau, D.-T.; et al. Association of Matrix Metalloproteinase-2 Genotypes With Prostate Cancer Risk. *Anticancer Res.* **2023**, *43*, 343–349. [CrossRef]
33. Liu, S.; Xu, C.; Wu, W.; Fu, Z.; He, S.; Qin, M.; Huang, J. Sphingosine Kinase 1 Promotes the Metastasis of Colorectal Cancer by Inducing the Epithelial-mesenchymal Transition Mediated by the FAK/AKT/MMPs Axis. *Int. J. Oncol.* **2018**, *54*, 41–52. [CrossRef]
34. Kesanakurti, D.; Chetty, C.; Dinh, D.H.; Gujrati, M.; Rao, J.S. Role of MMP-2 in the Regulation of IL-6/Stat3 Survival Signaling via Interaction with A5β1 Integrin in Glioma. *Oncogene* **2013**, *32*, 327–340. [CrossRef]
35. Liu, C.J.; Hsia, T.C.; Wang, R.F.; Tsai, C.W.; Chu, C.C.; Hang, L.W.; Wang, C.H.; Lee, H.Z.; Tsai, R.Y.; Bau, D.T. Interaction of Cyclooxygenase 2 Genotype and Smoking Habit in Taiwanese Lung Cancer Patients. *Anticancer Res.* **2010**, *30*, 1195–1199.
36. Wang, W.; Fan, X.; Zhang, Y.; Yang, Y.; Yang, S.; Li, G. Association between COX-2 Polymorphisms and Lung Cancer Risk. *Med. Sci. Monit.* **2015**, *21*, 3740–3747. [CrossRef] [PubMed]
37. Campa, D.; Zienolddiny, S.; Maggini, V.; Skaug, V.; Haugen, A.; Canzian, F. Association of a Common Polymorphism in the Cyclooxygenase 2 Gene with Risk of Non-Small Cell Lung Cancer. *Carcinogenesis* **2004**, *25*, 229–235. [CrossRef] [PubMed]
38. Wadowska, K.; Bil-Lula, I.; Trembecki, Ł.; Śliwińska-Mossoń, M. Genetic Markers in Lung Cancer Diagnosis: A Review. *Int. J. Mol. Sci.* **2020**, *21*, 4569. [CrossRef]
39. Bayramoglu, A.; Gunes, H.V.; Metintas, M.; Deg, I.; Mutlu, F.; Alataş, F. The Association of MMP-9 Enzyme Activity, MMP-9 C1562T Polymorphism, and MMP-2 and-9 and TIMP-1,-2,-3, and-4 Gene Expression in Lung Cancer. *Genet. Test. Mol. Biomark.* **2009**, *13*, 671–678. [CrossRef] [PubMed]
40. Sanli, M.; Akar, E.; Pehlivan, S.; Bakir, K.; Tuncozgur, B.; Isik, A.F.; Pehlivan, M.; Elbeyli, L. The Relationship of Metalloproteinase Gene Polymorphisms and Lung Cancer. *J. Surg. Res.* **2013**, *183*, 517–523. [CrossRef]
41. Hu, Z.; Huo, X.; Lu, D.; Qian, J.; Zhou, J.; Chen, Y.; Xu, L.; Ma, H.; Zhu, J.; Wei, Q.; et al. Functional Polymorphisms of Matrix Metalloproteinase-9 Are Associated with Risk of Occurrence and Metastasis of Lung Cancer. *Clin. Cancer Res.* **2005**, *11*, 5433–5439. [CrossRef]
42. Li, W.; Jia, M.X.; Wang, J.H.; Lu, J.L.; Deng, J.; Tang, J.X.; Liu, C. Association of Mmp9-1562c/t and Mmp13-77a/g Polymorphisms with Non-Small Cell Lung Cancer in Southern Chinese Population. *Biomolecules* **2019**, *9*, 107. [CrossRef]
43. Wang, J.; Cai, Y. Matrix Metalloproteinase 2 Polymorphisms and Expression in Lung Cancer: A Meta-Analysis. *Tumour. Biol.* **2012**, *33*, 1819–1828. [CrossRef] [PubMed]

44. González-Arriaga, P.; Pascual, T.; García-Alvarez, A.; Fernández-Somoano, A.; López-Cima, M.F.; Tardón, A. Genetic Polymorphisms in MMP 2, 9 and 3 Genes Modify Lung Cancer Risk and Survival. *BMC Cancer* **2012**, *12*, 121. [CrossRef]
45. Rollin, J.; Régina, S.; Vourc'h, P.; Iochmann, S.; Bléchet, C.; Reverdiau, P.; Gruel, Y. Influence of MMP-2 and MMP-9 Promoter Polymorphisms on Gene Expression and Clinical Outcome of Non-Small Cell Lung Cancer. *Lung Cancer* **2007**, *56*, 273–280. [CrossRef] [PubMed]
46. Zhou, Y.; Yu, C.; Miao, X.; Wang, Y.; Tan, W.; Sun, T.; Zhang, X.; Xiong, P.; Lin, D. Functional Haplotypes in the Promoter of Matrix Metalloproteinase-2 and Lung Cancer Susceptibility. *Carcinogenesis* **2005**, *26*, 1117–1121. [CrossRef]
47. Nakamura, T.; Ebihara, I.; Shimada, N.; Koide, H. Effect of Cigarette Smoking on Plasma Metalloproteinase-9 Concentration. *Clin. Chim. Acta* **1998**, *276*, 173–177. [CrossRef]
48. Cao, C.; Xu, N.; Zheng, X.; Zhang, W.; Lai, T.; Deng, Z.; Huang, X. Elevated Expression of MMP-2 and TIMP-2 Cooperatively Correlates with Risk of Lung Cancer. *Oncotarget* **2017**, *8*, 80560–80567. [CrossRef]
49. Drzewiecka-Jędrzejczyk, M.; Wlazeł, R.; Terlecka, M.; Jabłoński, S. Serum Metalloproteinase-2 and Tissue Inhibitor of Metalloproteinase-2 in Lung Carcinoma Patients. *J. Thorac. Dis.* **2017**, *9*, 5306–5313. [CrossRef] [PubMed]
50. Wadowska, K.; Błasiak, P.; Rzechonek, A.; Bil-Lula, I.; Śliwińska-Mossoń, M. New Insights on Old Biomarkers Involved in Tumor Microenvironment Changes and Their Diagnostic Relevance in Non-Small Cell Lung Carcinoma. *Biomolecules* **2021**, *11*, 1208. [CrossRef] [PubMed]
51. Gao, L.; Zhang, L.; Shi, X.; Zuo, L. MMP2 Gene Polymorphism and Tumor Susceptibility Study. *Res. Sq.* 2023; *preprint*. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Supplementary materials

Table S1. Observed and expected MMP-2 -735C/T and MMP-9 -1562C/T genotype frequencies in controls and cases in accordance with Hardy-Weinberg equilibrium.

	Observed quantity			Expected quantity			*p* value (Pearson's chi-square test)
	CC	CT	TT	CC	CT	TT	
MMP-2 -735C/T (rs2285053)							
Control – overall	77	18	5	74	24	2	0.041271
Control – non-smokers	39	5	3	35	11	1	0.012686
Control – smokers	37	12	2	38	12	1	0.600091
Cases – overall	83	26	1	81	26	2	0.718193
Cases – adenocarcinoma	39	10	0	36	12	1	0.485565
Cases – squamous cell carcinoma	28	7	0	26	8	1	0.576778
Cases – other lung neoplasms	16	9	1	19	6	1	0.330751
MMP-9 -1562C/T (rs3918242)							
Control – overall	70	25	4	69	28	3	0.664254
Control – non-smokers	35	10	2	33	13	1	0.533840
Control – smokers	34	14	2	35	14	1	0.867274
Cases – overall	76	30	3	76	30	3	0.997984
Cases – adenocarcinoma	39	9	1	34	14	1	0.303539
Cases – squamous cell carcinoma	22	12	0	24	9	1	0.417721
Cases – other lung neoplasms	15	9	2	18	7	1	0.200349

13.5. Załącznik 5 – Całkowity dorobek naukowy

Katarzyna Izabela Wadowska **Wrocław, 20.09.2023**

Wykaz publikacji

1. Publikacje w czasopismach naukowych z IF

Lp	Opis bibliograficzny	Punkty	IF
1	**Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Śliwińska-Mossoń Mariola: Analysis of MMP-2-735C/T (rs2285053) and MMP-9-1562C/T (rs3918242) polymorphisms in the risk assessment of developing lung cancer, **International Journal of Molecular Sciences**, 2023, vol. 24, nr 13, art.10576 [20 s.], DOI:10.3390/ijms241310576	140	5,6
2	**Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola: Hepcidin as a diagnostic biomarker in anaemic lung cancer patients, **Cancers**, 2023, vol. 15, nr 1, art.224 [24 s.], DOI:10.3390/cancers15010224	200	5,2
3	**Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola: New insights on old biomarkers involved in tumor microenvironment changes and their diagnostic relevance in non-small cell lung carcinoma, **Biomolecules**, 2021, vol. 11, nr 8, art.1208 [24 s.], DOI:10.3390/biom11081208	100	6,064
4	Śliwińska-Mossoń Mariola, **Wadowska Katarzyna**, Trembecki Łukasz, Bil-Lula Iwona: Markers useful in monitoring radiation-induced lung injury in lung cancer patients: a review, **Journal of Personalized Medicine**, 2020, vol. 10, nr 3, art.72 [15 s.], DOI:10.3390/jpm10030072	70	4,945
5	**Wadowska Katarzyna**, Bil-Lula Iwona, Trembecki Łukasz, Śliwińska-Mossoń Mariola: Genetic markers in lung cancer diagnosis: a review, **International Journal of Molecular Sciences**, 2020, vol. 21, nr 13, art.4569 [24 s.], DOI:10.3390/ijms21134569	140	5,924
	Podsumowanie	650	27,733

2. Rozdziały w monografii

Lp	Opis bibliograficzny	Punkty
1	Gajewska Beata, **Wadowska Katarzyna**, Śliwińska-Mossoń Mariola: Wpływ palenia papierosów na stężenie metaloproteinazy 2 i 9 w surowicy, W: Komórki, tkanki i narządy ludzkie - prawne problemy wykorzystania medycznego i pozamedycznego, (red.) Łukasz B. Pilarz, Lublin 2023, Wydawnictwo Naukowe TYGIEL Sp. z o. o., s. 75-83, ISBN 978-83-67104-93-7	20
	Podsumowanie	20

3. Abstrakty konferencyjne

Lp	Opis bibliograficzny
1	Bil-Lula Iwona, Pałyga A., **Wadowska Katarzyna**, Kuliczkowski Wiktor: FGF23 increases the diagnostic power of the test and facilitates the diagnosis of myocardial infarction - preliminary study, Clinical Chemistry and Laboratory Medicine, 2023, vol. 61, nr suppl.1, s.S547 poz.P0461, [WorldLab - EuroMedLab Roma 2023. Rome, Italy, May 21-25, 2023]
2	Gajewska Beata, Trembecki Łukasz, **Wadowska Katarzyna**, Śliwińska-Mossoń Mariola: Ocena zmian stężeń interleukiny-6 i czynnika martwicy nowotworów alfa w surowicy pacjentów z nowotworem płuc poddanych radioterapii, Biuletyn Polskiego Towarzystwa Onkologicznego NOWOTWORY, 2022, vol. 7, nr supl.1, s. 22, [Studenckie Onko-Forum 2022. Warszawa, 28 maja 2022 r.]
3	Gajewska Beata, **Wadowska Katarzyna**, Krysta Agnieszka, Śliwińska-Mossoń Mariola: Rola metaloproteinaz macierzy pozakomórkowej MMP-2 i MMP-9 w rozwoju insulinooporności: badania wstępne, Diagnostyka Laboratoryjna, 2022, vol. 58, nr suppl.1 , 68 poz.P-01, [XX Zjazd Naukowy Polskiego Towarzystwa Diagnostyki Laboratoryjnej. Kielce, 19-22 października 2022 roku. Streszczenia]
4	Gajewska Beata, **Wadowska Katarzyna**, Śliwińska-Mossoń Mariola: Wpływ palenia papierosów na stężenie metaloproteinazy 2 i 9 w surowicy, W: II Ogólnopolska Konferencja Naukowa „Od komórki do człowieka dyscypliny medyczne i niemedyczne". Lublin, 17 grudnia 2022 r. Abstrakty, (red.) Alicja Danielewska, Paulina Pomajda, Lublin 2022, Fundacja na rzecz promocji nauki i rozwoju Tygiel, s. 31-32, ISBN 978-83-67670-00-5

Lp	Opis bibliograficzny
5	**Wadowska Katarzyna**, Bil-Lula Iwona, Śliwińska-Mossoń Mariola: Badania diagnostyczne w proteinozie pęcherzyków płucnych, W: II. WSML - Wrocławskie Spotkanie Medycyny Laboratoryjnej. Wrocław, 26 marca 2021. Program konferencji i książka abstraktów 2021, 34 poz.P24, [[Dostęp 7.04.2021]. Dostępny w: https://medtube.pl/uploads/a/4/03252a48ceca6bebd6b26b609a92fb950fe3.pdf]
6	**Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola: Parametry metaboliczne u pacjentów z rakiem płuca ze współistniejącą cukrzycą typu 2, W: II. WSML - Wrocławskie Spotkanie Medycyny Laboratoryjnej. Wrocław, 26 marca 2021. Program konferencji i książka abstraktów 2021, 15 poz.P5, [[Dostęp 7.04.2021]. Dostępny w: https://medtube.pl/uploads/a/4/03252a48ceca6bebd6b26b609a92fb950fe3.pdf]
7	**Wadowska Katarzyna**, Bil-Lula Iwona, Śliwińska-Mossoń Mariola: Next-generation sequencing in lung cancer diagnosis and therapeutic strategies, W: 4th International Wroclaw Scientific Meetings. Wrocław, 09-10 October 2020, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2020, Wydawnictwo Naukowe TYGIEL sp. z o.o., s. 254-255, ISBN 978-83-66489-37-0
8	Tłokińska Paulina, **Wadowska Katarzyna**, Kepinska Marta: Akrylamid i jego metabolit jako substancje rakotwórcze we współczesnej diecie, W: Konferencja Naukowa "Wpływ związków toksycznych na zdrowie ludzi i zwierząt". Wrocław, 30.03.2017 r. Książka abstraktów 2017, 21 poz.2
9	**Wadowska Katarzyna**, Tłokińska Paulina, Bizoń Anna: Markery stresu oksydacyjnego na przestrzeni lat, W: Konferencja Naukowa "Wpływ związków toksycznych na zdrowie ludzi i zwierząt". Wrocław, 30.03.2017 r. Książka abstraktów 2017, 33 poz.3
10	**Wadowska Katarzyna**, Tłokińska Paulina, Kepinska Marta: Ocena jakości wrocławskiego powietrza w okresie grzewczym, W: Konferencja Naukowa "Wpływ związków toksycznych na zdrowie ludzi i zwierząt". Wrocław, 30.03.2017 r. Książka abstraktów 2017, 23 poz.5

Sumaryczny Impact Factor: 27,733

Punktacja Ministerialna: 670

22.09.2023 r. Beata Majewska

Uniwersytet Medyczny we Wrocławiu
Biblioteka Główna
DZIAŁ BIBLIOGRAFII I BIBLIOMETRII
ul. Marcinkowskiego 2-6, 50-368 Wrocław
tel. 71 784 19 25

13.6. Załącznik 6 – Oświadczenia współautorów publikacji włączonych do cyklu

1) Oświadczenie Katarzyny Wadowskiej
2) Oświadczenie dr hab. Marioli Śliwińska-Mossoń
3) Oświadczenie dr Piotra Błasiaka
4) Oświadczenie dr hab. Adama Rzechonka
5) Oświadczenie dr hab. Iwony Bil-Luli, prof. Uczelni
6) Oświadczenie lek. Łukasza Trembeckiego

Wrocław, 26.06.2023 r.
miejscowość, data

mgr Katarzyna Wadowska
tytuł, imię i nazwisko

Szkoła Doktorska
Katedra Analityki Medycznej
Zakład Chemii Klinicznej i Hematologii Laboratoryjnej
Wydział Farmaceutyczny
Uniwersytet Medyczny im. Piastów Śląskich
ul. Borowska 211A, 50-556 Wrocław
miejsce zatrudnienia

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

1. **Wadowska Katarzyna**, Bil-Lula Iwona, Trembecki Łukasz, Śliwińska-Mossoń Mariola, 2020, Genetic Markers in Lung Cancer Diagnosis: A Review, International Journal of Molecular Sciences, 21 (13), art. 4569, DOI: 10.3390/ijms21134569

   mój udział polegał na zebraniu i analizie piśmiennictwa, interpretacji informacji w nim zawartych, napisaniu pierwotnej wersji manuskryptu oraz współtworzeniu jego ostatecznej formy

2. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola, 2021, New Insights on Old Biomarkers Involved in Tumor Microenvironment Changes and Their Diagnostic Relevance in Non-Small Cell Lung Carcinoma, Biomolecules, 11 (8), art. 1208, DOI: 10.3390/biom11081208

   mój udział polegał na przygotowaniu bazy danych pacjentów, współudziale w doborze metod badawczych i ustaleniu warunków prowadzenia badań, wykonaniu części doświadczalnej oraz analizy statystycznej, interpretacji uzyskanych wyników, przechowywaniu wyników, zebraniu i analizie piśmiennictwa, napisaniu pierwotnej wersji manuskryptu oraz współtworzeniu jego ostatecznej formy, stworzeniu ryc.1, korespondencji z redakcją (autor korespondencyjny), a także na współudziale w pozyskiwaniu źródeł finansowania i kierowaniu projektem naukowym obejmującym badania opisane w tej pracy

3. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola, 2023, Hepcidin as a Diagnostic Biomarker in Anaemic Lung Cancer Patients, Cancers, 15 (1), art. 224, DOI: 10.3390/cancers15010224

   mój udział polegał na opracowaniu koncepcji i planu wykonania badań, przygotowaniu bazy danych pacjentów, doborze metod badawczych i ustaleniu warunków prowadzenia badań, wykonaniu części doświadczalnej oraz analizy statystycznej, interpretacji uzyskanych wyników, przechowywaniu wyników, zebraniu i analizie piśmiennictwa, napisaniu pierwotnej wersji manuskryptu oraz współtworzeniu jego ostatecznej formy, stworzeniu ryc.7, a także korespondencji z redakcją (autor korespondencyjny)

4. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Śliwińska-Mossoń Mariola, 2023, Analysis of MMP-2 -735C/T (rs2285053) and MMP-9 -1562C/T (rs3918242) Polymorphisms in the Risk Assessment of Developing Lung Cancer, International Journal of Molecular Sciences, 24 (13), art. 10576, DOI: 10.3390/ijms241310576

   mój udział polegał na opracowaniu koncepcji i planu wykonania badań, przygotowaniu bazy danych pacjentów, doborze metod badawczych i ustaleniu warunków prowadzenia badań, wykonaniu części doświadczalnej oraz analizy statystycznej, interpretacji uzyskanych wyników, przechowywaniu wyników, zebraniu i analizie piśmiennictwa, napisaniu pierwotnej wersji manuskryptu oraz współtworzeniu jego ostatecznej formy, stworzeniu ryc.2, a także kierowaniu projektem naukowym obejmującym badania opisane w tej pracy.

Jednocześnie wyrażam zgodę, aby wymienione powyżej artykuły zostały włączone do cyklu publikacyjnego będącego podstawą rozprawy doktorskiej mgr Katarzyny Wadowskiej.

Katarzyna Wadowska
....
podpis współautora

Uniwersytet Medyczny we Wrocławiu
Katedra Analityki Medycznej
ZAKŁAD CHEMII KLINICZNEJ
I HEMATOLOGII LABORATORYJNEJ
adiunkt
dr hab. Mariola Śliwińska-Mossoń
....
podpis promotora

Wrocław, 26.06.2023 r.
miejscowość, data

dr hab. n. med. Mariola Śliwińska-Mossoń
tytuł, imię i nazwisko

Katedra Analityki Medycznej
Zakład Chemii Klinicznej i Hematologii Laboratoryjnej
Wydział Farmaceutyczny
Uniwersytet Medyczny im. Piastów Śląskich
ul. Borowska 211A, 50-556 Wrocław
miejsce zatrudnienia

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

1. **Wadowska Katarzyna**, Bil-Lula Iwona, Trembecki Łukasz, Śliwińska-Mossoń Mariola, 2020, Genetic Markers in Lung Cancer Diagnosis: A Review, International Journal of Molecular Sciences, 21 (13), art. 4569, DOI: 10.3390/ijms21134569

   mój udział polegał na ustaleniu koncepcji i założeń pracy, planowaniu i nadzorze nad pisaniem pracy, zebraniu piśmiennictwa, ocenie merytorycznej manuskryptu, współtworzeniu ostatecznej wersji manuskryptu, korespondencji z redakcją (autor korespondencyjny) oraz pozyskaniu źródeł finansowania

2. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola, 2021, New Insights on Old Biomarkers Involved in Tumor Microenvironment Changes and Their Diagnostic Relevance in Non-Small Cell Lung Carcinoma, Biomolecules, 11 (8), art. 1208, DOI: 10.3390/biom11081208

   mój udział polegał na ustaleniu koncepcji i założeń pracy, planowaniu i nadzorze nad wykonanymi badaniami, współudziale w doborze metod badawczych i ustaleniu warunków prowadzenia badań, udziale w wykonaniu części oznaczeń do pracy, ocenie merytorycznej manuskryptu i krytycznej weryfikacji wniosków, współtworzeniu ostatecznej wersji manuskryptu oraz pozyskaniu źródeł finansowania

3. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola, 2023, Hepcidin as a Diagnostic Biomarker in Anaemic Lung Cancer Patients, Cancers, 15 (1), art. 224, DOI: 10.3390/cancers15010224

   mój udział polegał na ustaleniu koncepcji i założeń pracy, planowaniu i nadzorze nad wykonanymi badaniami, współudziale w doborze metod badawczych i warunków prowadzenia badań, udziale w wykonaniu części oznaczeń do pracy, ocenie merytorycznej manuskryptu i krytycznej weryfikacji wniosków, współtworzeniu ostatecznej wersji manuskryptu, pozyskaniu źródeł finansowania oraz kierowaniu projektem naukowym obejmującym badania opisane w tej pracy

4. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Śliwińska-Mossoń Mariola, 2023, Analysis of MMP-2 -735C/T (rs2285053) and MMP-9 -1562C/T (rs3918242) Polymorphisms in the Risk Assessment of Developing Lung Cancer, International Journal of Molecular Sciences,

   mój udział polegał na ustaleniu koncepcji i założeń pracy, planowaniu i nadzorze nad wykonanymi badaniami, współudziale w doborze metod badawczych i warunków prowadzenia badań, zebraniu piśmiennictwa, ocenie merytorycznej manuskryptu i krytycznej weryfikacji wniosków, współtworzeniu ostatecznej wersji manuskryptu, korespondencji z redakcją (autor korespondencyjny) oraz pozyskaniu źródeł finansowania.

Jednocześnie wyrażam zgodę, aby wymienione powyżej artykuły zostały włączone do cyklu publikacyjnego będącego podstawą rozprawy doktorskiej mgr Katarzyny Wadowskiej.

Mariola Śliwińska-Mossoń

podpis współautora

Uniwersytet Medyczny we Wrocławiu
Katedra Analityki Medycznej
ZAKŁAD CHEMII KLINICZNEJ
I HEMATOLOGII LABORATORYJNEJ
adiunkt
dr hab. Mariola Śliwińska-Mossoń

Mariola Śliwińska-Mossoń

podpis promotora

Wrocław, 26.06.2023 r.
miejscowość, data

dr n. med. Piotr Błasiak
tytuł, imię i nazwisko

Katedra i Klinika Chirurgii Klatki Piersiowej
Wydział Lekarski
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu
Dolnośląskie Centrum Onkologii, Pulmonologii i Hematologii
Dolnośląskie Centrum Torakochirurgii
ul. Grabiszyńska 105, 53-439 Wrocław
miejsce zatrudnienia

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

1. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola, 2021, New Insights on Old Biomarkers Involved in Tumor Microenvironment Changes and Their Diagnostic Relevance in Non-Small Cell Lung Carcinoma, Biomolecules, 11 (8), art. 1208, DOI: 10.3390/biom11081208

   mój udział polegał na opracowaniu koncepcji i założeń pracy, wyselekcjonowaniu pacjentów z rakiem płuca do badania, pobraniu i zabezpieczeniu materiału biologicznego do badań, współtworzeniu bazy danych pacjentów, korekcie ostatecznej wersji manuskryptu

2. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola, 2023, Hepcidin as a Diagnostic Biomarker in Anaemic Lung Cancer Patients, Cancers, 15 (1), art. 224, DOI: 10.3390/cancers15010224

   mój udział polegał na wyselekcjonowaniu pacjentów z rakiem płuca do badania, pobraniu i zabezpieczeniu materiału biologicznego do badań, współtworzeniu bazy danych pacjentów, korekcie ostatecznej wersji manuskryptu

3. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Śliwińska-Mossoń Mariola, 2023, Analysis of MMP-2 -735C/T (rs2285053) and MMP-9 -1562C/T (rs3918242) Polymorphisms in the Risk Assessment of Developing Lung Cancer, International Journal of Molecular Sciences, 24 (13), art. 10576, DOI: 10.3390/ijms241310576

   mój udział polegał na wyselekcjonowaniu pacjentów z rakiem płuca do badania, pobraniu i zabezpieczeniu materiału biologicznego do badań, współtworzeniu bazy danych pacjentów, korekcie ostatecznej wersji manuskryptu

Jednocześnie wyrażam zgodę, aby wymienione powyżej artykuły zostały włączone do cyklu publikacyjnego będącego podstawą rozprawy doktorskiej mgr Katarzyny Wadowskiej.

Uniwersytet Medyczny we Wrocławiu
KATEDRA I KLINIKA CHIRURGII
KLATKI PIERSIOWEJ
adiunkt
dr n. med. Piotr Błasiak

----
podpis współautora

Uniwersytet Medyczny we Wrocławiu
Katedra Analityki Medycznej
ZAKŁAD CHEMII KLINICZNEJ
I HEMATOLOGII LABORATORYJNEJ
adiunkt

----
podpis promotora

dr hab. Mariola Śliwińska-Mossoń

Wrocław, 26.06.2023 r.
miejscowość, data

dr hab. n. med. Adam Rzechonek
tytuł, imię i nazwisko

Dolnośląskie Centrum Onkologii, Pulmonologii i Hematologii
Dolnośląskie Centrum Torakochirurgii
ul. Grabiszyńska 105, 53-439 Wrocław
miejsce zatrudnienia

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

1. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola, 2021, New Insights on Old Biomarkers Involved in Tumor Microenvironment Changes and Their Diagnostic Relevance in Non-Small Cell Lung Carcinoma, Biomolecules, 11 (8), art. 1208, DOI: 10.3390/biom11081208

   mój udział polegał na opracowaniu koncepcji i założeń pracy, wyselekcjonowaniu pacjentów z rakiem płuca do badania, pobraniu i zabezpieczeniu materiału biologicznego do badań, współtworzeniu bazy danych pacjentów, korekcie ostatecznej wersji manuskryptu

2. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola, 2023, Hepcidin as a Diagnostic Biomarker in Anaemic Lung Cancer Patients, Cancers, 15 (1), art. 224, DOI: 10.3390/cancers15010224

   mój udział polegał na wyselekcjonowaniu pacjentów z rakiem płuca do badania, pobraniu i zabezpieczeniu materiału biologicznego do badań, współtworzeniu bazy danych pacjentów, korekcie ostatecznej wersji manuskryptu

3. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Śliwińska-Mossoń Mariola, 2023, Analysis of MMP-2 -735C/T (rs2285053) and MMP-9 -1562C/T (rs3918242) Polymorphisms in the Risk Assessment of Developing Lung Cancer, International Journal of Molecular Sciences, 24 (13), art. 10576, DOI: 10.3390/ijms241310576

   mój udział polegał na wyselekcjonowaniu pacjentów z rakiem płuca do badania, pobraniu i zabezpieczeniu materiału biologicznego do badań, współtworzeniu bazy danych pacjentów, korekcie ostatecznej wersji manuskryptu.

Jednocześnie wyrażam zgodę, aby wymienione powyżej artykuły zostały włączone do cyklu publikacyjnego będącego podstawą rozprawy doktorskiej mgr Katarzyny Wadowskiej.

dr n. med. Adam Rzechonek
chirurg ogólny
specjalista torakochirurg
Wilczyce, ul. Dworska 10
51-361 Wrocław
1176870

podpis współautora

Uniwersytet Medyczny we Wrocławiu
Katedra Analityki Medycznej
ZAKŁAD CHEMII KLINICZNEJ
I HEMATOLOGII LABORATORYJNEJ
adiunkt
dr hab. Mariola Śliwińska-Mossoń

podpis promotora

Wrocław, 26.06.2023 r.
miejscowość, data

dr hab. n. farm. Iwona Bil-Lula, prof. UMW
tytuł, imię i nazwisko

Katedra Analityki Medycznej
Zakład Chemii Klinicznej i Hematologii Laboratoryjnej
Wydział Farmaceutyczny
Uniwersytet Medyczny im. Piastów Śląskich
ul. Borowska 211A, 50-556 Wrocław
miejsce zatrudnienia

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

1. **Wadowska Katarzyna**, Bil-Lula Iwona, Trembecki Łukasz, Śliwińska-Mossoń Mariola, 2020, Genetic Markers in Lung Cancer Diagnosis: A Review, International Journal of Molecular Sciences, 21 (13), art. 4569, DOI: 10.3390/ijms21134569

   mój udział polegał na planowaniu i nadzorze nad pisaniem pracy, krytycznej analizie pracy, korekcie językowej, współtworzeniu ostatecznej wersji manuskryptu oraz pozyskaniu źródeł finansowania

2. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola, 2021, New Insights on Old Biomarkers Involved in Tumor Microenvironment Changes and Their Diagnostic Relevance in Non-Small Cell Lung Carcinoma, Biomolecules, 11 (8), art. 1208, DOI: 10.3390/biom11081208

   mój udział polegał na planowaniu i nadzorze nad wykonanymi badaniami oraz pisaniem pracy, krytycznej analizie pracy, korekcie językowej, administracji projektem, a także na pozyskaniu źródeł finansowania

3. **Wadowska Katarzyna**, Błasiak Piotr, Rzechonek Adam, Bil-Lula Iwona, Śliwińska-Mossoń Mariola, 2023, Hepcidin as a Diagnostic Biomarker in Anaemic Lung Cancer Patients, Cancers, 15 (1), art. 224, DOI: 10.3390/cancers15010224

   mój udział polegał na krytycznej analizie pracy, korekcie językowej, współtworzeniu ostatecznej wersji manuskryptu oraz na pozyskaniu źródeł finansowania.

Jednocześnie wyrażam zgodę, aby wymienione powyżej artykuły zostały włączone do cyklu publikacyjnego będącego podstawą rozprawy doktorskiej mgr Katarzyny Wadowskiej.

Uniwersytet Medyczny we Wrocławiu
KATEDRA ANALITYKI MEDYCZNEJ
kierownik
dr hab. Iwona Bil-Lula, prof. uczelni
podpis współautora

Uniwersytet Medyczny we Wrocławiu
Katedra Analityki Medycznej
ZAKŁAD CHEMII KLINICZNEJ
I HEMATOLOGII LABORATORYJNEJ
adiunkt
dr hab. Mariola Śliwińska-Mossoń

Wrocław, 26.06.2023 r.
miejscowość, data

lek. Łukasz Trembecki
tytuł, imię i nazwisko

Katedra Onkologii
Klinika Radioterapii
Wydział Lekarski
Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu
Dolnośląskie Centrum Onkologii, Pulmonologii i Hematologii
Zakład Radioterapii
pl. Hirszfelda 12, 53-413 Wrocław
miejsce zatrudnienia

OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

1. **Wadowska Katarzyna**, Bil-Lula Iwona, Trembecki Łukasz, Śliwińska-Mossoń Mariola, 2020, Genetic Markers in Lung Cancer Diagnosis: A Review, International Journal of Molecular Sciences, 21 (13), art. 4569, DOI: 10.3390/ijms21134569

   mój udział polegał na ustaleniu koncepcji i założeń pracy oraz ocenie merytorycznej manuskryptu.

Jednocześnie wyrażam zgodę, aby wymienione powyżej artykuły zostały włączone do cyklu publikacyjnego będącego podstawą rozprawy doktorskiej mgr Katarzyny Wadowskiej.

Trembecki Łukasz

podpis współautora

lek. Łukasz Trembecki
specjalista
radioterapii onkologicznej
...5193

Uniwersytet Medyczny we Wrocławiu
Katedra Analityki Medycznej
ZAKŁAD CHEMII KLINICZNEJ
I HEMATOLOGII LABORATORYJNEJ
adiunkt
M. Śliwińska-Mossoń
dr hab. Mariola Śliwińska-Mossoń