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ROZPRAWA DOKTORSKA

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Zmiany glikozylacji klasteryny oraz ekspresji wybranych parametrów równowagi oksydacyjno-antyoksydacyjnej jako potencjalne biomarkery obniżonej płodności męskiej

The changes in clusterin glycosylation and expression of selected parameters of oxidative-antioxidant balance as potential biomarkers of reduced male fertility

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Nie dokonuje odkrycia, kto nie bada niemożliwości.

Albert Einstein

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Spis treści

1. Cykl publikacji stanowiących podstawę rozprawy doktorskiej

Cykl publikacji będących podstawą ubiegania się o stopień doktora nauk farmaceutycznych stanowią trzy publikacje oryginalne oraz jeden artykuł przeglądowy, opublikowane w latach 2020-2022 w czasopismach o zasięgu międzynarodowym, posiadających wysoki współczynnik wpływu IF.

I. **Ewa Janiszewska**, Ewa Maria Kratz: Could the glycosylation analysis of seminal plasma clusterin become a novel male infertility biomarker? **Molecular Reproduction and Development, 2020**, vol. 87, nr 5, s. 515-524, DOI:10.1002/mrd.23340 - artykuł przeglądowy (załącznik nr 1).

IF_{2020}=2,609; MEiN=100 pkt.

II. **Ewa Janiszewska**, Izabela Kokot, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The possible association of clusterin fucosylation changes with male fertility disorders. **Scientific Reports, 2021**, vol. 11, art.15674 [16 s.], DOI:10.1038/s41598-021-95288-w - artykuł oryginalny (załącznik nr 2).

IF_{2020}=4,997 MEiN=140 pkt.

III. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Zuzanna Stelmasiak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The association between clusterin sialylation degree and levels of oxidative–antioxidant balance markers in seminal plasmas and blood sera of male partners with abnormal sperm parameters. **International Journal of Molecular Sciences, 2022**, vol. 23, nr 18, art.10598 [28 s.], DOI:10.3390/ijms231810598 - artykuł oryginalny (załącznik nr 3).

IF_{2021}=6,208; MEiN=140 pkt.

IV. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: Are there associations between seminal plasma advanced oxidation protein products and selected redox-associated

biochemical parameters in infertile male patients? A preliminary report. **Cells, 2022**, vol. 11, nr 22, art.3667 [15 s.], DOI:10.3390/cells11223667 - artykuł oryginalny (załącznik nr 4).

IF_{2021}=7,666; MEiN=140 pkt.

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2. Wstęp

Niepłodność to rosnący problem na świecie, szczególnie w krajach wysokorozwiniętych. Szacuje się, że na całym świecie 20-30% przypadków niepłodności spowodowanych jest czynnikiem męskim i żeńskim jednocześnie, a kolejne 20-30% przypadków to niepłodność związana z czynnikiem męskim [1]. W Polsce ponad 1,5 miliona par starających się o potomstwo boryka się z tym schorzeniem, z czego połowa przypadków jest związana z niepłodnością męską [2,3].

Rutynowo wykonywana analiza seminologiczna nie dostarcza pełnych informacji na temat ewentualnych patologii dotyczących męskiego układu rozrodczego, prowadzących do zaburzeń płodności. Nawet 30-50% wyników badań standardowych nasienia niepłodnych mężczyzn nie wykazuje odchyleń od wartości referencyjnych podanych przez Światową Organizację Zdrowia (WHO, *ang. World Health Organization*), co jest określane mianem niepłodności idiopatycznej [4,5]. Warto zwrócić uwagę na fakt, że badanie nasienia dotyczy głównie plemników oraz ich właściwości, natomiast istotny składnik ejakulatu, plazma nasienia, traktowana jest marginalnie. Plazma nasienia stanowi ok. 98% ejakulatu i jest mieszaniną m. in. białek oraz glikoprotein, biorących udział w kapacytacji, reakcji akrosomalnej, interakcjach między oocytem a plemnikiem, modulacji odpowiedzi immunologicznej w żeńskich drogach rodnych oraz neutralizacji wolnych rodników [6–8].

Biorąc pod uwagę istotną rolę plazmy nasienia w dojrzewaniu plemników oraz procesie zapłodnienia wewnątrzustrojowego, a także fakt, że nie poznano w pełni wszystkich mechanizmów prowadzących do obniżonego potencjału rozrodczego mężczyzn, poszukuje się nowych, potencjalnych biomarkerów diagnostycznych obniżonej płodności męskiej. Niniejsza rozprawa stanowi próbę odpowiedzi na pytanie, czy zmiany ekspresji glikanów klasteryny (CLU, *ang. clusterin*) oraz wybranych parametrów stresu oksydacyjnego w plazmie nasienia i/lub surowicy mogą stać się nowymi, potencjalnymi markerami diagnostycznymi obniżonej płodności męskiej.

3. Wybrane aspekty teoretyczne

3.1. Niepłodność męska - definicja, epidemiologia, etiopatogeneza

Światowa Organizacja Zdrowia definiuje niepłodność jako niemożność zajścia w ciążę pomimo regularnego współżycia płciowego (3–4 razy w tygodniu), utrzymywanego powyżej 12 miesięcy, bez stosowania jakichkolwiek środków zapobiegawczych [9]. Szacuje się, że globalnie problem niepłodności dotyczy ponad 187 milionów ludzi, z czego nawet w 50% przypadków jest spowodowana czynnikiem męskim [10]. Niepłodność męską WHO uznała za krytyczny problem zdrowia publicznego [11].

Wśród wielu przyczyn niepłodności męskiej wymienia się wady anatomiczne, zaburzenia genetyczne, żylaki powrózków nasiennych, czynniki toksyczne (m. in. estrogeny środowiskowe, alkohol, związki chemiczne obecne w pożywieniu) oraz stres. Nawet 50% przypadków niepłodności męskiej to niepłodność idiopatyczna – występująca bez uchwytnej przyczyny [5]. Wspólnym mianownikiem wielu przyczyn niepłodności męskiej jest stres oksydacyjny, definiowany jako zaburzenie równowagi oksydacyjno-antyoksydacyjnej w organizmie [12].

W 2021 roku WHO wydała szóstą edycję rekomendacji dotyczących badania nasienia u mężczyzn o obniżonej płodności. Wprowadzono pewne zmiany w ocenie badania ejakulatu, jednak wyniki analizy seminologicznej wciąż nie dostarczają odpowiedzi na pytanie o patogenezę zaburzeń męskiej płodności [13]. Istotne jest zatem poszukiwanie dodatkowych, czułych biomarkerów obniżonego potencjału reprodukcyjnego mężczyzn.

3.2. Klasteryna - budowa i funkcje

Jedną z głównych glikoprotein plazmy nasienia jest klasteryna (CLU, nazywana też apolipoproteiną J, ApoJ), o masie cząsteczkowej 75-80 kDa [14], która występuje w dwóch postaciach: dominującej wydzielniczej (*ang. secretory,* sCLU) oraz jądrowej (*ang. nuclear,* nCLU). Jest ona dimerem złożonym z podjednostek α oraz β połączonych mostkami disiarczkowymi. Główną formą klasteryny występującą u mężczyzn

w surowicy oraz plazmie nasienia jest sCLU, która jest wydzielana przez jądra, najądrza oraz pęcherzyki nasienne [15].

Udowodniono, że podjednostka α klasteryny posiada trzy miejsca N-glikozylacji, a podjednostka β cztery, jednak badania *in vitro* wykazały, że CLU równocześnie może mieć przyłączone glikany maksymalnie w sześciu miejscach N-glikozylacji [14]. Głównymi resztami cukrowymi wchodzącymi w skład glikanów CLU, oprócz tych stanowiących część rdzeniową, są: fukoza, kwas sjalowy oraz struktury oligosacharydowe typu Lewis: $Lewis^a$, $Lewis^b$, $Lewis^x$ i $Lewis^y$ [16,17].

Klasteryna bierze udział m. in. w transporcie lipidów, adhezji komórkowej, hamowaniu niszczenia komórek na drodze inhibicji kompleksu atakującego błonę (MAC, *ang, membrane attack complex*), a także w tolerancji immunologicznej na męskie antygeny w żeńskich drogach rozrodczych. Poprzez pełnienie roli białka opiekuńczego oraz właściwości podobne do tych jakie mają białka szoku termicznego, CLU wspiera organizm w utrzymaniu równowagi oksydacyjno-antyoksydacyjnej i jest uznawana za czuły biomarker stresu oksydacyjnego [18,19].

3.3. Glikozylacja jako jeden z głównych procesów potranslacyjnych determinujących właściwości oraz funkcje białek

Glikozylacja to jeden z najważniejszych procesów potranslacyjnych polegający na enzymatycznym dołączaniu reszt cukrowych do łańcuchów aminokwasowych białek [20]. W zależności od typu wiązania i miejsca przyłączania glikanów do aminokwasów w łańcuchu polipeptydowym wyróżnia się:

a) **N-glikany** – tworzą wiązanie N-glikozydowe między atomem azotu grupy amidowej reszty asparaginy w sekwencji: asparagina - dowolny aminokwas oprócz proliny – seryna lub treonina a N-acetyloglukozoaminą (GlcNAc);
b) **O-glikany** – tworzą wiązanie O-glikozydowe między seryną lub treoniną a N-acetylogalaktozoaminą (GalNAc).

N-glikany oraz O-glikany mogą tworzyć różnego rodzaju, niekiedy bardzo złożone struktury oligosacharydowe. W przypadku glikozylacji CLU wykazano,

że tworzy ona N-glikany typu kompleksowego, w których skład, w części antenowej, wchodzą najczęściej galaktoza (Gal), kwas sjalowy (SA, *ang. sialic acid*) oraz fukoza [16]. W badaniach nad strukturą glikanów klasteryny plazmy nasienia wykazano także istotną rolę struktur typu Lewis oraz fukozy w pełnieniu jej funkcji w procesie zapłodnienia w żeńskich drogach rozrodczych, m. in. w interakcjach główki plemnika z osłonką przejrzystą oocytu [21–23].

3.4. Stres oksydacyjny i jego wpływ na męską płodność

Stres oksydacyjny (OS, *ang. oxidative stress*) jest definiowany jako zaburzenie równowagi między powstawaniem reaktywnych form tlenu a mechanizmami obronnymi organizmu, odpowiedzialnymi za ich neutralizację oraz eliminację [12]. Stan ten prowadzi do uszkodzeń wielu struktur komórkowych, szczególnie dwuwarstwy fosfolipidowej błon komórkowych. W konsekwencji peroksydacja lipidów wyzwala kaskadę procesów zapalnych, prowadząc do nasilenia wewnątrzkomórkowych procesów oksydacyjnych. Zaobserwowano wpływ stresu oksydacyjnego na utlenianie lipidów oraz integralność błony komórkowej plemników, co znalazło odzwierciedlenie w zaburzeniach ich ruchliwości oraz zniszczeniu struktury DNA główki, a w rezultacie nasileniu apoptozy komórek plemnikowych, co prowadzi do zmniejszenia ich ruchliwości, liczby oraz do powstawania deformacji [24]. Z uwagi na fakt, że plemniki nie posiadają własnego systemu naprawy uszkodzeń oksydacyjnych, są one szczególnie narażone na OS [25]. Plazma nasienia zawiera enzymatyczne oraz nieenzymatyczne związki o charakterze antyoksydantów, wśród których znajdują się m. in. białka, jony wapnia, żelaza czy kwas moczowy [26–28].

4. Cel pracy

Pomimo aktualizacji wytycznych WHO dotyczących badania nasienia, metody diagnostyki niepłodności męskiej stosowane obecnie, w wielu przypadkach nie dostarczają odpowiedzi na pytanie o przyczynę zaburzeń rozrodczości. Rutynowo stosowana analiza seminologiczna nie obejmuje składników plazmy nasienia, która jest mieszaniną białek, glikoprotein, enzymów, jonów oraz lipidów niezbędnych do prawidłowego dojrzewania plemników oraz przebiegu procesu zapłodnienia. Biorąc pod uwagę fakt, że niepłodność męska to rosnący problem o charakterze globalnym, a klasteryna jest jedną z głównych glikoprotein plazmy nasienia, odgrywającą istotną rolę w procesie dojrzewania plemników oraz zapłodnienia, nasuwa się pytanie, czy plazma nasienia nie mogłaby stać się cennym materiałem biologicznym, zawierającym nowe biomarkery diagnostyczne niepłodności męskiej?

Celem pracy było nie tylko poszerzenie wiedzy na temat ekspresji określonych struktur cukrowych N-glikanów klasteryny obecnej w plazmach nasienia oraz surowicach niepłodnych mężczyzn: fukozy oraz kwasu sjalowego, ale także sprawdzenie, czy istnieją zależności między obydwoma analizowanymi płynami biologicznymi w ich ekspresji. Poszukiwanie różnic w ekspresji analizowanych parametrów, zarówno tych związanych z glikozylacją CLU, jak i markerów stresu oksydacyjnego, miało na celu znalezienie odpowiedzi na pytanie, czy któryś z nich może stać się dodatkowym markerem diagnostycznym, o satysfakcjonującej czułości i specyficzności, różnicującym grupy pacjentów niepłodnych z zaburzeniami dotyczącymi liczby, ruchliwości oraz morfologii plemników. Ponadto biorąc pod uwagę istotną rolę stresu oksydacyjnego oraz fakt, że CLU jest czułym biomarkerem OS, celem moich badań było również sprawdzenie zależności między stężeniem CLU, ekspresją jej glikanów a wybranymi wskaźnikami stresu oksydacyjnego, jak również parametrami biochemicznymi związanymi z zaburzeniami równowagi oksydacyjno-antyoksydacyjnej.

4.1. **Artykuł przeglądowy** – Czy analiza glikozylacji klasteryny obecnej w plazmie nasienia może stać się nowym biomarkerem niepłodności męskiej?

Celem dokonanej kwerendy bibliograficznej, której rezultatem było powstanie artykułu przeglądowego, było zebranie dostępnych, jak najbardziej aktualnych, informacji dotyczących budowy, ekspresji i glikozylacji klasteryny, analizowanych w kontekście męskiej płodności oraz jej zaburzeń. Szczególny nacisk położono na zebranie wiedzy na temat glikozylacji CLU, jak również jej udziału w regulacji zaburzeń równowagi oksydacyjno-antyoksydacyjnej. Klasteryna jest glikoproteiną biorącą udział w reakcji kapacytacji plemników, a także regulującą tolerancję immunologiczną na męskie antygeny w żeńskich drogach rozrodczych. Glikany CLU najczęściej wykazują ekspresję fukozy, kwasu sjalowego, a także struktur oligosacharydowych typu Lewis^{x} oraz Lewis^{y} [29]. Zebrane dane z piśmiennictwa dotyczące budowy glikanów CLU oraz potencjalnej roli związków cukrowych w interakcjach między oocytem a plemnikiem podczas zapłodnienia, pozwoliły na określenie kierunku badań dotyczących profilu i stopnia glikozylacji klasteryny.

4.2. **I artykuł oryginalny** – Potencjalny związek między zmianami fukozylacji klasteryny a zaburzeniami męskiej płodności

Celem badań przedstawionych i omówionych w artykule było określenie stopnia oraz profilu fukozylacji klasteryny obecnej w surowicach oraz plazmach nasienia niepłodnych pacjentów, z wykorzystaniem biotynylowanych lektyn specyficznych wobec fukozy oraz oznaczenie stężeń fukozylotransferazy 3 i 4 (odpowiednio: FUT3, *ang. fucosyltransferase-3* oraz FUT4, *ang. fucosyltransferase-4*). Dodatkowo sprawdzono, czy występowały korelacje między obydwoma płynami biologicznymi w profilu i stopniu fukozylacji CLU oraz stężeniach FUT3 i FUT4, a także czy występowały różnice w oznaczonych wartościach między badanymi grupami niepłodnych pacjentów [30]. Korelowano także otrzymane wyniki oznaczeń z wybranymi parametrami analizy seminologicznej. Weryfikowano również wartość diagnostyczną

oznaczanych parametrów w celu zaproponowania panelu biomarkerów różnicujących niepłodnych mężczyzn z odmiennymi zaburzeniami dotyczącymi plemników.

4.3. **II artykuł oryginalny** – Zależności między stopniem sjalilacji klasteryny a poziomami markerów równowagi oksydacyjno-antyoksydacyjnej w plazmach nasienia oraz surowicach niepłodnych mężczyzn

Głównym celem prowadzonych badań było określenie profilu oraz stopnia sjalilacji klasteryny obecnej w plazmach nasienia oraz surowicach niepłodnych mężczyzn, z wykorzystaniem biotynylowanych lektyn specyficznych wobec kwasu sjalowego, analizowanych w kontekście ekspresji wybranych parametrów stresu oksydacyjnego, oznaczanych w obu płynach biologicznych. Oznaczono stężenia sirtuin 3 oraz 5 (odpowiednio: SIRT3, *ang. sirtuin 3* oraz SIRT5,*ang. sirtuin 5*), oceniono poziom całkowitej zdolności antyoksydacyjnej sprawdzając całkowity status antyoksydacyjny (TAS, *ang. total antioxidant status*) oraz zdolność do redukcji jonów żelaza (FRAP, *ang. ferric reducing antioxidant power*). Ponadto sprawdzono, czy występowały korelacje między obydwoma płynami biologicznymi w profilu i stopniu sjalilacji CLU oraz stężeniach sirtuin i wybranych parametrów równowagi oksydacyjno-antyoksydacyjnej. Zweryfikowano również wartość diagnostyczną analizowanych parametrów oraz ich potencjalną przydatność do różnicowania grup niepłodnych mężczyzn o różnych zaburzeniach dotyczących plemników.

4.4. **III artykuł oryginalny** – Czy istnieją zależności między zaawansowanymi produktami utleniania białek a wybranymi parametrami biochemicznymi związanymi ze statusem oksydacyjno-redukcyjnym w plazmach nasienia niepłodnych mężczyzn?

Celem zaplanowanych, przeprowadzonych i opisanych w artykule badań było sprawdzenie, czy w plazmie nasienia istnieją zależności między stężeniami zaawansowanych produktów utleniania białek (AOPP, *ang. advanced oxidation protein*

products) a stężeniami wybranych parametrów biochemicznych: białka całkowitego, żelaza, kwasu moczowego, magnezu i wapnia oraz sprawdzenie, czy istnieją powiązania między wartościami oznaczonych parametrów z typami zaburzeń dotyczących plemników u niepłodnych mężczyzn będących uczestnikami badania.

5. Materiały i metody zastosowane w badaniach

5.1. Materiał badany

Materiałem badanym były dwa płyny biologiczne: plazmy nasienia (*n*=132) oraz surowice (*n*=91) pochodzące od pacjentów zgłaszających się do Centrum Ginekologii, Położnictwa i Neonatologii w Opolu (pozytywna opinia Komisji Bioetycznej KB-549/2019 oraz KB-591/2021) oraz Kliniki Leczenia Niepłodności InviMed w Warszawie (pozytywna opinia Komisji Bioetycznej KB-765/2018, KB-103/2019 oraz KB-117/2020). Uczestnicy badania to niepłodni mężczyźni w wieku od 29 do 44 lat. Kryteriami wykluczenia z badań było występowanie jednego lub więcej następujących zdarzeń: obecność ogólnoustrojowego stanu zapalnego manifestująca się podwyższonym stężeniem surowiczego białka C-reaktywnego (CRP, *ang. C-reactive protein*), obecność podwyższonej liczby leukocytów w nasieniu (leukospermia), a także współwystępowanie żeńskiej przyczyny niepłodności. W oparciu o wyniki badania ogólnego nasienia, wykonanego zgodnie z obowiązującymi standardami WHO z 2010 roku, pacjentów podzielono na cztery grupy: teratozoospermiczną (T; <4% plemników z prawidłową morfologią), astenoteratozoospermiczną (AT; <32% plemników wykazujących prawidłowy ruch postępowy oraz <4% plemników z prawidłową morfologią), oligoastenoteratozoospermiczną (OAT; koncentracja plemników poniżej 15×10^6/ml, <32% plemników wykazujących prawidłowy ruch postępowy oraz <4% plemników z prawidłową morfologią), oraz normozoospermiczną (N; koncentracja plemników $\geq15\times10^6$/ml, ≥4% plemników z prawidłową morfologią, całkowity ruch plemników ≥40% lub ruch postępowy plemników ≥32% 30 minut po ejakulacji). Dokładna charakterystyka grup badanych została przedstawiona w pracach oryginalnych, stanowiących podstawę niniejszej rozprawy doktorskiej [30–32].

Nasienie było oddawane na drodze masturbacji po 3-5 dniach abstynencji seksualnej, do sterylnych pojemników. Po upłynnieniu nasienia (maksymalnie 60 minut w 37°C), wykonywano standardową analizę seminologiczną, zgodnie z obowiązującymi na czas rozpoczęcia badań rekomendacjami WHO z 2010 roku. Z wykorzystaniem komputerowo wspomaganej analizy nasienia określono całkowitą liczbę plemników w ejakulacie, koncentrację plemników, odsetek ruchliwych plemników, odsetek

plemników poruszających się ruchem postępowym oraz odsetek plemników o prawidłowiej morfologii (SCA Motility and Concentration, Microptic SL, Barcelona, Hiszpania; wersja oprogramowania 6.5.0.5). Objętość ejakulatu, pH oraz żywotność plemników określono za pomocą metod manualnych. Po wykonanej analizie seminologicznej ejakulaty wirowano 3500×g przez 10 minut w temperaturze pokojowej, uzyskując plazmę nasienia, która następnie została poporcjowana i zamrożona w temperaturze -86°C do dnia rozpoczęcia badań.

Próbki krwi pobierano od uczestników badania na czczo, do probówek z aktywatorem krzepnięcia. Po zakończeniu procesu wykrzepiania krwi, próbki wirowano w temperaturze pokojowej przy 2000×g przez 10 minut, w celu uzyskania surowicy, którą następnie podzielono na mniejsze porcje i zamrożono w temperaturze -86°C do dnia wykonania oznaczeń.

Projekt badawczy będący tematem niniejszej rozprawy doktorskiej został pozytywnie zaopiniowany przez Komisję Bioetyczną działającą przy Uniwersytecie Medycznym im. Piastów Śląskich we Wrocławiu (nr KB-707/2019).

5.2. Metodyka badań

Opisane poniżej metody oznaczeń zastosowane w trakcie badań eksperymentalnych, zostały szczegółowo opisane w artykułach oryginalnych wchodzących w skład cyklu publikacji stanowiących podstawę niniejszej rozprawy doktorskiej. Analiza profilu glikozylacji (fukozylacji oraz sjalilacji) klasteryny obecnej w plazmach nasienia oraz surowicach pacjentów została przeprowadzona z zastosowaniem testów lektyno-ELISA (*ang. lectin based enzyme-linked immunosorbent assay*). Testy te umożliwiają półilościową ocenę ekspresji związków cukrowych, m. in. fukozy oraz kwasu sjalowego, na glikanach glikoprotein dzięki wykorzystaniu biotynylowanych lektyn roślinnych specyficznie wiążących się z w/w cukrami [33]. Szczegółowa procedura dotycząca analizy profilu i stopnia fukozylacji klasteryny plazmy nasienia oraz surowiczej u niepłodnych pacjentów została przedstawiona w pierwszej publikacji oryginalnej [30]. Szczegóły metodologiczne dotyczące wariantu testu lektyno-ELISA, zastosowanego do oceny profilu i stopnia sjalilacji klasteryny w obydwu płynach biologicznych zostały opisane w drugim artykule,

przedstawiającym wyniki przeprowadzonych badań [31]. Trzecia publikacja oryginalna zawiera opis wykonania manualnego oznaczenia stężeń zaawansowanych produktów utleniania białek, które jest modyfikacją metody opisywanej w piśmiennictwie [34], umożliwiającą zmniejszenie objętości użytego do oznaczeń materiału badanego [31]. Wszystkie oznaczenia z wykorzystaniem testów komercyjnych zostały wykonane zgodnie z protokołami dołączonymi przez producentów, z zastosowaniem zasad dobrej praktyki laboratoryjnej, a ponadto do każdej serii oznaczeń dołączano materiał kontrolny o znanej wartości stężenia lub absorbancji danego parametru. W przypadku metod manualnych, w tym testów lektyno-ELISA, każde oznaczenie dla danego pacjenta, jak również krzywą kalibracyjną w metodach ilościowych, wykonywano w dublecie w celu obniżenia ewentualnej nieprecyzji metody.

5.2.1. I artykuł oryginalny – Potencjalny związek zmian fukozylacji klasteryny z zaburzeniami męskiej płodności

Stężenie klasteryny w plazmach nasienia oznaczono za pomocą komercyjnego immunoenzymatycznego testu fazy stałej: Human Clusterin ELISA Kit (Bioassay Technology Laboratory, Szanghaj, Chiny). W badaniach wstępnych wykazano, że stężenie CLU w surowicach jest około tysiąckrotnie wyższe niż w plazmach nasienia (µg/ml vs. ng/ml), dlatego stężenie klasteryny w surowicach oznaczono z wykorzystaniem komercyjnego testu ELISA o szerszym zakresie liniowości: Human Clusterin ELISA Kit, Invitrogen (ThermoFischer Scientific, Frederick, USA). Stężenia fukozylotransferaz (FUT, *ang. fucosyltransferase*) 3 oraz 4 (odpowiednio: FUT3 oraz FUT4) oznaczono za pomocą komercyjnie dostępnych testów: Human Fucosyltransferase 3 ELISA Kit oraz Human Fucosyltransferase 4 ELISA Kit (Bioassay Technology Laboratory, Szanghaj, Chiny). Wszystkie odczyty stężeń analizowanych parametrów wykonano za pomocą czytnika mikropłytek Mindray-96A (Shenzhen Mindray Bio-Medical Electronics, Chiny).

Do oceny profilu i stopnia fukozylacji CLU wykorzystano zmodyfikowany test lektyno-ELISA z zastosowaniem biotynylowanych lektyn specyficznych wobec fukozy:

a) *Lotus tetragonolobus* agglutinin (LTA, Vector Labortories Inc., Burlingname, CA, USA) - lektyna wykrywająca fukozę przyłączoną wiązaniem α1,3 do N-acetyloglukozoaminy antenowej, charakterystyczną dla struktur oligosacharydowych typu Lewis^{x}, sjalo-Lewis^{x} oraz Lewis^{y}, aczkolwiek obecność końcowego kwasu sjalowego przyłączonego wiązaniem α2,3 do antenowej galaktozy ogranicza możliwość wiązania się LTA z fukozą przyłączoną do antenowej GlcNAc;
b) *Ulex europaeus* agglutinin (UEA, Vector Labortories Inc., Burlingname, CA, USA) - lektyna specyficznie reagująca z fukozą przyłączoną wiązaniem α1,2 do antenowej galaktozy, wchodzącej w skład dwufukozylowanych struktur oligosacharydowych typu Lewis^{y}. Wykazano, że obecność fukozy przyłączonej wiązaniem α1,2 do galaktozy obniża szanse na przyłączenie do glikanu końcowego kwasu sjalowego;
c) *Lens culinaris* agglutinin (LCA, Vector Labortories Inc., Burlingname, CA, USA) - lektyna ta rozpoznaje sekwencje zawierające α-połączone reszty mannozy, a obecność fukozy przyłączonej wiązaniem α1,6 do GlcNAc rdzenia oligosacharydowego znacznie zwiększa jej powinowactwo. Dlatego też lektyna ta jest wykorzystywana do wykrywania obecności fukozy rdzeniowej N-glikanów [35–37].

Po opłaszczeniu płytki ELISA (Nunc MaxiSorp, Thermo Fisher Scientific, Dania) kozimi przeciwciałami poliklonalnymi reagującymi specyficznie z ludzką klasteryną (Invitrogen, Thermo Fisher Scientific, Rockford, USA) nanoszono próby badane w odpowiednim rozcieńczeniu, dobranym w serii eksperymentów wstępnych tak, aby w 100 µl nanoszonego do dołka płytki roztworu próby badanej był 1 ng CLU w przypadku plazm nasienia lub 50 ng CLU dla surowic. W kolejnym etapie próbki inkubowano z biotynylowanymi lektynami, natomiast uwidocznienie reakcji następowało w oparciu o reakcję lektyn z ekstrawidyną znakowaną fosfatazą alkaliczną (Sigma-Aldrich, Saint Louis, USA), która następnie reagowała z p-NPP tworząc barwny produkt (para-nitrofenylofosforan disodowy) [30]. Wynik, odczytywany jako wartość absorbancji przy długości fali 405 nm i filtrze referencyjnym 630 nm, odzwierciedla względną reaktywność fukozy wchodzącej w skład glikanów klasteryny z odpowiednią, specyficzną lektyną. Odczyty stężeń lub absorbancji analizowanych parametrów

wykonano za pomocą czytnika mikropłytek Mindray-96A (Shenzhen Mindray Bio-Medical Electronics, Chiny).

5.2.2. II artykuł oryginalny – Zależności między stopniem sjalilacji klasteryny a poziomami markerów równowagi oksydacyjno-antyoksydacyjnej w plazmach nasienia oraz surowicach niepłodnych mężczyzn

Do oceny profilu sjalilacji CLU wykorzystano zmodyfikowany test lektyno-ELISA z zastosowaniem lektyn specyficznych wobec kwasu sjalowego:

a) *Sambucus nigra* agglutinin (SNA, Vector Labortories Inc., Burlingname, CA, USA) - lektyna wykrywająca kwas sjalowy przyłączony wiązaniem α2,6 do galaktozy antenowej części glikanu;
b) *Maackia amurensis* agglutinin (MAA), Vector Labortories Inc., Burlingname, CA, USA) - lektyna wykrywająca kwas sjalowy przyłączony wiązaniem α2,3 do galaktozy antenowej części glikanu [35].

Po opłaszczeniu płytki ELISA (Nunc MaxiSorp, Thermo Fisher Scientific, Dania) kozimi przeciwciałami poliklonalnymi anty-ludzka klasteryna (Invitrogen, Thermo Fisher Scientific, Rockford, USA) nanoszono próby badane w odpowiednim rozcieńczeniu, dobranym w serii eksperymentów wstępnych tak, aby w 100 µl roztworu próby badanej nanoszonego do dołka płytki był 1 ng CLU w przypadku plazm nasienia lub 50 ng CLU dla surowic. W kolejnym etapie próbki inkubowano z biotynylowanymi sjalo-specyficznymi lektynami, natomiast uwidocznienie reakcji następowało w oparciu o reakcję lektyn z ekstrawidyną znakowaną fosfatazą alkaliczną (Sigma-Aldrich, Saint Louis, USA), która następnie reagowała z p-NPP tworząc barwny produkt [30]. Wynik, odczytywany jako wartość absorbancji przy długości fali 405 nm i filtrze referencyjnym 630 nm, odzwierciedla względną reaktywność kwasu sjalowego wchodzącego w skład glikanów klasteryny z odpowiednią, specyficzną lektyną. Odczyty absorbancji analizowanych parametrów wykonano za pomocą czytnika mikropłytek Mindray-96A (Shenzhen Mindray Bio-Medical Electronics, Chiny).

Stężenia SIRT3 oraz SIRT5 oznaczono za pomocą komercyjnych immunoenzymatycznych testów fazy stałej, odpowiednio: Human Sirtuin 3 ELISA Kit oraz Human Sirtuin 5 ELISA Kit (Bioassay Technology Laboratory, Szanghaj, Chiny), zgodnie z protokołem producenta. Odczyty stężeń sirtuin wykonano przy użyciu czytnika mikropłytek Mindray-96A (Shenzhen Mindray Bio-Medical Electronics, Chiny).

Do oceny całkowitej zdolności antyoksydacyjnej wybrano całkowity status antyoksydacyjny (TAS, *ang. total antioxidant status*) oraz zdolność do redukcji jonów żelaza (FRAP, *ang. ferric reducing antioxidant power*). Oznaczenia stężeń TAS wykonano z użyciem komercyjnie dostępnych odczynników (Randox TAS Kit, Crumlin, Zjednoczone Królestwo) na analizatorze biochemicznym Konelab20i® (Thermo Scientific, Vantaa, Finlandia).

Stężenia FRAP oceniono za pomocą metody kolorymetrycznej, na podstawie protokołu opisanego przez Benzie i Strain [38] z wykorzystaniem 2,4,6-tripirydylo-S-triazyny (TPTZ). Pomiar absorbancji produktu końcowego reakcji został wykonany przy długości fali 593 nm, za pomocą spektrofotometru UV/VIS (UV-6300PC, VWR, Szanghaj, Chiny).

5.2.3. III artykuł oryginalny – Czy istnieją zależności między zaawansowanymi produktami utleniania białek a wybranymi parametrami biochemicznymi związanymi ze statusem oksydacyjno-redukcyjnym w plazmach nasienia niepłodnych mężczyzn?

Oznaczenie stężeń zaawansowanych produktów utleniania białek przeprowadzono w oparciu o metodę opisaną przez Witko-Sarsat i wsp [34] z modyfikacjami umożliwiającymi istotne zmniejszenie objętości materiału badanego. Szczegółowy opis procedury oznaczeń został przedstawiony w pracy oryginalnej [32]. Nowatorskim rozwiązaniem było tworzenie mieszaniny reakcyjnej na płytce ELISA (Nunc MaxiSorp, Thermo Fisher Scientific, Dania), co oprócz oszczędności objętości cennego materiału badanego jakim jest plazma nasienia umożliwiło przyspieszenie wykonania analizy. Próbki badane były rozcieńczane solą fizjologiczną buforowaną

fosforanem (PBS, *ang. phosphate-buffered saline*) i nanoszone na płytkę ELISA. Po dodaniu w odpowiednich odstępach czasowych roztworów jodku potasu oraz lodowatego kwasu octowego, niezwłocznie dokonywano pomiaru absorbancji przy długości fali 340 nm, z wykorzystaniem czytnika Multiskan Go ELISA (Thermo Fischer Scientific, Roskilde, Dania). Każde oznaczenie było wykonane w dublecie w celu zmniejszenia nieprecyzji metody. Do odczytu stężeń AOPP wykonano krzywą kalibracyjną w oparciu o roztwór o znanym stężeniu chloraminy T.

Stężenia białka całkowitego, żelaza, kwasu moczowego, wapnia oraz magnezu oznaczono z wykorzystaniem komercyjnie dostępnych odczynników (Thermo Scientific, Vantaa, Finlandia) na analizatorze biochemicznym Konelab20i® (Thermo Scientific, Vantaa, Finlandia).

5.3. Analiza statystyczna

Szczegóły dotyczące wykonanych analiz statystycznych opisano w każdym z artykułów oryginalnych. Analizę statystyczną przeprowadzono z zastosowaniem oprogramowania Statistica PL (wersja 13.3, StatSoft Polska Sp. z o. o.).

5.3.1. I artykuł oryginalny – Potencjalny związek zmian fukozylacji klasteryny z zaburzeniami męskiej płodności

Do oceny normalności rozkładu wyników uzyskanych dla poszczególnych parametrów wybrano test Shapiro-Wilka. Uzyskane wyniki przedstawiono jako wartości średnie wraz z odchyleniami standardowymi (SD, *ang. standard deviation*) oraz w formie graficznej w postaci median z przedziałami międzykwartylowymi (Q1-Q3). Do porównania otrzymanych wyników w parach między grupami niepłodnych mężczyzn (T, AT, OAT oraz N) wykorzystano test U Manna-Whitney'a. Analiza korelacji rang Spearmana pozwoliła na ustalenie, czy istnieją zależności między wartościami uzyskanych wyników oznaczeń. Do ustalenia wartości klinicznej badanych zmiennych wykorzystano analizę krzywych ROC (*ang. receiver operating characteristic*). Ponadto przeprowadzona analiza skupień przedstawiona w postaci dendrogramu pozwoliła na ustalenie, czy istnieje możliwość wyodrębnienia optymalnego zestawu parametrów

spośród wszystkich analizowanych, który mógłby stanowić dodatkowy panel diagnostyczny męskiej niepłodności. Za istotne uznano wartości, dla których $p<0,05$.

5.3.2. II artykuł oryginalny – Zależności między stopniem sjalilacji klasteryny a poziomami markerów równowagi oksydacyjno-antyoksydacyjnej w plazmach nasienia oraz surowicach niepłodnych mężczyzn

Test Shapiro-Wilka został zastosowany do oceny normalności rozkładu analizowanych zmiennych. Uzyskane wyniki przedstawiono jako wartości średnie wraz z odchyleniami standardowymi, a także w formie graficznej jako mediany z przedziałami międzykwartylowymi (Q1-Q3). Porównanie otrzymanych wyników w parach między grupami badanymi niepłodnych pacjentów (T, AT, OAT oraz N) wykonano z zastosowaniem testu U Manna-Whitney'a. Analiza korelacji rang Spearmana pozwoliła na sprawdzenie, czy istnieją zależności między stopniem sjalilacji klasteryny a stężeniami wybranych parametrów równowagi oksydacyjno-antyoksydacyjnej. Wartość kliniczną badanych zmiennych sprawdzono za pomocą analizy krzywych ROC oraz wielomianowej regresji logistycznej, w której grupa normozoospermiczna została zdefiniowana jako referencyjna. Dodatkowo, przeprowadzona analiza skupień przedstawiona w postaci dendrogramu umożliwiła sprawdzenie, czy spośród wszystkich analizowanych parametrów można wyodrębnić panel markerów, który mógłby wzbogacić diagnostykę męskiej niepłodności. Za istotne uznano wartości, dla których $p<0,05$.

5.3.3. III artykuł oryginalny – Czy istnieją zależności między zaawansowanymi produktami utleniania białek a wybranymi parametrami biochemicznymi związanymi ze statusem oksydacyjno-redukcyjnym w plazmach nasienia niepłodnych mężczyzn?

Do oceny normalności rozkładu wyników uzyskanych dla poszczególnych parametrów wybrano test Shapiro-Wilka. Uzyskane wyniki przedstawiono jako wartości

średnie wraz z odchyleniami standardowymi, a także w formie graficznej jako mediany z przedziałami Q1-Q3. Test U Manna-Whitney'a zastosowano do porównania otrzymanych wyników w parach między grupami badanymi (T, AT, OAT oraz N). Przeprowadzona analiza korelacji rang Spearmana dała odpowiedź na pytanie, czy istnieją zależności między stężeniami zaawansowanych produktów utleniania białek (AOPP, *ang. advanced oxidation protein products*) a stężeniami wybranych parametrów biochemicznych, związanych ze statusem oksydacyjno-redukcyjnym plazmy nasienia. Do ustalenia wartości klinicznej badanych zmiennych wykorzystano analizę krzywych ROC. Za istotne uznano wartości, dla których $p<0,05$.

6. Najważniejsze wyniki badań

6.1. I artykuł oryginalny – Potencjalny związek zmian fukozylacji klasteryny z zaburzeniami męskiej płodności

Stężenia klasteryny w plazmach nasienia były istotnie wyższe w grupie oligoastenoteratozoospermicznej (mediana: 66,01 ng/ml) w porównaniu do grup: normozoospermicznej, astenoteratozoospermicznej i teratozoospermicznej (wartości median odpowiednio: 18,98 ng/ml, p=0,000114; 19,02 ng/ml, p=0,000001 oraz 19,13 ng/ml, p=0,000003). Natomiast w surowicach stężenia klasteryny były istotnie niższe w grupie normozoospermicznej (mediana: 16.54 μg/ml) w porównaniu z grupami AT, OAT oraz T (wartości median odpowiednio: 36,16 μg/ml, p=0,001718; 37,90 μg/ml, p=0,000318 oraz 36,82 μg/ml, p=0,000183).

Stężenia w plazmach nasienia enzymu FUT3, którego działanie jest związane z powstawaniem struktur oligosacharydowych typu Lewis^x oraz Lewis^y, były istotnie wyższe w grupie T (mediana: 2,685 ng/ml) w porównaniu do grup N oraz AT (wartości median odpowiednio: 2,470 ng/ml, p=0,002763 oraz 2,462 ng/ml, p=0,030302). Ponadto stężenia FUT3 były istotnie wyższe w grupie OAT (mediana: 2,690 ng/ml) w porównaniu z grupami AT oraz N, z istotnością odpowiednio: p=0,038031 oraz p=0,011090.

Stężenia FUT4 w plazmach nasienia, enzymu mającego związek z ekspresją m. in. struktur oligosacharydowych typu Lewis^x oraz sialo-Lewis^x glikanów glikoprotein, były istotnie wyższe w grupie normozoospermicznej (mediana: 0,843 ng/ml) w porównaniu z grupą teratozoospermiczną (mediana: 0,777 ng/ml, p=0,032026). Ponadto stężenia FUT4 w grupie astenoteratozoospermicznej (mediana: 0,843 ng/ml) były istotnie wyższe w porównaniu do grupy teratozoospermicznej (p=0,018689). Nie wykazano istotnych różnic w stężeniach surowiczej FUT3 oraz FUT4 między grupami badanych pacjentów.

Względna reaktywność glikanów klasteryny obecnej w surowicy z lektyną UEA wykrywającą fukozę przyłączoną wiązaniem α1,2 do Gal części antenowej glikanu, była istotnie wyższa w grupie pacjentów normozoospermicznych (mediana: 0,05 AU) w porównaniu z pozostałymi grupami badanymi: AT, OAT oraz T (wartości median

odpowiednio: 0,023 AU, p=0,000253; 0,020 AU, p=0,00001 oraz 0,022 AU, p=0,000006). Podobną zależność zaobserwowano w przypadku względnej reaktywności glikanów surowiczej klasteryny z lektyną LCA – pacjenci normozoospermiczni wykazywali istotnie wyższą ekspresję fukozy rdzeniowej (mediana: 0,162 AU) w porównaniu do pozostałych grup badanych AT, OAT oraz T (wartości median odpowiednio: 0,055 AU, 0,054 AU oraz 0,077 AU, z istotnościami odpowiednio: p=0,000113, p=0,000029 oraz p=0,000225). Ponadto względna reaktywność glikanów surowiczej klasteryny z LCA była istotnie wyższa w grupie T w porównaniu do grup OAT oraz AT z istotnością odpowiednio p=0,03462 oraz p=0,00884.

Analiza korelacji między parametrami będącymi przedmiotem tej części badań a wybranymi parametrami analizy seminologicznej wykazała istnienie ujemnych korelacji między stężeniami klasteryny plazmy nasienia a całkowitą liczbą plemników (r=-0,409, p=0,001) oraz odsetkiem plemników poruszających się ruchem postępowym (r=-0,317, p=0,009). Stężenie FUT3 obecnej w plazmie nasienia ujemnie korelowało z odsetkiem plemników o prawidłowej morfologii (r=-0,300; p=0,014). Wykazano również istnienie korelacji między względną reaktywnością glikanów klasteryny plazmy nasienia z lektyną UEA a odsetkiem plemników o prawidłowej morfologii (r=0,270; p=0,027).

Analiza krzywych ROC umożliwiła ocenę użyteczności klinicznej analizowanych parametrów jako dodatkowych biomarkerów różnicujących niepłodnych mężczyzn z określonymi zaburzeniami dotyczącymi plemników. Analiza ta wykazała, że stężenie klasteryny, FUT3 oraz FUT4 w plazmie nasienia może być pomocne w różnicowaniu niepłodnych grup pacjentów, których wyniki analizy nasienia mogą zarówno odbiegać jak i znajdować w zakresie wartości uznanych za prawidłowe. Wartości stężeń klasteryny w plazmach nasienia umożliwiły różnicowanie grupy oligoastenoteratozoospermicznej od grupy normozoospermicznej (czułość i swoistość odpowiednio: 100% oraz 65%; AUC=0,774 (pole powierzchni pod krzywą ROC) – umiarkowana wartość kliniczna) teratozoospermicznej (czułość i swoistość odpowiednio: 56,8% oraz 91,3%; AUC=0,834 – umiarkowana wartość kliniczna) oraz astenoteratozoospermicznej (czułość i swoistość odpowiednio: 96,6% oraz 81,8%; AUC=0,903 – wysoka wartość kliniczna). Wartości stężeń fukozylotransferazy 3 w plazmach nasienia umożliwiły różnicowanie grupy normozoospermicznej od grupy oligoasternozoospermicznej (czułość i swoistość

odpowiednio: 56,8% oraz 91,3%; AUC=0,706 – umiarkowana wartość kliniczna) oraz teratozoospermicznej (czułość i swoistość odpowiednio: 61,8% i 91,3%; AUC=0,736 – umiarkowana wartość kliniczna). Wartości stężeń fukozylotransferazy 4 plazmy nasienia miały umiarkowaną wartość kliniczną i różniły się istotnie miedzy grupą teratozoospermiczną i astenoteratozoospermiczną (czułość i swoistość odpowiednio: 56,3% i 84,4%; AUC=0.710).

Otrzymane wartości stężeń klasteryny w surowicach pozwoliły na różnicowanie grupy normozoospermicznej od pozostałych grup pacjentów: astenoteratozoospermicznej (czułość i swoistość odpowiednio: 100% oraz 75%; AUC=0,821 – umiarkowana wartość kliniczna), oligoastenoteratozoospermicznej (czułość i swoistość odpowiednio: 96,3% oraz 75%; AUC=0,819 – umiarkowana wartość kliniczna), oraz teratozoospermicznej (czułość i swoistość odpowiednio: 100% oraz 75%; AUC=0,823 – umiarkowana wartość kliniczna). Stężenia surowiczej FUT4 umożliwiły różnicowanie grupy normozoospermicznej od astenoteratozoospermicznej (czułość i swoistość odpowiednio: 73,3% oraz 64,3%; AUC=0,710 – umiarkowana wartość kliniczna). Względna reaktywność glikanów klasteryny obecnej w surowicy z lektyną UEA umożliwiła różnicowanie grupy normozoospermicznej od astenoteratozoospermicznej (czułość i swoistość odpowiednio: 86,7% oraz 75%; AUC=0,869 – umiarkowana wartość kliniczna), oligoastenoteratozoospermicznej (czułość i swoistość odpowiednio: 92,6% oraz 81,3%; AUC=0,878 – umiarkowana wartość kliniczna) oraz teratozoospermicznej (czułość i swoistość odpowiednio: 87,1% oraz 81,3%; AUC=0,879 – umiarkowana wartość kliniczna). Względna reaktywność glikanów klasteryny obecnej w surowicy z lektyną LCA reagującą specyficznie z fukozą rdzeniową różniła się istotnie między grupą normozoospermiczną a grupami: astenoteratozoospemiczną (czułość i swoistość odpowiednio: 93,3% oraz 87,5%; AUC=0,881 – umiarkowana wartość kliniczna), oligoastenoteratozoospermiczną (czułość i swoistość odpowiednio: 88,9% oraz 75%; AUC=0,861 – umiarkowana wartość kliniczna) oraz teratozoospermiczną (czułość i swoistość odpowiednio: 90,3% oraz 75%; AUC=0,818 – umiarkowana wartość kliniczna). Ponadto otrzymane wartości względnej reaktywności glikanów surowiczej CLU z LCA umożliwiły także różnicowanie grupy teratozoospermicznej od astenoteratozoospermicznej (czułość i swoistość odpowiednio: 93,3% oraz 54,8%; AUC=0,737 – umiarkowana wartość kliniczna).

Do analizy skupień wybrano parametry, które istotnie różnicowały badane grupy pacjentów oraz w analizie krzywych ROC ich wartości AUC były wyższe od 0,700. Spośród parametrów plazmy nasienia, dla których AUC≥0,706, wybrano stężenia CLU, FUT3 i FUT4, natomiast dla parametrów surowiczych kryterium włączenia do analizy były wartości AUC≥0,710, a wybrane parametry to stężenia CLU i FUT4 oraz względne reaktywności glikanów klasteryny z UEA oraz LCA. Otrzymane wyniki przeprowadzonej analizy skupień pozwoliły na zaproponowanie dwóch powyższych paneli biomarkerów, odpowiednio dla plazmy nasienia oraz dla surowicy, które mogą mieć potencjalne zastosowanie w diagnostyce niepłodności.

6.2. **II artykuł oryginalny** – Zależności między stopniem sjalilacji klasteryny a poziomami markerów równowagi oksydacyjno-antyoksydacyjnej w plazmach nasienia oraz surowicach niepłodnych mężczyzn

Względna reaktywność glikanów klasteryny plazmy nasienia ze sjalospecyficzną lektyną MAA, wykrywającą końcowy kwas sjalowy przyłączony do Gal wiązaniem α2,3, była istotnie niższa w grupie OAT (mediana: 0,171 AU) w porównaniu do pozostałych grup: N, T, AT (wartości median odpowiednio: 0,376 AU, p=0,00512; 0,74 AU, p<0,000001 oraz 0,933 AU, p=0,000003). Względna reaktywność glikanów surowiczej klasteryny z lektyną SNA, specyficzną wobec kwasu sjalowego przyłączonego do Gal anteny cukrowej za pomocą wiązania α2,6, była istotnie wyższa w grupie N (mediana: 0,881 AU) w porównaniu do pozostałych grup: T, AT, OAT (wartości median odpowiednio: 0,428 AU, 0,412 AU oraz 0,495 AU) z istotnością odpowiednio: p=0,000009, p=0,000073 oraz p=0,000123. Wartości współczynnika sjalilacji (MAA/SNA) w plazmach nasienia były istotnie niższe w grupie OAT (mediana: 0,756) w porównaniu do grupy T (mediana: 3,781; p=0,000005) oraz AT (mediana: 4,455; p=0,000054).

Stężenia SIRT3 w plazmach nasienia były istotnie wyższe w grupie normozoospermicznej (mediana: 9,35 ng/ml; p<0,000001) w porównaniu do grup T oraz OAT (wartości median odpowiednio: 2,64 ng/ml oraz 2,11 ng/ml). Także stężenia SIRT3 były istotnie wyższe w grupie AT (mediana: 10,9 ng/ml) w porównaniu w grupami

T (p=0,000001) oraz OAT (p<0,000001). Stężenia SIRT3 w surowicach były natomiast istotnie niższe w grupie normozoospermicznej (mediana: 2,73 ng/ml) w porównaniu do grup AT oraz OAT (wartości median odpowiednio: 8,94 ng/ml; p=0,000859 oraz 6,27 ng/ml; p=0,002728).

Wartości stężeń SIRT5 obecnej w plazmach nasienia były istotnie niższe w grupie OAT (mediana: 1,34 ng/ml) w porównaniu do pozostałych grup badanych: N, T, AT (wartości median odpowiednio: 7,28 ng/ml, 6,89 ng/ml oraz 5,72 ng/ml, z istotnością odpowiednio: p=0,000019, p=0,000423 oraz p=0,000001). Nie wykazano istotnych różnic w stężeniach SIRT5 w surowicach między badanymi grupami niepłodnych mężczyzn.

Badania dotyczące całkowitej zdolności antyoksydacyjnej wykazały istnienie istotnych różnic między analizowanymi grupami niepłodnych mężczyzn jedynie w stężeniach FRAP - stężenia tego parametru w surowicach pacjentów normozoospermicznych (mediana: 1,49 mmol/l) były istotnie wyższe w porównaniu do grupy astenoteratozoospermicznej (mediana: 1,2 mmol/l; p=0,005187).

Analiza korelacji między analizowanymi parametrami wykazała istnienie ujemnej korelacji między współczynnikiem sjalilacji a względną reaktywnością glikanów CLU plazmy nasienia z SNA (r=−0,5556; p<0.001). Współczynnik sjalilacji korelował natomiast dodatnio ze względnymi reaktywnościami glikanów CLU z MAA, zarówno w plazmach nasienia (r= 0,8698, p<0,001) jak i surowicach (r= 0,9529; p<0,001).

Analiza krzywych ROC dla badanych przeze mnie parametrów plazmy nasienia wykazała, że względna reaktywność glikanów CLU z MAA, współczynnik sjalilacji, stężenia SIRT3 oraz SIRT5 mogą stanowić dodatkowe markery różnicujące pacjentów z nieprawidłowościami dotyczącymi plemników od niepłodnych mężczyzn normozoospermicznych. Otrzymane wartości względnej reaktywności glikanów CLU plazmy nasienia z MAA umożliwiły różnicowanie grupy oligoastenoteratozoospermicznej od grup: normozoospermicznej (czułość i swoistość odpowiednio: 79,3% oraz 72,4%; AUC=0,715 – umiarkowana wartość kliniczna), teratozoospermicznej (czułość i swoistość odpowiednio: 79,3% oraz 91,2%; AUC=0,890 – umiarkowana wartość kliniczna) oraz astenoteratozoospermicznej (czułość i swoistość odpowiednio: 75,9% oraz 94,4%; AUC=0,883 – umiarkowana wartość kliniczna).

Powyższe obserwacje zostały potwierdzone w analizie krzywych ROC w wariancie, w którym porównywano grupę OAT z pozostałymi pacjentami traktowanymi jako jedna grupa (czułość i swoistość odpowiednio: 75,9% oraz 85,2%; AUC=0,826 – umiarkowana wartość kliniczna). Współczynnik sjalilacji dla plazm nasienia umożliwił różnicowanie grupy OAT od grupy T (czułość i swoistość odpowiednio: 89,7% oraz 67,6%; AUC=0,836 – umiarkowana wartość kliniczna) oraz AT (czułość i swoistość odpowiednio: 55,2% oraz 100%; AUC=0,837 – umiarkowana wartość kliniczna). Zależność tę potwierdzono, porównując grupę OAT z pozostałymi pacjentami analizowanymi łącznie, jako jedna grupa (czułość i swoistość odpowiednio: 89,7% oraz 55,6%; AUC=0,777 – umiarkowana wartość kliniczna). Stężenia SIRT3 w plazmach nasienia różniły się istotnie między grupą OAT a grupami: normozoospermiczną (czułość i swoistość odpowiednio: 82,1% oraz 94,6%; AUC=0,951 – wysoka wartość kliniczna) oraz astenozoospermiczną (czułość i swoistość odpowiednio: 100% oraz 81,8%; AUC=0,948 – wysoka wartość kliniczna). Wartości stężeń SIRT3 pozwoliły także na różnicowanie grupy AT oraz T (czułość i swoistość odpowiednio: 81,8% oraz 100%; AUC=0,906 – wysoka wartość kliniczna), a także grupy T oraz N (czułość i swoistość odpowiednio: 100% oraz 75,7%; AUC=0,887 – umiarkowana wartość kliniczna). Opisane zależności zostały potwierdzone w drugim wariancie analizy krzywych ROC, w którym każda z badanych grup pacjentów została porównana z pozostałymi pacjentami traktowanymi jako jedna grupa. Na uwagę zasługuje fakt, że stężenie SIRT3 jest jedynym parametrem plazmy nasienia, dla którego średnia wartość AUC ma obiecującą, umiarkowaną, wartość kliniczną (AUC=0,786) we wszystkich porównywanych ze sobą konfiguracjach parametrów. Oznaczenie stężeń SIRT5 w plazmach nasienia umożliwiło różnicowanie grupy OAT od grup: normozoospermicznej (czułość i swoistość odpowiednio: 96,4% oraz 73%; AUC=0,812 – umiarkowana wartość kliniczna) oraz teratozoospermicznej (czułość i swoistość odpowiednio: 96,4% oraz 69,4%; AUC=0,759 – umiarkowana wartość kliniczna). Na uwagę zasługuje fakt, że stężenie tej sirtuiny obecnej w plazmie nasienia, związanej z równowagą oksydacyjno-antyoksydacyjną, istotnie różniło się w grupie OAT w porównaniu z grupą AT, a jego wartość kliniczna była wysoka (czułość i swoistość odpowiednio: 96,4% oraz 81,8%; AUC=0,916). Oznaczone wartości stężeń SIRT5, w oparciu o przeprowadzoną analizę krzywych ROC, pozwoliły także na różnicowanie grupy OAT od pozostałych

pacjentów traktowanych łącznie (czułość i swoistość odpowiednio: 96,4% oraz 73,7%; AUC=0,816 – umiarkowana wartość kliniczna).

Przeprowadzona analiza krzywych ROC dla wartości parametrów oznaczonych w surowicach wykazała, że ekspresja SNA- i MAA-reaktywnych kwasów sjalowych na glikanach CLU, stężenia SIRT3 oraz FRAP mogą być dodatkowymi markerami różnicującymi niepłodnych pacjentów zarówno normozoospermicznych, jak i tych, których wyniki analizy seminologicznej wykazują nieprawidłowości. Wartości względnych reaktywności glikanów surowiczej CLU ze sjalospecyficzną lektyną SNA pozwoliły na różnicowanie grupy normozoospermicznej od grupy: astenoteratozoospermicznej (czułość i swoistość odpowiednio: 86,7% oraz 81,3%; AUC=0,892 – umiarkowana wartość kliniczna), oligoastenoteratozoospermicznej (czułość i swoistość odpowiednio: 88,9% oraz 68,8%; AUC=0,837 – umiarkowana wartość kliniczna) oraz teratozoospermicznej (czułość i swoistość odpowiednio: 87,1% oraz 81,3%; AUC=0,872 – umiarkowana wartość kliniczna). Wartości względnych reaktywności glikanów surowiczej CLU z lektyną MAA różniły się istotnie między grupą normozoospermiczną a grupami: AT (czułość i swoistość odpowiednio: 60% oraz 87,5%; AUC=0,756 – umiarkowana wartość kliniczna) oraz T (czułość i swoistość odpowiednio: 51,6% oraz 87,5%; AUC=0,716 – umiarkowana wartość kliniczna). Podobne zależności zaobserwowano dla wartości względnych reaktywności glikanów surowiczej CLU z SNA oraz MAA, gdy analizę krzywych ROC wykonano porównując pacjentów normozoospermicznych z pacjentami zaklasyfikowanymi do pozostałych grup analizowanymi łącznie (SNA: czułość i swoistość odpowiednio: 83,1% oraz 82,2%, AUC=0,863 – umiarkowana wartość kliniczna; MAA: czułość i swoistość odpowiednio: 87,5% oraz 54,8%, AUC=0,700 – umiarkowana wartość kliniczna). Oznaczenie stężeń SIRT3 w surowicach umożliwiło różnicowanie grupy AT od grupy N (czułość i swoistość odpowiednio: 100% oraz 61,1%, AUC=0,830 – umiarkowana wartość kliniczna) oraz T (czułość i swoistość odpowiednio: 93,3% oraz 51,6%, AUC=0,740 – umiarkowana wartość kliniczna), a także grupy OAT od grupy N (czułość i swoistość odpowiednio: 100% oraz 61,1%, AUC=0,761 – umiarkowana wartość kliniczna). Ponadto stężenia surowiczej SIRT3 różniły się także między grupami AT oraz N a pozostałymi pacjentami (dla grupy AT czułość i swoistość odpowiednio: 93,3% oraz 52,6%, AUC=0,714 – umiarkowana wartość kliniczna; dla grupy N czułość i swoistość odpowiednio: 61,1%

oraz 94,5%, AUC=0,749 – umiarkowana wartość kliniczna). Stężenia FRAP w surowicach różniły się istotnie między grupą AT a grupą N (czułość i swoistość odpowiednio: 80% oraz 77,8%, AUC=0,78 – umiarkowana wartość kliniczna) oraz grupą T (czułość i swoistość odpowiednio: 60% oraz 77,4%, AUC=0,701 – umiarkowana wartość kliniczna).

Do analizy skupień wybrano te parametry plazmy nasienia, które istotnie różnicowały badane grupy pacjentów oraz w analizie krzywych ROC ich wartości AUC były wyższe od 0,715: względna reaktywność glikanów CLU z MAA, współczynnik sjalilacji, stężenia SIRT3 oraz SIRT5. Parametry surowicy, które wybrano do analizy skupień, w analizie krzywych ROC charakteryzowały się wartościami AUC≥0,701 i były to: względne reaktywności glikanów CLU z SNA i MAA, stężenia SIRT3 oraz FRAP. Przeprowadzona analiza skupień potwierdziła, że dwa powyższe panele biomarkerów, odpowiednio dla plazmy nasienia oraz dla surowicy, mogą mieć potencjalne zastosowanie w diagnostyce męskiej niepłodności.

Zastosowany model wielomianowej regresji logistycznej potwierdził potencjalną przydatność diagnostyczną oznaczania stężeń SIRT3 w plazmach nasienia w celu różnicowania grupy normozoospermicznej od grupy OAT (p=0,003) oraz T (p<0,001). Wykazano również, że oznaczane w plazmie nasienia stężenie SIRT5 jest dobrym predyktorem różnicującym grupę normozoospermiczną od teratozoospermicznej z istotnością p=0,006. Wielomianowa regresja logistyczna zastosowana dla parametrów oznaczonych w surowicach wykazała, że względna reaktywność glikanów surowiczej klasteryny z SNA jest dobrym markerem, przydatnym do różnicowania grupy niepłodnych mężczyzn normozoospermicznych od pozostałych grup pacjentów: AT (p=0,003), OAT (p=0,007) oraz T (p=0,002). Z kolei wartości stężeń FRAP stanowią dobry marker pomocny w różnicowaniu grupy N od grupy AT (p=0.040).

6.3. **III artykuł oryginalny** – Czy istnieją zależności między zaawansowanymi produktami utleniania białek a wybranymi parametrami biochemicznymi związanymi ze statusem oksydacyjno-redukcyjnym w plazmach nasienia niepłodnych mężczyzn?

Wartości stężeń AOPP oznaczone w plazmach nasienia niepłodnych mężczyzn normozoospermicznych (mediana: 483,19 μmol/l) były istotnie wyższe od tych obserwowanych w grupach AT oraz OAT (wartości median odpowiednio: 163,51 μmol/l, p=0,000035 oraz 134,26 μmol/l, p=0,000002). Dodatkowo, stężenia AOPP w grupie teratozoospermicznej (mediana: 505 μmol/l) również były istotnie wyższe w porównaniu z grupą AT (p=0,000043) oraz OAT (p=0,000002). Stężenia białka całkowitego były istotnie niższe w grupie T (mediana: 3,02 g/dl) w porównaniu do grup AT oraz OAT (wartości mediany odpowiednio: 4,13 g/dl, p=0,007072 oraz 3,66 g/dl, p=0,030200). Stężenia żelaza w plazmach nasienia były istotnie niższe w grupie OAT (mediana: 12,80 μg/dl) w porównaniu do grupy T (mediana: 16,44 μg/dl, p=0,020539) oraz N (mediana: 22,01 μg/dl, p=0,031121). Nie wykazano istotnych różnic w wartościach stężeń kwasu moczowego, magnezu oraz wapnia między badanymi grupami niepłodnych mężczyzn. Wyniki przeprowadzonej analizy korelacji wykazały istnienie silnej, dodatnio skorelowanej, zależności między stężeniami wapnia oraz magnezu (r=0,8714, p<0,001), wapnia oraz żelaza (r=0,8120, p<0,001), magnezu oraz żelaza (r=0,7885, p<0,001), a także zaobserwowano istnienie umiarkowanej dodatniej korelacji między stężeniami AOPP a stężeniami żelaza (r=0,5393, p<0,001), wapnia (r=0,5262, p<0,001) i magnezu (r=0,4511, p<0,001).

Ocena przydatności diagnostycznej analizowanych parametrów dokonana w oparciu o wyniki przeprowadzonej analizy krzywych ROC wykazała, że oznaczenie stężeń AOPP w plazmach nasienia może być pomocne w różnicowaniu normozoospermicznych niepłodnych pacjentów od grup AT (czułość i swoistość odpowiednio: 100% oraz 69,7%; AUC=0,837 – umiarkowana wartość kliniczna) oraz OAT (czułość i swoistość odpowiednio: 96,4% oraz 75,8%; AUC=0,855 – umiarkowana wartość kliniczna). Ponadto parametr ten umożliwia różnicowanie grupy T

od grupy AT (czułość i swoistość odpowiednio: 100% oraz 76,7%; AUC=0,861 – umiarkowana wartość kliniczna) oraz OAT (czułość i swoistość odpowiednio: 100% oraz 80%; AUC=0,861 – umiarkowana wartość kliniczna). Oznaczenie stężeń białka całkowitego w plazmach nasienia umożliwiło różnicowanie grupy AT od grupy T (czułość i swoistość odpowiednio: 70% oraz 80%; AUC=0,728 – umiarkowana wartość kliniczna), natomiast wartości stężeń żelaza różniły się istotnie między grupą normozoospermiczną a grupą oligoastenoteratozoospermiczną (czułość i swoistość odpowiednio: 95,7% oraz 50%; AUC=0,725 – umiarkowana wartość kliniczna).

7. Podsumowanie

Biorąc pod uwagę fakt, że glikozylacja jest jedną z kluczowych modyfikacji potranslacyjnych białek, warunkującą wiele funkcji biologicznych glikoprotein, oraz że oddziaływania między glikanami osłonki przejrzystej oraz glikoproteinami plazmy nasienia mają istotne znaczenie w procesie zapłodnienia, badania nad profilem i stopniem glikozylacji najważniejszych glikoprotein plazmy nasienia mogą rzucić nowe światło na przyczyny zaburzeń męskiego potencjału rozrodczego. Przeprowadzone badania, których wyniki omówiono w niniejszej rozprawie, wykazały, że plazma nasienia, która w rutynowej diagnostyce niepłodności męskiej nie znalazła jak dotąd zastosowania, może stanowić cenne źródło dodatkowych biomarkerów o potencjalnym zastosowaniu diagnostycznym. Dodatkowo, pozyskane informacje na temat ekspresji analizowanych przeze mnie markerów oraz wzajemnych korelacji między nimi, mogą stanowić przyczynek do lepszego poznania molekularnych mechanizmów prowadzących do zaburzeń prowadzących do obniżenia męskiego potencjału rozrodczego. Należy zwrócić uwagę na fakt, że w artykułach wchodzących w skład cyklu publikacji stanowiących podstawę niniejszej rozprawy doktorskiej po raz pierwszy dokonano analizy profilu i stopnia fukozylacji oraz sjalilacji CLU oraz oceny zdolności redukcji jonów żelaza (FRAP) zarówno w plazmach nasienia jak i w surowicach niepłodnych pacjentów, co pozwoliło na przeprowadzenie porównań między tymi dwoma płynami biologicznymi.

Zaproponowane w artykułach oryginalnych zestawy parametrów mogą stanowić dodatkowe panele biomarkerów pomocnych w różnicowaniu grup niepłodnych

pacjentów. Wyniki moich badań poszerzyły zakres informacji dotyczących profilu i stopnia fukozylacji oraz sjalilacji klasteryny obecnej w plazmach nasienia oraz surowicach niepłodnych mężczyzn. Ponadto wyniki przeprowadzonych badań wzbogaciły dotychczasową wiedzę dotyczącą korelacji między analizowanymi przeze mnie parametrami plazm nasienia jak i surowic krwi pacjentów z nieprawidłowymi parametrami ejakulatu. W dalszej perspektywie, przeprowadzenie analogicznych badań również w grupie normozoospermicznych, zdrowych mężczyzn o udowodnionej płodności i porównanie ich wyników z tymi otrzymanymi dla grupy normozoospermicznych niepłodnych pacjentów, stworzy możliwość zaproponowania panelu biomarkerów związanych z męską niepłodnością idiopatyczną.

8. Wnioski

8.1. **I artykuł oryginalny** – Potencjalny związek zmian fukozylacji klasteryny z zaburzeniami męskiej płodności

1) Zaobserwowane różnice między grupą OAT a pozostałymi grupami badanymi w stężeniach klasteryny oraz fukozylotransferaz 3 i 4 obecnych w plazmach nasienia, nasuwają przypuszczenie, że obniżona liczba plemników w nasieniu może mieć związek ze zmienioną ekspresją wymienionych parametrów.
2) Oznaczenie stężeń klasteryny, jak również FUT3 oraz FUT4 w plazmie nasienia może stanowić dodatkowy panel biomarkerów pozwalających na różnicowanie niepłodnych mężczyzn, których wyniki analizy seminologicznej zarówno są prawidłowe, jak i odbiegają od wartości uznanych za referencyjne.
3) Różnice w stężeniach surowiczej CLU, ekspresji fukozy rdzeniowej jak również fukozy przyłączonej wiązaniem α1,2 do Gal anten cukrowych glikanów CLU mogą stanowić dodatkowe markery różnicujące normozoospermicznych niepłodnych mężczyzn od pacjentów z nieprawidłową liczbą, ruchliwością oraz morfologią plemników, czego nie zaobserwowano dla analogicznych parametrów badanych w plazmie nasienia.
4) Różnice obserwowane w stężeniach surowiczej klasteryny oraz fukozylotransferazy 3, jak również w ekspresji fukozy przyłączonej wiązaniem α1,2 w glikanach CLU między niepłodną grupą pacjentów

normozoospermicznych a pozostałymi grupami badanymi mogą sugerować, że zaburzenia liczby, ruchliwości oraz morfologii plemników nie są jedyną przyczyną problemów z męską płodnością.

8.2. **II artykuł oryginalny** – Zależności między stopniem sjalilacji klasteryny a poziomami markerów równowagi oksydacyjno-antyoksydacyjnej w plazmach nasienia oraz surowicach niepłodnych mężczyzn

1) Zmiany dotyczące stopnia sjalilacji klasteryny obecnej w plazmach nasienia są prawdopodobnie związane z nieprawidłową syntezą oraz dojrzewaniem plemników, co w konsekwencji prowadzi do zmniejszenia potencjału rozrodczego mężczyzn w wieku reprodukcyjnym. Dodatkowo, zmiany stopnia sjalilacji CLU zaobserwowano także w surowicach niepłodnych pacjentów.
2) Ekspresja końcowego, SNA-reaktywnego, kwasu sjalowego przyłączonego wiązaniem α2,6 do Gal anten cukrowych glikanów klasteryny obecnej w plazmie nasienia jest niezależna od wartości parametrów nasienia ocenianych podczas jego standardowej analizy. Natomiast dla surowic zaobserwowano, że występowanie zaburzeń dotyczących liczby, morfologii i/lub ruchliwości plemników znajdowało odzwierciedlenie w obniżeniu ekspresji kwasu sjalowego przyłączonego wiązaniem α2,6 do glikanów surowiczej CLU, a co za tym idzie względna reaktywność glikanów klasteryny z SNA może stanowić dodatkowy biomarker niepłodności męskiej, różnicujący z dużą czułością oraz swoistością niepłodnych normozoospermicznych pacjentów od mężczyzn z nieprawidłowymi wynikami badania nasienia.
3) Względna reaktywność glikanów CLU plazmy nasienia z MAA, lektyną wykrywającą kwas sjalowy przyłączony wiązaniem α2,3 do glikanów CLU, oraz współczynnik sjalilacji (MAA/SNA), wraz ze stężeniem SIRT3 oraz SIRT5 mogą stanowić dodatkowy panel biomarkerów użytecznych w diagnostyce niepłodności męskiej.
4) Istnienie pozytywnych korelacji między stężeniami SIRT3 oraz SIRT5 w obydwu analizowanych płynach biologicznych może wskazywać na synergistyczne

działanie tych enzymów w zaburzeniach równowagi oksydacyjno-antyoksydacyjnej niepłodnych mężczyzn. Ponadto wykazane istnienie ujemnych korelacji między stężeniami CLU oraz SIRT3 i SIRT5 może sugerować odmienne mechanizmy działania tych biologicznie aktywnych cząsteczek w utrzymaniu równowagi oksydacyjno-antyoksydacyjnej.

5) Względne reaktywności glikanów CLU plazmy nasienia ze sjalospecyficzną lektyną MAA, wartość współczynnika sjalilacji, stężenia SIRT3, SIRT5 oraz FRAP mogą być pomocne w różnicowaniu pacjentów z grupy OAT od pozostałych grup badanych.
6) Stopień ekspresji SNA- i MAA-reaktywnego kwasu sjalowego na glikanach surowiczej CLU oraz stężenia SIRT3 i FRAP w surowicy mogą być pomocne w różnicowaniu pacjentów normozoospermicznych od mężczyzn z nieprawidłowymi parametrami nasienia.

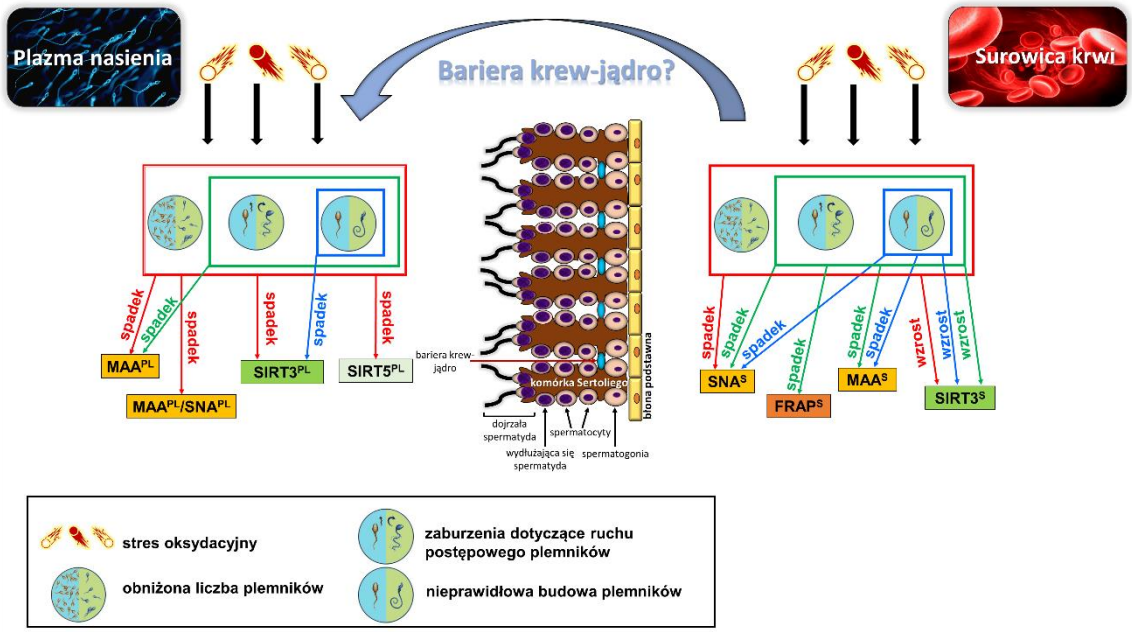
8.3 **III artykuł oryginalny** – Czy istnieją zależności między zaawansowanymi produktami utleniania białek a wybranymi parametrami biochemicznymi związanymi ze statusem oksydacyjno-redukcyjnym w plazmach nasienia niepłodnych mężczyzn?

1) Obniżone stężenia zaawansowanych produktów utleniania białek w plazmach nasienia były obserwowane u pacjentów z zaburzeniami dotyczącymi ruchliwości plemników. Dlatego stężenie AOPP oznaczane w plazmie nasienia może stanowić obiecujący parametr różnicujący mężczyzn z zaburzeniami związanymi z ruchliwością plemników od tych z innymi typami zaburzeń dotyczących plemników, aczkolwiek zależności między tworzeniem AOPP a upośledzeniem ruchu plemników mogą być złożone.
2) Istotnie podwyższone stężenia białka całkowitego w grupach oligoastenoteratozoospermicznej oraz astenoteratozoospermicznej w porównaniu z grupą teratozoospermiczną nasuwają przypuszczenie, że zaburzenia dotyczące budowy plemników mogą być związane z obniżonym poziomem białka całkowitego w plazmie nasienia.

3) Obniżone stężenie żelaza w plazmach nasienia może mieć wpływ na zmniejszoną liczbę plemników.
4) Wykazane istnienie silnych dodatnich korelacji między stężeniami żelaza, magnezu i wapnia w plazmach nasienia mężczyzn z nieprawidłowościami dotyczącymi plemników stanowią potwierdzenie, że pierwiastki te odgrywają bardzo ważną rolę w prawidłowym procesie dojrzewania plemników, a także w reakcji kapacytacji i reakcji akrosomalnej.

8.4 Wnioski końcowe

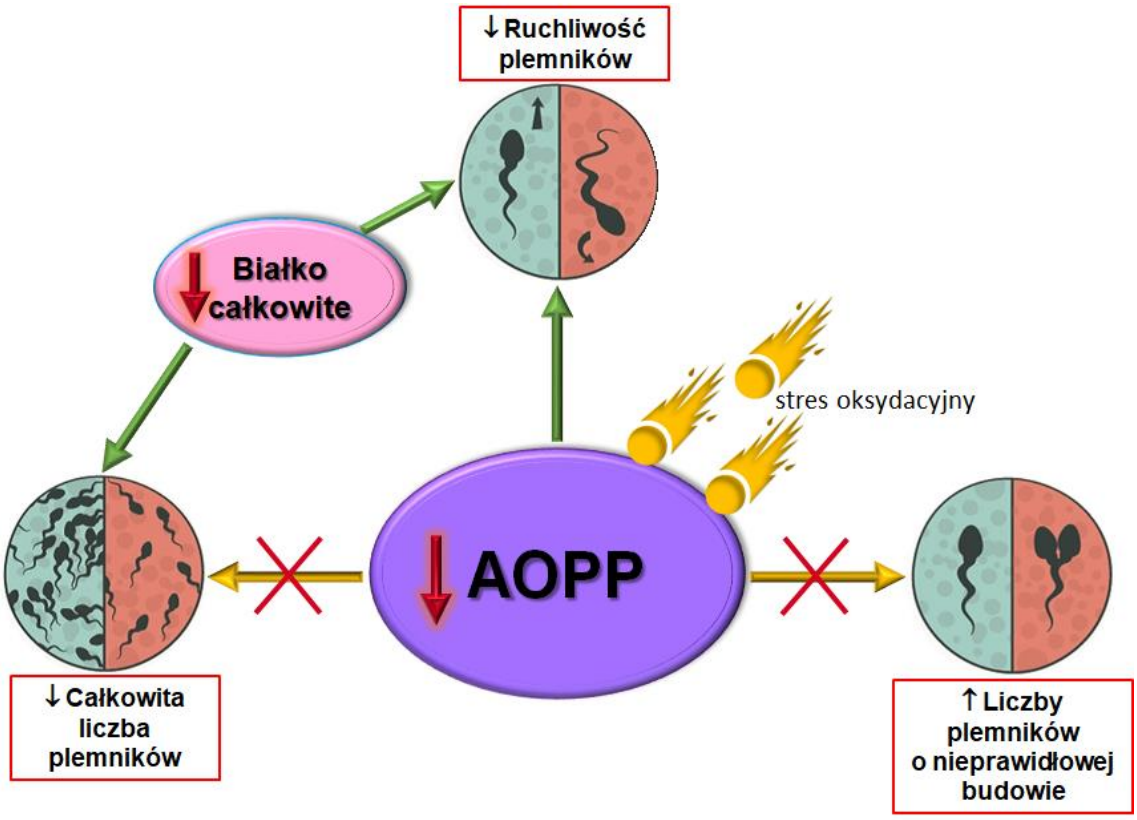
1) Klasteryna, obecna w plazmach nasienia oraz surowicach niepłodnych mężczyzn, wykazuje różnice w profilu fukozylacji w analizowanych płynach biologicznych, a różnice w stężeniach surowiczej CLU, ekspresji fukozy rdzeniowej jak również fukozy przyłączonej wiązaniem α1,2 do Gal anten cukrowych glikanów CLU mogą stanowić dodatkowy panel markerów różnicujących normozoospermicznych niepłodnych mężczyzn od pacjentów z nieprawidłową liczbą, ruchliwością oraz morfologią plemników.
2) Profil i stopień sjalilacji CLU różni się między badanymi grupami oraz płynami biologicznymi i prawdopodobnie jest także związany z zaburzeniami równowagi oksydacyjno-antyoksydacyjnej u niepłodnych mężczyzn. Nasuwają się zatem pytania, mogące stać się inspiracją do dalszych badań: jaki jest związek między profilem i stopniem sjalilacji CLU plazmy nasienia i surowicy oraz czy CLU plazmy nasienia pochodzi tylko z męskich gruczołów rozrodczych, czy też bariera krew-jądro jest przepuszczalna dla cząsteczek CLU? W oparciu o wyniki analiz statystycznych wyodrębniono panel biomarkerów plazmy nasienia, przydatny w różnicowaniu pacjentów z grupy OAT od pozostałych grup badanych, w skład którego wchodzą: względne reaktywności glikanów CLU plazmy nasienia ze sjalospecyficzną lektyną MAA, wartość współczynnika sjalilacji, stężenia SIRT3, SIRT5 oraz FRAP. Ponadto stopień ekspresji SNA- i MAA-reaktywnego kwasu sjalowego na glikanach surowiczej CLU oraz stężenia SIRT3 i FRAP w surowicy mogą stanowić dodatkowy panel parametrów, pomocny w różnicowaniu pacjentów normozoospermicznych od mężczyzn z nieprawidłowymi parametrami nasienia (Rysunek 1).



Rysunek 1. Zależności między sjalilacją klasteryny a wybranymi parametrami stresu oksydacyjnego.

SNA^{PL}- względna reaktywność glikanów klasteryny z SNA w plazmie nasienia; MAA^{PL}- względna reaktywność glikanów klasteryny z MAA w plazmie nasienia; MAA^{PL}/SNA^{PL} – współczynnik sjalilacji klasteryny w plazmie nasienia; SNA^{S}- względna reaktywność glikanów klasteryny z SNA w surowicy; MAA^{S}- względna reaktywność glikanów klasteryny z MAA w surowicy; MAA^{S}/SNA^{S} – współczynnik sjalilacji klasteryny w surowicy; $SIRT3^{PL}$- stężenie sirtuiny 3 w plazmie nasienia; $SIRT5^{PL}$- stężenie sirtuiny 5 w plazmie nasienia; $FRAP^{PL}$- zdolność do redukcji jonów żelaza w plazmie nasienia; $SIRT3^{S}$- stężenie sirtuiny 3 w surowicy; $SIRT5^{S}$- stężenie sirtuiny 5 w surowicy; $FRAP^{S}$- zdolność do redukcji jonów żelaza w surowicy. Rysunek jest modyfikacją pierwotnej wersji mojego autorstwa, zrobioną i udostępnioną przez Promotora niniejszej dysertacji.

3) Stężenie AOPP oznaczane w plazmach nasienia może stanowić obiecujący parametr różnicujący mężczyzn z zaburzeniami związanymi z ruchliwością plemników od pacjentów z innymi typami zaburzeń dotyczących plemników, natomiast zmiany stężenia białka całkowitego w plazmach nasienia mogą mieć związek z zaburzeniami budowy plemników. Ponadto obniżone stężenie żelaza w plazmach nasienia może mieć związek z obniżoną liczbą plemników (Rysunek 2).



Rysunek 2. Zależności między stężeniami zaawansowanych produktów oksydacji białek (AOPP) oraz białka całkowitego w plazmach nasienia a wybranymi parametrami standardowej analizy nasienia.

Modyfikacja na podstawie Janiszewskiej i wsp. [32].

9. Streszczenie

Wprowadzenie: Niepłodność męska to narastający problem społeczny na świecie a jej mechanizmy molekularne nie zostały w pełni poznane. Nadal brakuje nowego, czułego biomarkera obniżonego potencjału rozrodczego mężczyzn, który mógłby uzupełnić standardową analizę nasienia, co byłoby szczególnie cenne w przypadkach niepłodności idiopatycznej. Skład proteomu plazmy nasienia, zarówno ilościowy (stężenia poszczególnych białek i glikoprotein), jak i jakościowy (m. in. zmiany ekspresji glikanów glikoprotein) jest jednym z kluczowych czynników wpływających na dojrzewanie plemników oraz proces zapłodnienia wewnątrzustrojowego. Wśród wielu przyczyn niepłodności męskiej szczególną uwagę zwraca się również na zaburzenia równowagi oksydacyjno-oksydacyjnej, określane mianem stresu oksydacyjnego (OS). Jedną z głównych glikoprotein plazmy nasienia jest klasteryna (CLU), pełniąca istotne funkcje w męskim układzie rozrodczym oraz będąca czułym biomarkerem OS. Zmiany stężenia i/lub profilu i stopnia glikozylacji CLU mogą stać się przyczynkiem do wzbogacenia procesu diagnostycznego zaburzeń męskiej płodności.

Cel badań: Celem badań było sprawdzenie zależności między stężeniem CLU, ekspresją jej glikanów a wybranymi wskaźnikami OS: TAS (*ang. total antioxidant status*), FRAP (*ang. ferric reducing antioxidant power)*, stężeniami sirtuiny 3 oraz 5 (SIRT3 oraz SIRT5), w plazmach nasienia oraz surowicach niepłodnych mężczyzn. Ponadto sprawdzono, czy istnieją zależności między stężeniami wybranych parametrów biochemicznych plazmy nasienia, związanych ze statusem oksydacyjno-redukcyjnym, a stężeniami zaawansowanych produktów utleniania białek - AOPP (*ang. advanced oxidation protein products*). Dodatkowym aspektem było zaproponowanie zestawów parametrów, zarówno plazmy nasienia jak i surowiczych, mogących stać się dodatkowymi biomarkerami, różnicującymi grupy niepłodnych pacjentów z zaburzeniami dotyczącymi liczby, ruchliwości oraz morfologii plemników. Celem przeprowadzonych badań było także porównanie dwóch płynów ustrojowych, plazmy nasienia i surowicy, pod kątem zawartości w nich wybranych do oznaczeń parametrów, z równoczesną analizą ich potencjalnego zastosowania w diagnostyce męskiej niepłodności.

Materiał i metody: Materiałem badanym były 132 plazmy nasienia oraz 91 surowic krwi, pochodzące od niepłodnych pacjentów, które na podstawie wyników standardowej oceny nasienia podzielono na grupy: teratozoospermiczną (T), astenoteratozoospermiczną (AT), oligoastenoteratozoospermiczną (OAT), oraz normozoospermiczną (N). Stężenia CLU oznaczono metodą ELISA. Ekspresję fukozy sprawdzono za pomocą zmodyfikowanego testu lektyno-ELISA z wykorzystaniem biotynylowanych lektyn specyficznych wobec fukozy (*Lotus tetragonolobus* agglutinin - LTA, *Ulex europaeus* agglutin - UEA, *Lens culinaris agglutinin* - LCA). Ekspresję struktur oligosacharydowych typu Lewis sprawdzono oznaczając stężenia fukozylotransfeazy 3 i 4 metodą ELISA. Ekspresję kwasu sjalowego sprawdzono z użyciem zmodyfikowanego testu lektyno-ELISA z użyciem specyficznych biotynylowanych lektyn (*Sambucus nigra* agglutinin - SNA, *Maackia amurensis* agglutinin - MAA). Stężenia SIRT3 oraz SIRT5 oznaczono metodą ELISA. Stężenia TAS, FRAP, AOPP, żelaza, kwasu moczowego, wapnia oraz magnezu oznaczono metodą kolorymetryczną. Analizę statystyczną wykonano z wykorzystaniem oprogramowania Statistica 13.3 PL, za istotne uznając wyniki, dla których wskaźnik istotności $p<0,05$.

Wyniki: Stężenia CLU w plazmach nasienia pacjentów z grupy OAT były istotnie wyższe w porównaniu do pozostałych grup niepłodnych mężczyzn. Surowicze stężenia CLU były natomiast istotnie niższe w grupie OAT w porównaniu z pozostałymi grupami badanymi. Stężenia FUT3 w plazmach nasienia były istotnie wyższe w grupie T w porównaniu do grup N oraz AT, a także istotnie wyższe w grupie OAT w porównaniu z grupami AT oraz N. Stężenia FUT4 w plazmach nasienia były istotnie wyższe u niepłodnych mężczyzn norozoospermicznych w porównaniu z pacjentami z grupy T oraz istotnie wyższe w grupie AT w porównaniu z grupą T. Względne reaktywności glikanów CLU obecnej w surowicach z fukozospecyficzną lektyną UEA były istotnie wyższe w grupie N w porównaniu z pozostałymi grupami badanymi. Podobnie, względne reaktywności glikanów CLU obecnej w surowicach z lektyną specyficzną wobec fukozy rdzeniowej - LCA, były istotnie wyższe w grupie N w porównaniu z pozostałymi grupami pacjentów. Ponadto wartość tego parametru była istotnie wyższa w grupie T w porównaniu z grupami OAT oraz AT. Analiza krzywych ROC oraz analiza skupień umożliwiły zaproponowanie panelu parametrów, przydatnych w różnicowaniu pacjentów z zaburzeniami dotyczącymi plemników. W plazmach nasienia były to: stężenia CLU,

FUT3 i FUT4, natomiast w surowicach: stężenia CLU i FUT4 oraz względne reaktywności glikanów CLU z fukozospecyficznymi lektynami UEA oraz LCA. Względne reaktywności glikanów CLU plazmy nasienia ze sjalospecyficzną lektyną MAA były istotnie niższe w grupie OAT w porównaniu do pozostałych grup pacjentów, natomiast ekspresja kwasu sjalowego SNA-reaktywnego na glikanach surowiczej CLU była istotnie wyższa w grupie OAT w porównaniu do pozostałych grup badanych. Stężenia SIRT3 w plazmach nasienia były istotnie wyższe w grupie N w porównaniu do grup T oraz OAT, a także stężenia tej sirtuiny były istotnie wyższe w grupie AT w porównaniu z grupami T oraz OAT. Stężenia surowiczej SIRT3 były istotnie niższe w grupie N w porównaniu z grupami AT oraz OAT. Stężenia SIRT5 w plazmach nasienia były istotnie niższe w grupie OAT w porównaniu do pozostałych grup pacjentów. Stężenia FRAP w surowicach pacjentów z grupy N były istotnie wyższe niż w grupie AT. Na podstawie analizy krzywych ROC oraz analizy skupień zaproponowano panel parametrów pozwalających na różnicowanie grupy OAT od pozostałych grup badanych, którymi są: względne reaktywności glikanów CLU plazmy nasienia ze sjalospecyficzną lektyną MAA, wartość współczynnika sjalilacji MAA/SNA, stężenia SIRT3, SIRT5 oraz FRAP. Analiza krzywych ROC oraz analiza skupień umożliwiły zaproponowanie zestawu surowiczych parametrów, przydatnych w różnicowaniu niepłodnych normozoospermicznych mężczyzn od pacjentów z nieprawidłowościami dotyczącymi plemników. Były to: względne reaktywności glikanów surowiczej CLU ze sjalospecyficznymi lektynami SNA i MAA oraz stężenia SIRT3 i FRAP. Stężenia AOPP w plazmach nasienia pacjentów z grupy N były istotnie wyższe w porównaniu z grupami AT oraz OAT, a także stężenia tego parametru były istotnie wyższe w grupie T w porównaniu do grup AT oraz OAT. Stężenia białka całkowitego w plazmach nasienia były istotnie niższe w grupie T w porównaniu do grup AT oraz OAT, natomiast stężenia żelaza w plazmach nasienia były istotnie niższe w grupie OAT w porównaniu do grup T oraz N. Analiza krzywych ROC wykazała, że oznaczenie stężeń AOPP w plazmach nasienia może stanowić dodatkowy parametr różnicujący niepłodnych normozoospermicznych mężczyzn od pacjentów z zaburzeniami dotyczącymi ruchliwości oraz budowy plemników, a także może stanowić obiecujący parametr różnicujący mężczyzn z zaburzeniami związanymi z ruchliwością plemników od tych z innymi typami zaburzeń dotyczących plemników.

Wnioski: Wyniki przeprowadzonych badań w znaczący sposób przyczyniły się do pogłębienia wiedzy na temat glikozylacji klasteryny obecnej w plazmach nasienia oraz surowicach mężczyzn z nieprawidłowościami dotyczącymi plemników. Różnice obserwowane w ekspresji lub stężeniach analizowanych parametrów sugerują, że zaburzenia liczby, ruchliwości oraz morfologii plemników nie są jedyną przyczyną niepłodności męskiej. Zmiany stężeń klasteryny oraz fukozylotransferaz 3 i 4 w plazmach nasienia mogą mieć związek z obniżoną liczbą plemników w nasieniu. Różnice w stężeniach surowiczej CLU, ekspresji fukozy rdzeniowej oraz fukozy przyłączonej wiązaniem α1,2 do galaktozy anten cukrowych glikanów CLU mogą stanowić dodatkowe markery różnicujące normozoospermicznych niepłodnych mężczyzn od pacjentów z nieprawidłową liczbą, ruchliwością oraz morfologią plemników. Względna reaktywność glikanów surowiczej klasteryny z SNA może stanowić dodatkowy biomarker niepłodności męskiej, różnicujący z dużą czułością oraz swoistością niepłodnych normozoospermicznych pacjentów od mężczyzn z nieprawidłowymi wynikami badania nasienia. Stężenia AOPP oznaczane w plazmach nasienia mogą stanowić obiecujący parametr różnicujący mężczyzn z zaburzeniami związanymi z ruchliwością plemników od tych z innymi nieprawidłowościami dotyczącymi plemników, natomiast zmiany stężenia białka całkowitego w tym materiale biologicznym mogą mieć związek z zaburzeniami budowy plemników. Ponadto obniżone stężenia żelaza w plazmach nasienia korelowały dodatnio ze zmniejszoną liczbą plemników. W przyszłości poszerzenie spektrum badań o grupę normozoospermiczną mężczyzn o udowodnionej płodności da szansę na wytypowanie biomarkerów charakterystycznych dla męskiej niepłodności idiopatycznej.

10. Summary

Introduction: Male infertility is a growing problem around the world and the mechanisms of its development are not completely known yet. The early and sensitive biomarker of decreased male reproductive potential that could support the standard semen analysis, which would particularly be valuable in cases of idiopathic infertility, is still missing. The composition of seminal plasma proteome, the quantative (proteins and glycoproteins concentrations) as well as qualitative (*e.g.* the changes in expression of glycoproteins glycans) is one of the key factor affecting spermatozoa maturation as well as intrauterine fertilization process. Among many male infertility reasons, special attention is paid also to oxidant-antioxidant imbalance, caused by oxidative stress (OS). One of the main seminal plasma glycoproteins is clusterin (CLU), playing important functions in the male reproductive tract, also known as sensitive OS biomarker. Changes in the concentration and/or profile and degree of CLU glycosylation may reach the diagnostic process of male fertility disorders.

Aim of the study: The aim of the study was to check if there are relationships between CLU concentration, its glycans' expression, and selected OS parameters: TAS (*total antioxidant status*), FRAP (*ferric reducing antioxidant power)*, sirtuin 3 and 5 concentrations (SIRT3 and SIRT5, respectively) in seminal plasmas and sera of infertile men. Moreover, the correaltions between concentrations of selected seminal plasma biochemical parameters associated with redox status and advanced oxidation protein products (AOPP) were also checked. Another aspect of the study was to select a set of seminal plasma and serum parameters which may become an additional biomarkers differentiating groups of infertile men with disorders in sperm count, motility and morphology. The aim of the study was also to compare two body fluids, seminal plasma and serum, in terms of the content of parameters selected for determination, with the simultaneous analysis of their potential use in the diagnostics of male infertility.

Materials and methods: The study material consisted of 132 seminal plasmas and 91 blood sera from infertile patients which were divided into groups on the basis of the results of standard semen analysis: teratozoospermic (T), asthenoteratozoospermic (AT), oligoasthernoteratozoospermic (OAT) and normozoospermic (N). CLU concentrations were determined using ELISA method. Fucose expression was checked

in the modified lectin-ELISA test with biotinylated lectins specific to fucose (*Lotus tetragonolobus* agglutinin - LTA, *Ulex europaeus* agglutin - UEA, *Lens culinaris* agglutinin - LCA). The expression of Lewis oligosaccharide structures was checked by determining of fucosyltransferases 3 and 4 concentrations (FUT3 and FUT4, respectively) by ELISA test. Sialic acid expression was checked in the modified lectin-ELISA test with biotinylated lectins specific to sialic acid (*Sambucus nigra* agglutinin - SNA, *Maackia amurensis* agglutinin - MAA). The concentrations of SIRT3 and SIRT5 were determined using ELISA method. The concentrations of TAS, FRAP, AOPP, iron, uric acid, calcium and magnesium were determined using colorimetric methods. Statistical analysis of the obtained results was carried out using the Statistica 13.3 PL software, as significant considering the results for which the *p* values were lower than 0.05.

Results: Seminal plasma CLU concentrations were significantly higher in the OAT group in comparison to the other examined groups of infertile men. Serum CLU concentrations were significantly lower in the OAT group in comparison to the other examined groups. Seminal plasma FUT3 concentrations were significantly higher in the T group in comparison to the N and AT as well as these concentrations were significantly higher in the OAT group in comparison to the AT and N group. Seminal plasma FUT4 concentrations were significantly higher in the infertile N group in comparison to the T group and significantly higher in the AT group in comparison to the T group. Relative reactivities of serum CLU glycans with fucose-specific lectin UEA were significantly higher in the N group in comparison to the other examined groups. Relative reactivities of serum CLU glycans with core fucose-specific lectin LCA were also significantly higher in the N group in comparison to the other examined groups. Moreover, this parameter was significantly higher in the T group in comparison to the OAT and AT group. ROC curve analysis as well as cluster analysis enabled the selection the panel of parameters, useful in the differentiation the groups of patients with sperm cells disorders. For seminal plasma there were: concentrations of CLU, FUT3 and FUT4, whereas for serum there were: concentrations of CLU and FUT4, relative reactivities of CLU glycans with UEA and LCA. The relative reactivities of seminal plasma CLU glycans with sialic acid specific lectin MAA were significantly lower in the OAT group in comparison to the other examined groups, whereas the relative reactivities of serum

CLU glycans with sialic acid specific lectin SNA were significantly higher in the OAT group in comparison to the other examined groups. Seminal plasma SIRT3 concentrations were significantly higher in the N group in comparison to the T and OAT groups as well as the concentrations of SIRT3 in seminal plasmas were significantly higher in the AT group in comparison to the T and OAT groups. Serum SIRT3 concentrations were significantly lower in the N group in comparison to the AT and OAT groups. Seminal plasma SIRT5 concentrations were significantly lower in the OAT group in comparison to the other examined groups. Serum FRAP concentrations in the N group were significantly higher in comparison to the AT group. Based on the ROC curve analysis as well as cluster analysis, the panel of following seminal plasma parameters enabling the differentiation of OAT group from other analyzed groups was proposed: relative reactivities of CLU glycans with sialic acid specific lectin MAA, sialylation ratio, SIRT3, SIRT5 and FRAP concentrations. ROC curve analysis and cluster analysis enabled the selection of panel of serum parameters useful in the differentiation of infertile normozoospermic men from patients with abnormalities in the results of standard semen analysis. There were: relative reactivities of serum CLU glycans with sialic acid specific lectins SNA and MAA, SIRT3 and FRAP concentrations. Seminal plasma AOPP concentrations in the N group were significantly higher in comparison to the AT and OAT groups as well as the AOPP concentrations were significantly higher in the T group in comparison to the AT and OAT groups. Seminal plasma total protein concentrations were significantly lower in the T group in comparison the AT and OAT groups, while iron concentrations were significantly lower in the OAT group in comparison to the T and N groups. The ROC curve analysis showed that the determination of AOPP concentrations in seminal plasmas may constitute an additional parameter differentiating infertile normozoospermic men from patients with sperm motility and morphology disorders, and may also be a promising parameter differentiating men with sperm motility disorders from those with other types of disorders regarding sperm.

Conclusions: The results of conducted studies considerably contributed to the extending the knowledge concerning the glycosylation of clusterin present in seminal plasmas and sera of infertile men with sperm abnormalities. The differences observed in the expression or concentrations of analyzed parameters suggest that sperm count, motility and morphology disorders are not the only cause of male infertility.

The differences in the seminal plasma CLU, FUT3 and FUT4 concentrations may be associated with the lowered sperm count. The differences in the serum CLU concentrations, the expression of core fucose and this linked via α1,2-glycosidic bound to antennary galactose of CLU glycans may constitute additional markers differentiating the normozoospermic infertile men from patients with abnormal sperm count, motility and morphology. Relative reactivities of serum CLU glycans with SNA may be used as an additional male infertility biomarker, differentiating infertile normozoospermic patients from men with sperm abnormalities with a high sensitivity and specificity. AOPP concentrations determined in seminal plasmas may be a promising parameter differentiating men with sperm motility disorders from those with other sperm abnormalities, while changes in total protein concentrations in this biological fluid may be related to sperm morphology disorders. In addition, decreased seminal plasma iron concentrations correlated positively with reduced sperm count. Expanding the spectrum of research with a normozoospermic group of men with proven fertility in the future will give a chance to select biomarkers related to idiopathic male infertility.

11. Piśmiennictwo

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13. Wykaz skrótów

AOPP	zaawansowane produkty utleniania białek, *ang. advanced oxidation protein products*
AT	astenoteratozoospermia
AU	jednostka absorbancji, *ang. absorbance unit*
AUC	pole powierzchni pod krzywą ROC, *ang. area under the curve*
CLU	klasteryna, *ang. clusterin*
CRP	białko C-reaktywne, *ang. C-reactive protein*
ELISA	immunoenzymatyczny test fazy stałej, *ang. enzyme-linked immunosorbent assay*
FRAP	zdolność do redukcji jonów żelaza, *ang. ferric reducing antioxidant power*
FUT3	fukozylotransferaza 3, *ang. fucosyltransferase 3*
FUT4	fukozylotransferaza 4, *ang. fucosyltransferase 4*
Gal	galaktoza
GalNAc	N-acetylogalaktozoamina
GlcNAc	N-acetyloglukozoamina
LCA	*Lens culinaris* agglutinin – lektyna z soczewicy jadalnej
LTA	*Lotus tetragonolobus* agglutinin – lektyna z głąbigroszku szkarłatnego
MAA	*Maackia amurensis* agglutinin – lektyna z makii amurskiej
MAC	kompleks atakujący błonę, *ang. membrane attack complex*
N	normozoospermia
nCLU	jądrowa forma klasteryny, *ang. nuclear clusterin*
OAT	oligoastenoteratozoospermia
OS	stres oksydacyjny, *ang. oxidative stress*
ROC	krzywa oceny jakości klasyfikatora, *ang. receiver operating characteristic*
SA	kwas sjalowy, *ang. sialic acid*
sCLU	wydzielnicza forma klasteryny, *ang. secretory clusterin*
SD	odchylenie standardowe, *ang. standard deviation*
SIRT3	sirtuina 3, *ang. sirtuin 3*
SIRT5	sirtuina 5, *ang. sirtuin 5*

SNA	*Sambucus nigra* agglutinin – lektyna z bzu czarnego
T	teratozoospermia
TAS	całkowity status antyoksydacyjny, *ang. Total Antioxidant Status*
TPTZ	2,4,6-tripirydylo-S-triazyna
UEA	*Ulex europaeus* agglutinin – lektyna z kolcolistu zachodniego
WHO	Światowa Organizacja Zdrowia, *ang. World Health Organization*

14. Spis rysunków

15. Spis załączników

Załącznik 1 – artykuł przeglądowy:

Janiszewska, E.; Kratz, E.M. Could the glycosylation analysis of seminal plasma clusterin become a novel male infertility biomarker? Mol. Reprod. Dev. 2020, 87, 515–524, doi:10.1002/mrd.23340.

Załącznik 2 – I artykuł oryginalny:

Janiszewska, E.; Kokot, I.; Gilowska, I.; Faundez, R.; Kratz, E.M. The possible association of clusterin fucosylation changes with male fertility disorders. Sci. Rep. 2021, 11, 1–16, doi:10.1038/s41598-021-95288-w.

Załącznik 3 – II artykuł oryginalny:

Janiszewska, E.; Kokot, I.; Kmieciak, A.; Stelmasiak, Z.; Gilowska, I.; Faundez, R.; Kratz, E.M. The Association between Clusterin Sialylation Degree and Levels of Oxidative–Antioxidant Balance Markers in Seminal Plasmas and Blood Sera of Male Partners with Abnormal Sperm Parameters. Int. J. Mol. Sci. 2022, 23, doi:10.3390/ijms231810598.

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Janiszewska, E.; Kokot, I.; Kmieciak, A.; Gilowska, I.; Faundez, R.; Kratz, E.M. Are There Associations between Seminal Plasma Advanced Oxidation Protein Products and Selected Redox-Associated Biochemical Parameters in Infertile Male Patients? A Preliminary Report. Cells 2022, 11, 3667, doi:10.3390/cells11223667.

Załącznik 5 – Całkowity dorobek naukowy

Załącznik 6 – Oświadczenia współautorów publikacji wchodzących w skład cyklu

15.1. **Załącznik 1 –** artykuł przeglądowy:

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REVIEW ARTICLE

Could the glycosylation analysis of seminal plasma clusterin become a novel male infertility biomarker?

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Abstract

Male infertility is becoming a rapidly growing problem around the world, mainly in the highly developed countries. Seminal proteome composition seems to be one of the crucial factors of the proper course of fertilization - clusterin (CLU) is among the most important ones. CLU, as one of the crucial seminal plasma glycoproteins, plays a very important role in sperm capacitation and immune tolerance in the female reproductive tract. CLU is also known as a sensitive marker of oxidative stress. It has six N-glycosylation sites and also exhibits chaperone activity. An analysis of changes in the profile and degree of CLU glycosylation may shed some new light on the molecular mechanisms of the fertilization process and may be used as an additional diagnostic marker of male fertility. This study constitutes a review of the recently available literature concerning human seminal CLU, including changes in its glycosylation, analyzed in the context of human reproduction.

KEYWORDS
clusterin, male fertility, semen quality, seminal clusterin glycosylation

1 | INTRODUCTION

Infertility is becoming an increasing problem of people around the world, mainly in the highly developed countries. The World Health Organization (WHO) defines infertility as a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (WHO, 2010). Approximately, 8–12% of reproductive-aged couples worldwide are unsuccessfully trying to have children, among which the male factor alone contributes to about 50% cases of childlessness (Inhorn & Patrizio, 2015; Mascarenhas, Flaxman, Boerma, Vanderpoel, & Stevens, 2012; Ombelet, Cooke, Dyer, Serour, & Devroey, 2008). Standard semen analysis is one of the primary tests commonly performed worldwide; it classifies sperm on the basis of its features (e.g., ejaculate pH and volume, spermatozoa count, motility, and morphology). Decreased sperm cells count (oligozoospermia), abnormal morphology (teratozoospermia), or lowered motility (asthenozoospermia) are the only clinical hints of male fertility. However, the standard ejaculate analysis often does not suffice to determine the actual cause of male infertility. Nearly, 15% of male infertility cases are regarded as idiopathic, and semen analysis conducted in these cases results in no pathological values (Ferens-Sieczkowska, Kowalska, & Kratz, 2013). In this regard, there is still a need to find new biomarkers of male infertility. Several years ago, Lewis concluded that international collaborations should be initiated to develop clinically relevant molecular and functional tests with agreed protocols and clinical thresholds as a matter of urgency; however, the problem of more detailed male fertility diagnostics is not completely solved, and, therefore, is still actual (Lewis, 2007).

The fertilization process is very complex and depends on many factors, from the general health status, through reproductive organs' formation and gametogenesis, to a multitude of biochemical reactions in the female reproductive tract, with a relevant role played by seminal plasma glycoproteins (Koperwas & Głowacka, 2017; Kratz & Achcińska, 2011). Semen is an organic fluid consisting of testicular components, including spermatozoa (5%) and seminal plasma, which is a product of male accessory sex glands (95%). Seminal plasma consists of many various glycoproteins, lipids, and inorganic ions, and plays a crucial role in the natural fertilization process, taking part in capacitation, acrosome reaction, maternal immune response modulation, and oocyte–sperm interaction (Milardi et al., 2012).

Proteomic analysis is a good tool, valuable in identifying seminal plasma compounds, describing their structure and alterations at the molecular level in many various pathological states. Conventional methods, such as two-dimensional gel electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, or mass spectrometry (MS), make it possible to identify larger proteins. Advanced technologies (e.g., matrix-assisted laser desorption ionization-time of flight, liquid chromatography–MS techniques) have an increased sensitivity to the detection of many biological fluid compounds (Selvam & Agarwal, 2018). The recently observable technological development has enabled the progress of scientific studies in the field.

Our review is based on literature research of approximately 3,000 entries, with the use of the PubMed and Google Scholar databases. It includes the recent data, published from 1990 to present, mostly in English, and was conducted using the following search terms or their combinations: clusterin (CLU), seminal plasma CLU, seminal CLU glycosylation, CLU glycosylation, oxidative stress, seminal plasma compounds, male fertility, and semen quality. This study included research on human and some animal models. Finally, 67 items from original papers and reviews, which in our opinion seemed to be most useful for our analysis, were selected. This study constitutes a review of the recently available literature concerning human seminal CLU, paying special attention to changes in its glycosylation, analyzed in the context of human reproduction. To emphasize the role of changes in the glycosylation profile and degree of human semen plasma glycoproteins, we briefly discussed some of them, which further indicates the need to better understand the role of CLU glycans in the process of human procreation, particularly in the context of male fertility.

2 | GLYCOSYLATION CHANGES IN SELECTED SEMINAL PLASMA GLYCOPROTEINS

The problem of seminal plasma proteins was a subject of scientific research over a century ago. The first publication concerning seminal plasma proteins comes from 1888, and was written by Posner (1888). In 1942, the first electrophoretic separation of seminal plasma proteins was conducted. Ross, Moore, and Miller (1942) found four peptide fractions (discussed in the study conducted by Samanta, Parida, Dias, & Agarwal, 2018). Nowadays, it is possible to identify a much greater number of proteins. Drabovich, Saraon, Jarvi, and Diamandis (2014) distinguished almost 3,200 proteins present in seminal plasma, which constitutes probably the largest library of seminal plasma proteins. One of the most important groups of these compounds are glycoproteins, which contain at least one covalently attached oligosaccharide chain in their structure (Przybyło, 1998). Protein glycosylation has a great impact on protein function and is of crucial importance (Drake et al., 2010; Larkin & Imperiali, 2011; Varki & Lowe, 2009) due to the fact that many adhesive properties of cells result from the protein–carbohydrate interaction, and are crucial in the species-specific recognition of the oocyte and sperm cell. Oligosaccharide structures, especially Lewis^x (Le^x), present on the surface of the reproductive cells, are particularly important in the course of gamete fusion (Pang et al., 2011; Wassarman, 2011). One of the functions of the glycan part of glycoproteins is protecting them from proteolytic fragmentation, therefore any changes in the oligosaccharide structures, as well as the degree of their expression, are crucial for the functioning and biological activity of semen proteins (Kratz, Faundez, & Katnik-Prastowska, 2011; Kątnik-Prastowska, Kratz, Faundez, & Chelmonska-Soyta, 2006).

In our previous studies, we observed changes in the glycosylation profile and the degree of some selected seminal plasma glycoproteins, which were examined in relation to the results of standard ejaculate analysis. For example, fibronectin (FN), a cell adhesion molecule, which contains only about 5–9% of carbohydrates, is present in many human body fluids including semen. After ejaculation, FN dimers accompanied by semenogelin participate in the liquefaction process of the gel structure of semen (discussed in the study by Ferens-Sieczkowska et al., 2013). Kątnik-Prastowska et al. (2006) documented that lower expression of the α-2,3-sialylated FN glycoforms and the appearance of the asialo-FN glycoforms were associated with high concentrations of FN in human seminal plasma with abnormal semen parameters. Another glycoprotein analyzed by us, one of the acute-phase proteins, the α-1-acid glycoprotein (AGP), was highly glycosylated (up to 45%). The increase in the sialyl-Lewis^x antigen in AGP can be used as a marker of genital tract inflammation, manifested by leukocytospermia (Kratz et al., 2011).

Kratz, Poland, van Dijk, and Kątnik-Prastowska (2003) also concluded that the presence of high concentrations of aberrantly glycosylated AGP molecules in seminal plasma might be indicative for a chronic inflammatory condition within the male reproductive tract. Authors documented that occurrence of high AGP concentrations in seminal plasmas of men with decreased fertility does not depend on abnormal sperm parameters or morphology and that the AGP molecules differ markedly in the composition of their glycans from seminal plasma samples with very low AGP concentrations: the higher extent of glycans branching, their lower sialylation and the presence of fucose typical for Lewis^a glycan structures were observed. This may affect glycosylation-dependent processes, like sperm-egg cell interaction and complement activation, and, thus, may contribute to the occurrence of male infertility (Kratz et al., 2003). Moreover, Poland et al. (2002) also documented an association between the high level of human seminal plasma AGP and high branching of its glycans as well as the expression of Lewis^a oligosaccharide structures. Glycosylation of seminal immunoglobulin G (IgG) and secretory immunoglobulin A (sIgA) was also investigated by us. In our studies, we have shown that the differences in the IgA secretory component (SC) sialylation are associated with fertility problems related to leukocytospermia (Kratz & Ferens-Sieczkowska, 2014). Moreover, we have also documented that the analysis of IgG and SC fucosylation status and the determination of IgG and sIgA concentrations in seminal plasma might constitute a valuable diagnostic tool for the evaluation of male infertility associated with leukocytospermia with accompanying inflammation (Kratz, Ferens-Sieczkowska, Faundez, & Kątnik-Prastowska, 2014). Olejnik, Kratz, Zimmer, and Ferens-Sieczkowska (2015) based on the results of

their study hypothesized that antennary fucosylation may be a common and important trait of seminal plasma glycoproteins, and hyperfucosylated glycans may disturb their natural course of mutual interactions. Those proteins which express the probable alterations to the highest extent, when identified, may serve as helpful glycomarkers in the examination of semen status. After the examination of infertile men seminal plasma glycome, authors concluded that oligosaccharide branching, O-glycan expression, and the sialylation extent may be considered as important features that influence maintaining sperm in the condition that enables fusion with the oocyte. According to Kratz, Kałuża, Zimmer, and Ferens-Sieczkowska (2015) opinion, glycosylation differences may be crucial, especially if they concern glycoprotein which plays a direct role in the fertilization process. Table 1 summarizes the studies on main seminal proteins glycosylation in relation to seminal disorders, typical for male infertility.

Despite many new scientific reports concerning male infertility and seminal plasma glycoproteins, a new biomarker indicating male fertility disorders is still missing. Recently, scientists have been inter alia considering implementation of CLU glycosylation in this approach.

3 | THE ROLE OF CLU GLYCOSYLATION

Glycoprotein clusterin (CLU), one of the crucial ejaculate glycoproteins, is also known as apolipoprotein J, gp80, Gp-III, or Ku70-binding protein. Glycosylation is a complex enzymatic process and many factors may affect this posttranslational modification. These factors include spatial conformation of glycosylated peptide, availability of an oligosaccharide precursor bound with a lipid carrier, accessibility, concentration of enzymes (glycosydases and glycosyltransferases), and their specificity. Both the physical and chemical properties of the intracellular environment affect those processes (Przybyło, 1999). Under physiological conditions, the glycosylation pattern is rather stable and plays a crucial role in proper oocyte-spermatozoa interaction. In all probability, the glycosylation pattern is altered in various pathological states. Thus, glycoproteomic research concerning glycosylation changes of important seminal plasma glycoprotein—CLU—seems to provide a great chance for discovering a new biomarker of male infertility (Ferens-Sieczkowska et al., 2013); we hope that it will also be helpful in understanding the molecular mechanisms accompanying the fertilization process.

TABLE 1 Glycosylation changes of selected human seminal plasma glycoproteins

Glycoprotein	Parameter of glycosylation	Seminal disorder	Reference
AGP	• N-glycans branching • Lewis^a antigen	Abnormal semen parameters	Poland et al. (2002)
	• N-glycans branching • SA α2,3/α2,6 linked	Abnormal semen parameters	Kratz et al. (2003)
	• Lewis^x antigen • Lewis^y antigen • SA α2,3/α2,6 linked	Leukocytospermia	Kratz et al. (2011)
FN	• SA α2,3/α2,6 linked	Abnormal semen parameters	Kątnik-Prastowska et al. (2006)
	• Lewis^x antigen • Lewis^y antigen • SA α2,3/α2,6 linked	Leukocytospermia	Kratz et al. (2011)
sIgA, SC	• SA α2,3/α2,6 linked	Leukocytospermia	Kratz and Ferens-Sieczkowska (2014)
	• Lewis^x antigen • Lewis^y antigen • Core Fuc	Leukocytospermia	Kratz et al. (2014)
IgG	• Lewis^x antigen • Lewis^y antigen • Core Fuc	Leukocytospermia	Kratz et al. (2014)
Panel of glycoproteins: FN, IgG, PSA, hCG, PAP, ATIII, TF, α1-AT, AGP, and GdS	• Lewis^x antigen • Core Fuc	Abnormal semen parameters	Olejnik et al. (2015)
Seminal plasma glycome	• SA α2,3/α2,6 linked • Complete and truncated to single GalNAc (Tn antigen) forms of O-glycans • N-glycans branching	Abnormal semen parameters	Kratz et al. (2015)

Abbreviations: α1-AT, α1-antitrypsin; AGP, α1-acid glycoprotein; ATIII: antithrombin III; FN, fibronectin; Fuc, fucose; GalNAc, N-acetylgalactosamine; GdS, glycodelin S; hCG, human chorionic gonadotropin; IgG, immunoglobulin G; PAP, prostatic acid phosphatase; PSA, prostate specific antigen; SA, sialic acid; SC, secretory component of sIgA; sIgA, secretory immunoglobulin A; TF, transferrin.

CLU has six possible N-glycosylation sites. Saraswat et al. (2016) carried out extensive research concerning the presence and structure of human seminal plasma glycoproteins. In the report, the authors showed that CLU had four N-glycosylation sites. Interestingly, the glycoprotein also had the highest number of glycan compositions. The authors found 43 different variants of secretory clusterin (sCLU) for these N-glycosylation sites. All CLU glycans were complex type, with terminal galactoses or sialylated. They contained fucose, Lewis^x/Lewis^a, blood group H, or Lewis^y/Lewis^b oligosaccharide structures (Figure 1). In some regions of CLU, huge microheterogeneity was observed (Saraswat et al., 2016). We have not found any further data concerning glycans alterations in the seminal plasma CLU of infertile men so far.

The primary structure of CLU is very conserved—about 30% of sCLU consists of carbohydrates (Li, Zoubeidi, Beraldi, & Gleav, 2013). Glycosylation is crucial for maintaining the properties of CLU. Rohne, Prochnow, Wolf, Renner, and Koch-Brandt (2014) proved that only CLU with proper core carbohydrate structure can perform its chaperone activity. Fully deglycosylated sCLU had reduced those features down to 90%. Sabatté et al. (2011) reported that seminal CLU carried highly fucosylated Lewis^x and Lewis^y oligosaccharide structures, which are great ligands for C-type lectin receptor selectively expressed on dendritic cells (DCs) and macrophages as well (DC-specific intercellular adhesion molecule-3-grabbing nonintegrin [SIGN]). Clark et al. (2012) identified CLU as one of the endogenous ligands for immune C-type lectins, DC-SIGN in the seminal plasma of fertile men. In contrast to the studies mentioned above, the serum CLU forms were highly sialylated complex type N-glycans without the Lewis antigens. The above observations may be helpful in the prevention of sexually transmitted infectious diseases in the future. The fact that serum CLU does not demonstrate such features is interesting. Merlotti et al. (2015) proved that seminal plasma CLU plays an important role in the utilization of damaged proteins. CLU inhibits aggregation of the destroyed proteins and forwards them to the DCs through DC-SIGN. The interactions between DCs and CLU result in DCs stimulation. Merlotti et al. (2015) presumed that it might be essential in the induction of immune tolerance in the female reproductive tract.

Studies concerning the expression of seminal proteins in patients suffering from unexplained male infertility, who had undergone in vitro fertilization (Kanannejad & Gharesi-Fard, 2018), have also been conducted. A comparison between the results of successful and failed fertilization procedures showed that there were significant differences in the expression of three proteins, CLU among them. Moreover, Kanannejad and Gharesi-Fard (2018) observed that CLU was overexpressed in the semen of patients in whom in vitro fertilization was successful.

4 | CLU STRUCTURE AND FUNCTION IN THE MALE REPRODUCTIVE TRACT

The term "CLU" derives from its ability to aggregate various types of cells. CLU was first identified in 1983 in ram testis fluid (Blaschuk, Burdzy, & Fritz, 1983; Fritz, Burdzy, Setchell, & Blaschuk, 1983). There are two forms of CLU: secretory (sCLU) and nuclear (nCLU). The secretory form is ~80 kDa heterodimer and the molecular weight of nCLU is ~55 kDa. nCLU occurs in the nucleus after some cytotoxic events (Leskov, Klokov, Li, Kinsella, & Boothman, 2003; Naponelli & Bettuzzi., 2017). It should be noted that the concentration of sCLU in seminal plasma fluid is several times higher than in the serum (Jenne et al., 1991).

Synthesis of seminal sCLU takes place in the testis, epididymis, and seminal vesicles (O'Bryan et al., 1990). In humans, the sCLU gene

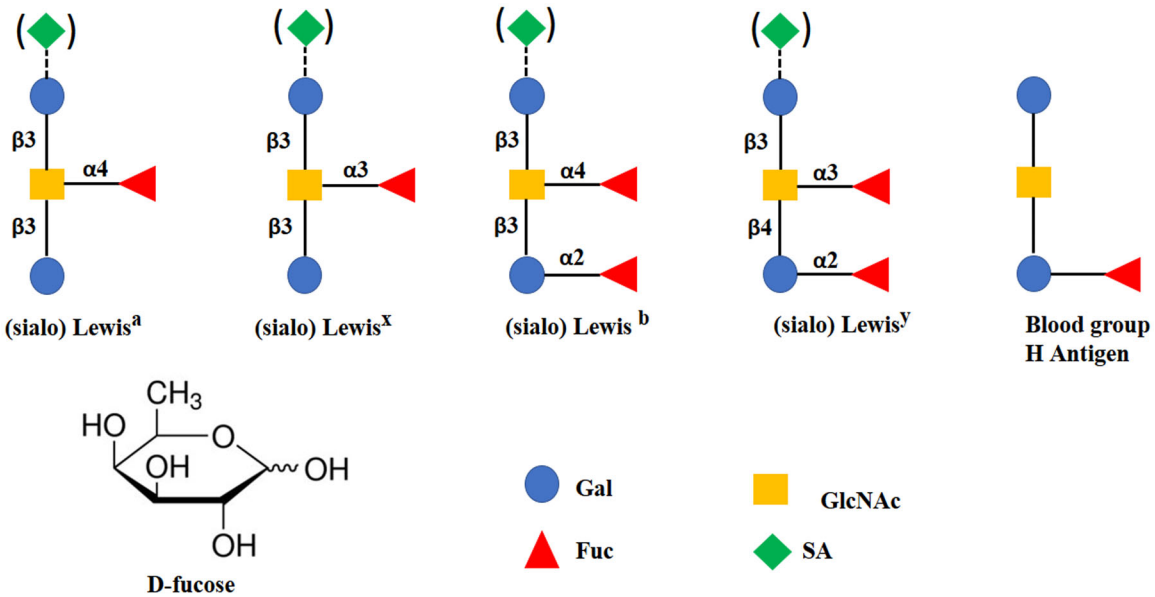

FIGURE 1 Typical oligosaccharide structures of human clusterin. On the basis of Cabezas-Cruz & de la Fuente (2017) and Magalhães, Ismail, & Reis (2010). Fuc, fucose; Gal, galactose; GlcNAc, N-acetylglucosamine; SA, sialic acid

is located in chromosome 8, and its structure is well preserved, ordered into nine exons and eight introns. The precursor of sCLU (pre-sCLU) is targeted to the endoplasmic reticulum by an initial 22 amino acids leader peptide. Sixty kilodalton pre-sCLU is then proteolytically cleaved into α- and β-subunits and further glycosylated, forming mature disulfide-linked heterodimeric secretory CLU. The α and β subunits have three and four N-glycosylation sites, respectively; however, an in vitro observation proved a maximum of six N-glycosylation sites in the whole molecule of the protein (Li et al., 2013; Figure 2). The most important functions of CLU are summarized in Figure 3.

Matukumalli, Tangirala, and Rao (2017) proved that the properties of CLU differed depending on its subcellular localization. The authors documented that CLU composed of both (α and β) subunits lowered the lipid levels, whereas β-chains caused intracellular lipid accumulation two times higher in comparison to the controls. The α-subunit did not present those features. Moreover, the weight of rabbits treated with β-chains of CLU increased by 40% compared to the control, despite no change in the eating habits (Matukumalli et al., 2017).

There are several scientific reports concerning the role of CLU as a sensitive oxidative stress biomarker. This is particularly important because sperm DNA oxidation damage constitutes one of the causes of infertility. CLU chaperone function is similar to function of small heat shock proteins (HSPs) due to its ability to bind unfolded proteins and inhibit their aggregation independently from the presence of ATP. Moreover, CLU contains structures called "molten-like globule" domains which are natively disorganized and probably contain amphipathic α-helices. It enables them to bind a number of various molecules. CLU takes part in the identification of damaged proteins and their disposal through endocytosis (Trougakos, 2013).

CLU is known as an important factor determining the invasiveness of many neoplasms, and the increased sCLU level enhances tumor cell proliferation via gelatinase A (metalloproteinase-2, MMP-2) and E-cadherin interactions (Zhong et al., 2018). It seems very likely that the CLU present in the male ejaculate may also interact with the gelatinases in it. The presented information seems to be significant, taking into consideration the fact that MMP-2 and MMP-9 (MMP-9 and gelatinase B), present in the seminal plasma, are crucial for proper semen liquefaction after ejaculation. Extended exposure to oxidative agents (i.e., reactive oxygen species, ionizing radiation) causes an increase in MMPs activity, altering the balance between MMPs and their tissue inhibitors (tissue inhibitors of MMPs [TIMPs]; Kratz & Piwowar, 2017). Tentes et al. (2007) detected an elevated concentration of inactive proenzyme form of MMP-9 in the seminal plasma of men with the lowered sperm concentration. In our previous study (Kratz et al., 2016), we observed an elevated MMP-9 concentration in the seminal plasma of infertile men and an increased MMP-9/TIMP1 ratio. Accordingly, the evaluation of associations between the seminal plasma sCLU, MMPs, and oxidative stress parameters appears to be another promising direction in male infertility research; however, this interesting possible association must be confirmed in the future study.

Zalata, El-Samanoudy, Shaalan, El-Baiomy, and Mostafa (2015) raised the issue of the harmful effects of cell phone radiation on the quality of human sperm. Due to the antioxidative features of CLU, the expression of seminal CLU gene and protein concentration was tested under normal conditions, and after 1 hr of exposure to cell phone radiation. Sperm motility, acrosin activity, and sperm DNA fragmentation were also assessed before and after the exposure. Cell phone radiation significantly increased not only the concentration of

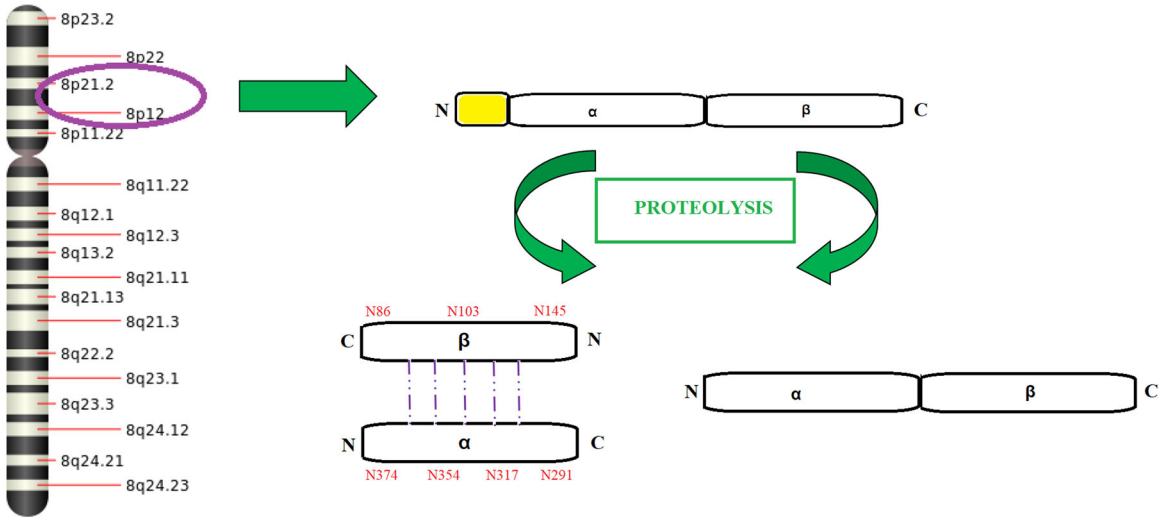

FIGURE 2 Schematic representation of human clusterin formation. The CLU gene is located in chromosome 8 (both particular loci are marked by a purple ellipse). The product of translation is a precursor protein consisting of 449 amino acids, including 22 amino acids leader peptide (marked yellow). As a result of proteolysis, the leader peptide is cleaved. Secretory form of CLU (sCLU) is rearranged in the golgi apparatus and endoplasmic reticulum. The mature form of clusterin consists of two subunits (α and β) combined by five disulfide bounds (purple lines). The nuclear form of CLU does not undergo the cleavage process, and consists of two subunits without disulfide bounds. N-glycosylation sites of sCLU are labeled in red. On the basis of Genetics Home Reference; Park, Mathis, and Lee (2014). CLU, clusterin

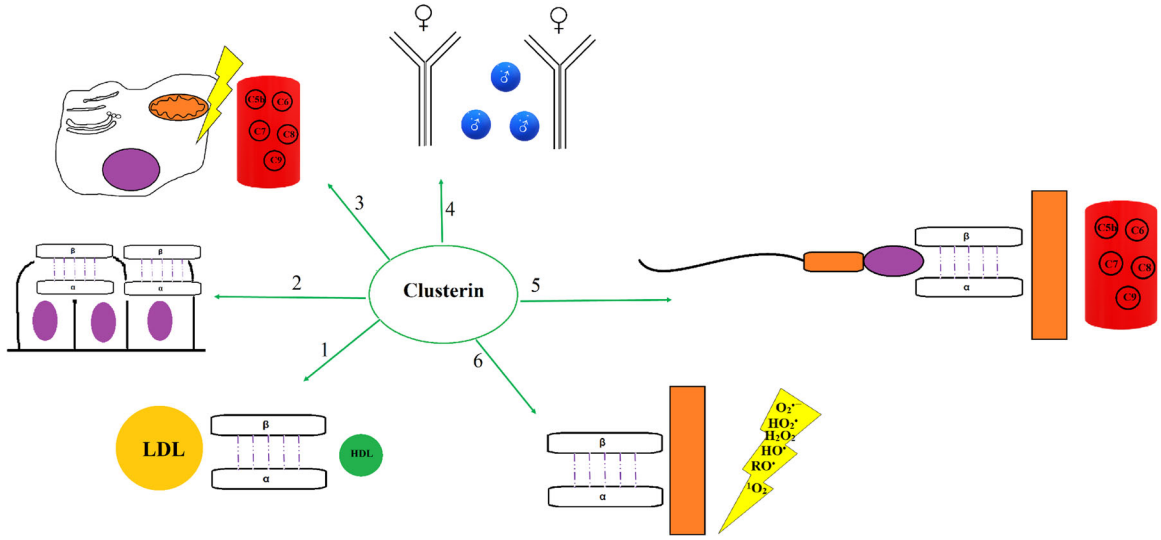

FIGURE 3 Schematic representation of the main CLU functions: 1—lipid transportation; 2—cell adhesion and cytoprotection; 3—cell damage induced by membrane attack complex (MAC), consisting of complement proteins known as C5b, C6, C7, and C8, inhibition; 4—immune tolerance for male antigens in the female reproductive tract induction; 5—sperm cells protection from the MAC; and 6—oxidative stress regulation (via chaperone activity). The main forms of reactive oxygen species are highlighted in yellow. On the basis of Jones and Jomary (2002) and Rohne, Prochnow, and Koch-Brandt (2016). HDL, high-density lipoprotein; LDL, low-density lipoprotein

CLU, but also the expression of the CLU gene. Moreover, sperm cell parameters verified before the beginning of the experiment were considerably worse than those of the samples not exposed to the radiation (Zalata et al., 2015).

5 | CLU CONCENTRATION IN SEMINAL PLASMA

The exact function of seminal CLU is not known well yet. Fukuda, Miyake, Enatsu, Matsushita, and Fujisawa (2016) proved that the concentration of sCLU in the seminal plasma fluid in infertile patients is significantly decreased in comparison to healthy normozoospermic men (47.9 ± 20.9 ng/mL). Moreover, the level of sCLU in non-obstructive azoospermic men was lower (18.4 ± 9.5 ng/mL) than in oligozoospermic patients (28.2 ± 8.6 ng/mL; Fukuda et al., 2016). It is consistent with the experiment conducted by Salehi et al. (2013), in which the authors demonstrated a negative correlation between the CLU concentration (sCLU in the infertile group: 14.48 ± 9.74 ng/mL vs. sCLU in group of fertile men: 48.31 ± 38.59 ng/mL) and abnormal morphology of spermatozoa, protamine insufficiency, and DNA fragmentation. However, certain papers contradict the ones mentioned above. Zalata et al. (2012) found that the expression of CLU RNA and the CLU gene was higher in the seminal plasma of infertile men. Zalata et al. (2012) showed that it correlated negatively with, inter alia, sperm count, motility, linear velocity, and significantly correlated positively with the percentage of abnormal forms of spermatozoa and sperm DNA fragmentation. Hashemitabar, Sabbagh, Orazizadeh, Ghadiri, and Bahmanzadeh (2015) compared the concentration of sperm tail proteins in normozoospermic and astenozoospermic men. The experiment showed increased levels of 14 proteins in astenozoospermic patients, CLU among them. Martinez-Heredia, de Mateo, Vidal-Taboada, Ballesca, and Oliva (2008) found that the expression of CLU precursor was increased in the ejaculates of astenozoospermic patients. Moreover, Muciaccia et al. (2012) proved that there was a significant difference between semen CLU immunolabeling in hypertensive men and those without elevated blood pressure. The proportion of abnormal spermatozoa labeled with CLU was elevated in hypertensive men in comparison with healthy donors. Standard semen analysis did not show abnormal results in both groups of patients. Interestingly, in the hypertensive group of patients, there was no correlation between patients' age and sperm cell mortality, whereas there was a statistically significant correlation with sperm DNA fragmentation. This direction of research may be important, taking into account the fact that hypertension also constitutes a very common problem, not only among older people but also, increasingly frequently, among those of childbearing potential (Muciaccia et al., 2012).

Han et al. (2012) investigated whether the CLU occurred in normal human spermatozoa. It had been assumed that this glycoprotein was indeed present only in pathological sperm cells; however, CLU was also found in its native structure in the inner plasma membrane of normal human spermatozoa. Han et al. (2012) concluded that there was no significant difference between the structure of the CLU present in normal and abnormal spermatozoa. Cui, Sharma, and Agarwal (2016) documented that CLU expression in semen was higher at the early stages of the sperm maturation process. An increased CLU level at later stages of sperm development suggests its abnormal ripening and corresponds to the decreased sperm aggregation and motility, causing infertility. Cui et al. (2016)

TABLE 2 A general overview of the main relationships between human and animal seminal clusterin expression and seminal parameters and/or fertility disorders

In human			
Medical issue	**CLU expression**	**Seminal parameter**	**Reference**
Cell phone radiation for one hour	• Increased CLU concentration • Increased CLU gene expression	• Increased sperm DNA fragmentation • Reduced sperm motility	Zalata et al. (2015)
Male infertility	• Decreased CLU concentration	• Increased percentage of abnormal spermatozoa • Sperm protamine insufficiency • Increased DNA fragmentation	Salehi et al. (2013)
Male infertility	• Increased CLU RNA expression • Increased CLU gene expression	• Decreased sperm count, motility, and linear velocity • Increased sperm DNA fragmentation • Increased percentage of abnormal spermatozoa	Zalata et al. (2012)
Male infertility and hypertension	• Different CLU immunolabeling in the group of hypertensive men	• Increased abnormal spermatozoa count labeled by CLU • Age-dependent increased sperm DNA fragmentation	Muciaccia et al. (2012)
Stages of sperm cell maturation in fertile men	• Increasing CLU expression at the early stages of sperm cells maturation • Decreased CLU expression at the later stage of sperm cells maturation	• Seminal parameters within reference ranges according to the WHO (2010) criteria	Cui et al. (2016)
Unexplained infertile men undergoing successful in vitro fertilization	• Overexpression of seminal CLU determined by MS (1.5 times higher in comparison with the unsuccessful IVF)	• Statistically significant lower sperm concentration (within reference ranges according to the WHO (2010) criteria)	Kanannejad and Gharesi-Fard (2018)
Successful fertilization	• sCLU as a transporting factor for sperm adhesion molecule 1 (SPAM1)	• Proper maturity of sperm	Griffiths et al. (2009)
In animals			
Veterinary issue	**CLU expression**	**Seminal parameter**	**Reference**
Stallion			
Successful fertilization	• Increased seminal CLU concentration	• Increased sperm and spermatozoa abnormalities	Griffin et al. (2019)
Bull			
Successful fertilization	• Clusterin-positive sperm	• Decreased pre- and postfreeze spermatozoa motility • Increased sperm and spermatozoa abnormalities	Ibrahim et al. (2000)
Boar			
Successful fertilization	• Very low concentration of CLU mRNA	• Normal spermatozoa	Zannoni et al. (2017)
Mouse			
Successful fertilization	• sCLU as a transporting factor for SPAM1	• Proper maturity of sperm	Griffiths et al. (2009)

Abbreviations: CLU, clusterin; IVF, in vitro fertilization; mRNA, messenger RNA; MS, mass spectrum; sCLU, secretory clusterin.

also suggested that seminal plasma proteins in infertile men can be modified or altered at different stages of sperm maturation.

There are several articles concerning the role of CLU in the fertilization processes in animals. Stallion CLU is the second major sperm protein. Inverse correlation between the sCLU concentration and the spermatozoa quality was observed and there was a direct association between the seminal CLU concentration and the percentage of abnormalities observed in sperm (Griffin, Baker, Aitken, Swegen, & Gibb, 2019). Ibrahim, Gilbert, Loseth, and Crabo (2000) used immunostaining for CLU detection in bull sperm. CLU-positive sperm correlated negatively with the pre- and postfreeze spermatozoa motility; the researchers also estimated a relative conception rate, which is a valuable tool for fertility determination. Just like in the case of stallion fertility, a positive correlation between CLU-positive sperm and spermatozoa abnormalities were observed. Zannoni et al. (2017) explored the presence of HSP transcripts, inter alia CLU, in the spermatozoa of boar with proven fertility. The concentration of CLU messenger RNA was very low in comparison with

the other studied proteins, for example, the HSP70 and HSP90. The results of western blot analysis demonstrated the presence of CLU only in the positive control, no signal was observed in the normal spermatozoa samples (Zannoni et al., 2017). Griffiths, Galileo, Aravindan, and Martin-DeLeon (2009) investigated the role of sCLU as a transporting factor for sperm adhesion molecule 1 (SPAM1) which is considered to be the maturity marker of sperm. The results of the experiment (conducted on a mice model and in human sperm) clearly indicate that CLU is crucial for transporting SPAM1 from the luminal fluids to the spermatozoa plasma membrane (Griffiths et al., 2009).

Considering the research results discussed in our work on the multidirectional activity and expression of CLU in both human and animal ejaculates, the role of CLU in human reproduction and its effect on male fertility seem obvious. In summary, the CLU present in both human and animal semen effects: increased sperm DNA fragmentation, increased percentage of abnormal spermatozoa, decreased sperm count, motility and linear velocity, their proper maturity, and sperm protamine insufficiency. Although not all mechanisms of this glycoprotein interaction, for example, on sperm, are fully understood, taking into account the fact that many of these types of interactions, key for the proper development of male gametes and the course of the fertilization process, involve glycan part of glycoproteins, it is very likely that the participation of CLU in maintaining a man's reproductive health is also associated with this type of interaction. A general overview of the main relationships between human and animal seminal CLU expression and seminal parameters and/or fertility disorders is shown in Table 2.

6 | CONCLUSION

Many publications indicate that changes in the concentration of seminal plasma proteins may have an influence on male fertility. Several papers referring to the role of the seminal proteome in the human reproduction process have been reported. CLU and its role in the pathogenesis of many diseases of affluence, such as Alzheimer's disease, neoplasms, atherosclerosis, and ischemia, were widely described (Liang et al., 2015; Przybyło, 1999). Few studies addressed the issue of seminal plasma CLU as a potential biomarker of male infertility. The recently published papers were focusing mainly on the quantity of that protein. So far, there is little information regarding the changes in CLU glycosylation analyzed in this context. Considering the fact that the glycosylation process is responsible for regulating crucial physiological processes and oxidative damage the DNA is one of the causes of infertility, further research in the area is needed. Analysis of seminal sCLU glycosylation in fertile and infertile men seem very promising and may constitute a helpful diagnostic tool for millions of infertile men, and it will additionally shed more light on the issue of molecular interactions involving glycoprotein glycans participating in the fertilization process.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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The possible association of clusterin fucosylation changes with male fertility disorders

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In the seminal plasma (n = 118) and serum (n = 90) clusterin (CLU) the fucosylation and the expression of selected fucosyltransferases (FUTs) were analyzed. Samples from infertile men were divided into groups based on the results of the standard semen analysis: normozoospermic (N), teratozoospermic (T), asthenoteratozoospermic (AT) and oligoasthenoteratozoospermic (OAT). The CLU fucosylation was analyzed using lectin-ELISAs with biotinylated lectins specific to α1,3-, α1,2-linked antennary fucose, and α1,6-linked core fucose (LTA, UEA, and LCA, respectively). The concentrations of FUT3 and FUT4, reflecting the expression of Le oligosaccharide structures, were measured using ELISA tests. The differences in serum CLU and FUT4 concentrations, and in the expression of core fucose and antennary fucose α1,2-linked in CLU glycans between the N group and other groups examined suggest that the disturbances in sperm count, motility, and morphology are not the only cause of male infertility. Lack of similarities between levels of examined parameters in blood serum and seminal plasma may suggest the differences in mechanisms leading to glycoproteins glycosylation. It confirmed the observed differences in concentrations of seminal plasma CLU, FUT3, and FUT4 between the OAT group and N, T, AT groups, indicating that decreased sperm count may be related to these parameters expression. The serum CLU concentrations and expression of core fucose and fucose α1,2-linked in CLU, seem to be good markers differentiating normozoospermic men from those with abnormal sperm parameters, which was not observed for seminal plasma.

Abbreviations

WHO	World Health Organization
CLU	Clusterin
FUT3	Fucosyltransferase 3
FUT4	Fucosyltransferase 4
LTA	*Lotus tetragonolobus* agglutinin
UEA	*Ulex europaeus* agglutinin
LCA	*Lens culinaris* agglutinin
Le	Lewis oligosaccharide structures
$Le^{a/x/b/y}$	$Lewis^{a/x/b/y}$ oligosaccharide structures
ELISA	Enzyme-linked immunosorbent assay
Lectin-ELISA	ELISA test with lectins
sLe	Sialyl-Lewis oligosaccharide structures

Male infertility issue. Male infertility is a growing problem, especially among developed countries[1]. Globally, 20–30% of infertility cases are because of the male factor alone, and another 20–30% due to male and female factors[2]. In the European region, including Poland, it is estimated that nearly 15% of pairs are trying to get pregnant unsuccessfully, and the male factor alone contributes to approximately 50% of cases[3]. Routinely performed semen analysis according to the WHO criteria published in 2010 is often not enough to determine the true cause

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of male fertility problems. Some pathological values in the ejaculate are only medical hints of male fertility[4]. Moreover, seminal analysis of approximately 15 percent of infertile men result in no pathological values, and these cases are regarded as idiopathic[5,6]. It is worth noting that there is no detailed data from the past decade concerning this issue. Hence, the early specific male infertility biomarkers, including those helpful in idiopathic infertility diagnosis, are still missing. The molecular mechanisms responsible for male fertility problems are still not fully understood.

Clusterin as one of the glycoproteins present in human semen. Seminogram provides information about the spermatozoa, which makes up only 5% of the human ejaculate. The remaining 95% constitutes seminal plasma, playing an important role in the proper sperm cell production and maturation. This mixture of lipids, inorganic ions, and glycoproteins is responsible for the physiological fertilization process[7–9]. Proteomic studies reported that the seminal plasma of fertile men contains over 6000 proteins, among which the majority belongs to glycoproteins[10]. Milardi et al.[11] identified 83 proteins present in seminal plasma samples of fertile men, and one of them was clusterin (CLU, also called Apolipoprotein J, ApoJ).

Serum CLU is synthesized in most human cells existing in all body fluids and seminal plasma CLU derived from testis, epididymis and seminal vesicles[12]. Clusterin is one of the main seminal plasma glycoproteins, contains about 30% carbohydrates[13] and occurs in two main forms: secretory (sCLU, molecular weight ~ 80 kDa) and nuclear (nCLU, molecular weight ~ 55 kDa). The latter form appears in the nucleus due to some cytotoxic events; seminal plasma contains the secretory form of CLU[14,15]. Some authors maintain that clusterin concentration in seminal plasma is several times higher than in serum[12]; however, the literature data concerning this issue is unclear. The human's sCLU gene is located in chromosome 8, and its structure is well preserved, ordered into nine exons and eight introns. An initial 22 amino acids leader peptide transfers the precursor of sCLU (pre-sCLU) to the endoplasmic reticulum (ER). In subsequent stages, the 60-kDa pre-sCLU undergoes proteolysis, forming α- and β-subunits and is glycosylated, creating a mature disulfide-linked heterodimeric secretory CLU. It is believed that the α subunit has three N-glycosylation sites, and β subunit has four N-glycosylation sites; however, an in vitro observation verified a maximum of six N-glycosylation sites in the whole particle of this protein[16,17].

Clusterin performs many various functions in the human body, especially in the reproductive system. It is associated with high-density lipoproteins, takes part in lipid transport, participates in cell adhesion and cytoprotection at the tissue–fluid interfaces[18,19]. In the male reproductive tract, clusterin is engaged in the semen liquefaction through interactions with epididymal protease inhibitor (eppin) present on the spermatozoa surface[18]. It is worth noting that sperm oxidation damage is recognized as one of the causes of male infertility, and CLU is a sensitive biomarker of oxidative stress. Clusterin performs chaperone function, which is similar to the function of small heat shock proteins. It is related to the fact that CLU binds unfolded proteins and inhibits their aggregation regardless of the presence of ATP[19]. CLU is also liable for immune tolerance for male antigens in the female reproductive tract. Merlotti et al.[20] documented that seminal CLU is a novel DC-SIGN (dendritic cell-specific ICAM-3-grabbing nonintegrin) ligand and can perform its role properly only in the highly fucosylated form. They hypothesized that taking into account the fact that female tolerance for male antigens requires *inter alia* DC's, seminal clusterin may be one of the crucial elements of female tolerance induction[20].

Protein glycosylation is one of the most common and most important posttranslational modifications affecting many molecular processes which determines protein function[21–23] since the protein-carbohydrate interaction is responsible for many of cells' adhesive properties and play a key role in the recognition between the sperm cell and oocyte[24]. Saraswat et al.[25] carried out a huge N-glycoproteomic study and distinguished fifty glycoproteins, among which the clusterin was one of the most interesting. The authors found four N-glycosylation sites for this glycoprotein and emphasized that CLU had the highest glycan content. The authors proposed 43 various glyco-variants of clusterin; all of them were the complex type with terminal sialic acid or galactose. CLU glycans contained fucose, Le^x/Le^a, blood group H or Le^y/Le^b oligosaccharide structures[25]. Considering that fucose is one of the most important glyco-element of seminal clusterin, comprehensive studies concerning CLU fucosylation may contribute to the knowledge of the role of fucosylated glycans in the development of male infertility. Our aim was to check a clusterin fucosylation profile and degree in seminal plamas and blood sera of infertile men using a modified semi-quantitative lectin-ELISA assay. Moreover, we would like to check if there are differences in the profile of fucosylation and fucose expression in clusterin glycoepitopes between groups of examined patients, as well as between seminal plasma and blood serum of infertile men.

Patient samples

Seminal plasma and serum samples were collected from infertile male patients attending the Clinical Center of Gynecology, Obstetrics, and Neonatology in Opole (Poland) and Fertility Clinics InviMed in Warsaw (Poland). Each patient gave informed consent for this study. The study was conducted according to the guidelines of the 2nd Declaration of Helsinki and approved by the Bioethics Committee of Wroclaw Medical University (No. KB 549/2019 and No. KB 707/2019).

The ejaculates were collected by masturbation into sterile containers after 3–5 days of sexual abstinence. After liquefaction (maximum 60 min at 37 °C), standard semen analysis was carried out using computer-assisted sperm analysis (total number of sperm in ejaculate, sperm concentration, total motility, progressive motility, and morphology), SCA Motility and Concentration, software version 6.5.0.5. (Microptic SL, Barcelona, Spain) and manually (semen volume, pH and viability). All input data in this method were consistent with current WHO semen analysis criteria. Ejaculates were centrifuged at 3500×*g* for 10 min at room temperature, aliquoted, and frozen at − 86 °C until use. Serum samples were obtained by peripheral blood collection and after coagulation centrifuged at 2000×*g* for 10 min at room temperature. Serum aliquots were also stored at − 86 °C until examination. None of the serum samples were haemolysed.

Based on the standard semen analysis (sperm concentration, progressive motility, morphology of spermatozoa), seminal samples (n = 118) were divided into groups: asthenoteratozoospermic (AT, n = 22; ≥ 32% of sperm had abnormal progression and lower than 4% of spermatozoa had normal morphology), oligoasthenoteratozoospermic (OAT, n = 29; sperm count lower than 15×10^6 mL^{-1}, ≥ 32% of sperm had abnormal progression and lower than 4% of spermatozoa had normal morphology), teratozoospermic (T, n = 38; lower than 4% of spermatozoa had normal morphology) and normozoospermic (N, n = 29, normal values of ejaculate parameters). Corresponding blood serum samples (n = 90) were divided into asthenoteratozoospermic (AT, n = 16), oligoasthenoteratozoospermic (OAT, n = 27), teratozoospermic (T, n = 31) and normozoospermic (N, n = 16) groups. In the normozoospermic ejaculates, the concentration of spermatozoa was higher than 15×10^6 mL^{-1}, and > 4% of sperm exhibited normal morphology, with total motility of ≥ 40% or progressive motility ≥ 32% (0.5 h after ejaculation). None of the seminal samples were leukospermic.

Methods

Clusterin concentration. Seminal plasma and blood serum clusterin concentrations were determined using Human Clusterin ELISA kit from Bioassay Technology Laboratory (catalog No. E1189Hu; Shanghai, China) and Human Clusterin Elisa Kit from Invitrogen (ThermoFisher Scientific, catalog No. EHCLU; Frederick, USA), respectively, according to the manufacturer instructions, without any modifications. The coefficients of variations (CV%) for both tests were defined by the manufacturers. The intra-assay CV% for seminal plasma CLU ELISA kit was defined as < 8% and inter-assay CV% was ranged by < 10%. The intra-assay CV% for blood serum CLU ELISA kit was ranged by < 10% and inter-assay CV% was defined as < 12%.

Fucosyltransferases concentration. FUT-3 concentration was measured using Human Galactoside 3(4)-L-Fucosyltransferase ELISA Kit from Bioassay Technology Laboratory (catalog No. E4361Hu; Shanghai, China), and FUT-4 concentration was measured using Human Fucosyltransferase 4 ELISA kit from Bioassay Technology Laboratory (catalog No. E4612Hu; Shanghai, China) according to the manufacturer instructions, without any modifications. The coefficients of variations (CV%) for both tests were defined by the manufacturers. The intra-assay coefficients of variations were defined as < 8% and inter-assay coefficients of variations ranged by < 10% for both tests. Levels of these enzymes correspond to the expression of $Le^{a/x/b/y}$/$sLe^{a/x}$ and Le^x/sLe^x oligosaccharide structures, respectively[26,27].

Determination of fucose expression in the seminal plasma and blood serum clusterin. Three biotinylated fucose-specific lectins: *Lotus tetragonolobus* agglutinin (LTA, catalog No. B-1325, Vector Laboratories Inc., Burlingame, CA, USA), *Ulex europaeus* agglutinin (UEA, catalog No. B-1065, Vector Laboratories Inc., Burlingame, CA, USA) and *Lens culinaris* agglutinin (LCA, catalog No. B-1045, Vector Laboratories Inc., Burlingame, CA, USA) were used to determine fucose expression in the lectin-ELISA procedure according to the Kratz et al.[24] with modifications described below. The specificity of lectins is not absolute, and they can react with more than one oligosaccharide residue. *Lotus tetragonolobus* agglutinin and *Ulex europaeus* agglutinin detect fucoses linked to the galactose or antennary N-acetylglucosamine by α1,3 glycosidic bond and α1,2 glycosidic bond, respectively[28]. *Lens culinaris* agglutinin was used for core fucose detection[29].

Lectin-ELISA procedure. Schematic representation of the whole lectin-ELISA procedure was shown in Supplementary Fig. 1S.

ELISA-plate coating. ELISA plates (Nunc MaxiSorp, Thermo Fisher Scientific, Denmark) were coated with goat anti-human clusterin polyclonal antibodies (Invitrogen, Thermo Fisher Scientific, catalog No. PA1-26903; Rockford, USA). The antibodies were diluted 1:5000 in 10 mM TBS, pH = 8.5. After 2 h of incubation at 37 °C, the plate was washed three times with the same buffer. For LCA, due to the high absorbance of blank in the preliminary experiments, oxidation of oligosaccharides of anti-human clusterin polyclonal antibodies, which coated ELISA plate, was performed. Sodium meta-periodate solution (100 mM $NaIO_4$, 100 mM $NaHCO_3$, pH = 8.1) was added, and after 90-min incubation at room temperature in the dark, the plate was washed with 10 mM TBS pH = 7.5. The next step, free binding sites were blocked by 10 mM TBS, 0.1% Tween20, 1% BSA (blocking buffer, pH = 7.5). After 2 h of incubation at 37 °C, plates were stored at 4 °C overnight. In the lectin-ELISA procedure for LTA and UEA, the step of oligosaccharide oxidation was unnecessary and therefore omitted. After Ab-coating and washing steps, the free binding sites of ELISA wells were blocked by blocking buffer as described above for LCA.

Sample dilution. Seminal plasma and sera samples were diluted in 10 mM TBS, 0.1% Tween20, pH = 7.5 buffer to obtain proper clusterin concentration per well: 1 ng/100 µL for seminal plasma and 50 ng/100 µL for serum samples. Samples were incubated at 37 °C for two hours with gentle shaking. All samples were analyzed in duplicate to minimize imprecision. Two pairs of blank were added to each lectin-ELISA experiment—they contained all reagents, but instead of patients' samples, 10 mM TBS, 0.1% Tween20, pH = 7.5 (washing buffer) was used. After each next step of examination, wells were washed using a washing buffer.

Clusterin reduction. Due to the limited availability of some clusterin glycans, we decided to include one step of the lectin-ELISA procedure and unfold seminal plasma and serum clusterin bound with antibodies, using dithiothreitol (DTT). DTT was diluted in 0.1 M Tris–HCl, pH = 8.0 to obtain concentration 2 mg/mL. After

70 min. incubation at 37 °C plates were washed three times using 10 mM TBS, pH = 7.5. This step was applied in the lectin-ELISA procedure for all lectins used.

Lectin-fucose interactions. Biotinylated lectins were used to detect α1,3-linked, α1,2-linked antennary fucose, and α1,6-linked core fucose (LTA, UEA, LCA, respectively). All lectins dilutions were established in the series of preliminary experiments using 10 mM TBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2 \times 6H_2O$, 1 mM $MnCl_2 \times 4H_2O$, 1% BSA and 0.1% Tween20, pH = 7.5. *Lotus tetragonolobus* lectin was diluted 1:100, *Ulex europaeus* agglutinin 1:250, and *Lens culinaris* agglutinin 1:2,000, and next, plates were incubated one hour at 37 °C with gentle shaking.

Clusterin-lectin complexes detection. ExtrAvidin alkaline phosphatase labeled (Sigma-Aldrich, catalog No. E2636; Saint Louis, USA) diluted 1:10,000 in the washing buffer was used to quantify clusterin-lectin complexes. After 1 h of incubation at 37 °C, the color reaction with disodium para-nitrophenyl phosphate was induced. The absorbances were measured with Mindray MR-96A Microplate Reader (Shenzhen Mindray Bio-Medical Electronics, China) at 405 nm with a reference filter of 630 nm. The results were expressed in the absorbance units (AU) after subtracting the absorbances of the blank samples.

Statistical analysis. Statistical analysis was performed using Statistica 13.3PL software (StatSoft Inc., Tulsa, OK, USA). Shapiro–Wilk's test was used to analyze normality of all parameters distribution, and values obtained for all relative reactivities with lectins were presented as mean ± SD (SD—standard deviation) and the graphs as median with interquartile range (Q1–Q3). The U Mann–Whitney test was used to compare all relative reactivities with lectins used, and CLU and FUTs concentrations between normozoospermic group versus each group of patients with abnormal semen parameters, and between AT, OAT and T groups. The Spearman's rank correlation was used to check the associations between measured relative reactivities with lectins, CLU concentrations, and FUTs levels, also for the comparison of both biological materials examined. Moreover, the correlations between selected sperm parameters, being a part of standard ejaculate analysis, and seminal plasma parameters analyzed by us in the study were investigated. The diagnostic significance of lectins relative reactivities with CLU glycans, CLU, and FUTs concentrations was analyzed using receiver operating characteristic (ROC) curves. Based on the AUC, the clinical value of laboratory test can be defined as: 0–0.5—zero, 0.5–0.7—limited, 0.7–0.9—moderate and > 0.9—high[30]. The Youden index method was used for the determination of cut-off points. Moreover, for seminal plasma and serum samples in which all parameters were determined, and only for those parameters for which the AUC values, determined in ROC analysis, were moderate or high, cluster analysis was performed: the results were presented as a dendrogram, beginning from one cluster in which all examined subjects were gathered. The study was performed for 93 seminal plasmas and 83 serum samples for which all selected parameters were determined. All subjects were divided into groups exclusively based on differences or identities in the values of selected factors. In the next step, the subjects were clustered. Patients who presented similarities in terms of the values of all the analyzed traits were grouped together, and a separate cluster was formed for those with different values. To summarize, the greater distance of separation means the greater differences in subject characteristics. The similarities between samples were calculated using an Euclidean metric on the original data points, with no reference to the clinical status of the subjects. The *p*-Values less than 0.05 were considered significant.

Results

The mean values of analyzed parameters are summarized in Table 1, and Fig. 1 shows the results of determinations only for those parameters whose values significantly differed between the study groups.

Clusterin concentration. Seminal plasma clusterin concentrations were significantly higher in the OAT group (median value: 66.01 ng/mL) in comparison to the normozoospermic (median value: 18.98 ng/mL), asthenoteratozoospermic (median value: 19.02 ng/mL) and teratozoospermic group (median value: 19.13 ng/mL) with significances of $p = 0.000114$, $p = 0.000001$ and $p = 0.000003$, respectively. In contrast, CLU concentrations in sera were significantly lower in the normozoospermic group (median value: 16.54 µg/mL) in comparison to the asthenoteratozoospermic (median value: 36.16 µg/mL; $p = 0.001718$), oligoasthenoteratozoospermic (median value: 37.90 µg/mL; $p = 0.000318$) and teratozoospermic (median value: 36.82 µg/mL; $p = 0.000183$) groups (Fig. 1).

Fucosyltransferases concentrations. The concentrations of seminal plasma FUT3 which participate in the formation of variety of Le oligosaccharide structures in glycoproteins, were significantly higher in teratozoospermic patients (median value: 2.685 ng/mL) in comparison to the normozoospermic (median value: 2.470 ng/mL) and asthenoteratozoospermic (median value: 2.462 ng/mL) samples with significances of $p = 0.002763$ and $p = 0.030302$, respectively. Seminal plasma FUT3 concentrations were also significantly higher in the OAT patients (median value: 2.690 ng/mL) in comparison to the AT group (median value: 2.462 ng/mL; $p = 0.038031$) and N group (median value: 2.470 ng/mL; $p = 0.011090$) (Table 1).

The concentrations of seminal FUT4, which may be associated with the expression of i.a. Le^x/sLe^x oligosaccharide glycan structures, were significantly higher in the normozoospermic samples (median value: 0.843 ng/mL) in comparison to the teratozoospermic group (median value: 0.777 ng/mL) with a significance of $p = 0.032026$. Moreover, FUT4 concentrations in AT group (median value: 0.843 ng/mL) were significantly higher than in the T group with a significance of $p = 0.018689$. No significant differences for serum FUT3 and FUT4 concentrations between groups analyzed by us were found (Table 1). For serum FUT3 concentrations, the median values were

	Group			
	N $N^{PL}=29$ $N^{S}=16$	T $N^{PL}=38$ $N^{S}=31$	AT $N^{PL}=22$ $N^{S}=16$	OAT $N^{PL}=29$ $N^{S}=27$
Parameter	**MEAN ± SD**	**MEAN ± SD**	**MEAN ± SD**	**MEAN ± SD**
CLU^{PL} (ng/mL)	36.46 ± 27.66 $p=0.000114^{**}$	33.08 ± 23.44 $p=0.000003^{**}$	29.43 ± 19.95 $p=0.000001^{**}$	66.59 ± 9.66
CLU^{S} (µg/mL)	21.53 ± 11.91 $p=0.001718^{*}$ $p=0.000318^{**}$ $p=0.000183^{***}$	37.53 ± 7.20	38.25 ± 7.63	36.73 ± 7.55
$FUT3^{PL}$ (ng/mL)	2.506 ± 0.317 $p=0.011090^{**}$ $p=0.002763^{***}$	2.698 ± 0.292	2.484 ± 0.506 $p=0.038031^{**}$ $p=0.030302^{***}$	3.135 ± 1.853
$FUT3^{S}$ (ng/mL)	3.260 ± 1.478	4.422 ± 3.937	4.640 ± 2.724	5.138 ± 4.385
$FUT4^{PL}$ (ng/mL)	0.839 ± 0.110 $p=0.032026^{***}$	0.757 ± 0.159	0.871 ± 0.250 $p=0.018689^{***}$	0.822 ± 0.149
$FUT4^{S}$ (ng/mL)	1.120 ± 0.593	1.508 ± 1.423	2.034 ± 1.537	2.246 ± 2.052
LTA^{PL} (AU)	0.647 ± 0.630	0.642 ± 0.497	0.590 ± 0.476	0.648 ± 0.516
LTA^{S} (AU)	0.200 ± 0.124	0.175 ± 0.068	0.170 ± 0.065	0.203 ± 0.118
UEA^{PL} (AU)	0.315 ± 0.230	0.360 ± 0.353	0.485 ± 0.433	0.439 ± 0.414
UEA^{S} (AU)	0.070 ± 0.055 $p=0.000253^{*}$ $p=0.00001^{**}$ $p=0.000006^{***}$	0.024 ± 0.012	0.024 ± 0.008	0.024 ± 0.011
LCA^{PL} (AU)	0.323 ± 0.220	0.409 ± 0.238	0.416 ± 0.210	0.377 ± 0.239
LCA^{S} (AU)	0.162 ± 0.078 $p=0.000113^{*}$ $p=0.000029^{**}$ $p=0.000225^{***}$	0.092 ± 0.080	0.064 ± 0.038 $p=0.008840^{***}$	0.069 ± 0.048 $p=0.034620^{***}$

Table 1. The values of selected parameters measurements in seminal plasma and serum groups of patients with fertility problems. CLU^{PL}—seminal plasma CLU concentration; CLU^{S}—serum CLU concentration; $FUT3^{PL}$—seminal plasma FUT3 concentration; $FUT3^{S}$—serum FUT3 concentration; $FUT4^{PL}$—seminal plasma FUT4 concentration; $FUT4^{S}$—serum FUT4 concentration; LTA^{PL}—relative reactivity of seminal plasma CLU glycans with *Lotus tetragonolobus* agglutinin; LTA^{S}—relative reactivity of serum CLU glycans with *Lotus tetragonolobus* agglutinin; UEA^{PL}—relative reactivity of seminal plasma CLU glycans with *Ulex europaeus* agglutinin; UEA^{S}—relative reactivity of serum CLU glycans with *Ulex europaeus* agglutinin; LCA^{PL}—relative reactivity of seminal plasma CLU glycans with *Lens culinaris* agglutinin; LCA^{S}—relative reactivity of serum CLU glycans with *Lens culinaris* agglutinin. Significant differences versus: *AT group, **OAT group, ***T group. A two-tailed *p*-Value of less than 0.05 was considered significant.

following: 2.923; 2.821; 3.499 and 2.838 ng/mL in normozoospermia, T, AT and OAT groups, respectively. The median values of FUT4 concentrations were: 1.080; 1.013; 1.462 and 1.013 ng/mL in normozoospermia, T, AT and OAT groups, respectively.

Fucose expression in the glycans of serum and seminal plasma clusterin. There were no significant differences between seminal plasma groups in relative reactivities of CLU glycans with fucose-specific lectins LTA, UEA, and LCA (Table 1). The median value of LTA relative reactivity with seminal CLU glycans was 0.371 AU for normozoospermia, and 0.542; 0.439 and 0.653 AU, for T, AT and OAT groups, respectively. The median values of UEA relative reactivity with CLU glycans were the following: 0.283 AU in normozoospermia, 0.284; 0.283 and 0.277 AU in T, AT and OAT groups, respectively. The median value of LCA relative reactivity with seminal CLU glycans was 0.276 AU for normozoospermic group, and 0.341; 0.415 and 0.326 AU for T, AT and OAT groups, respectively.

No significant differences were observed between the studied groups in the values of relative reactivities of serum CLU glycans with LTA, and the medians of the obtained values were as follows: 0.171 AU in normozoospermia, 0.144 AU in teratozoospermia, 0.160 AU for asthenoteratozoospermia and 0.174 AU in oligoasthenoteratozoospermia. Relative reactivities of CLU glycans with UEA in sera of normozoospermic patients (median value: 0.05 AU) were significantly higher in comparison to the other groups: AT (median value: 0.023 AU; $p=0.000253$), OAT (median value: 0.020 AU; $p=0.00001$) and T (median value: 0.022 AU; $p=0.000006$). Similar dependency was observed for relative reactivity of CLU glycans with LCA in serum samples—normozoospermic patients (median value: 0.162 AU) had significantly higher core-fucose expression in comparison to the AT (median value: 0.055 AU; $p=0.000113$), OAT (median value: 0.054 AU; $p=0.000029$) and T (median

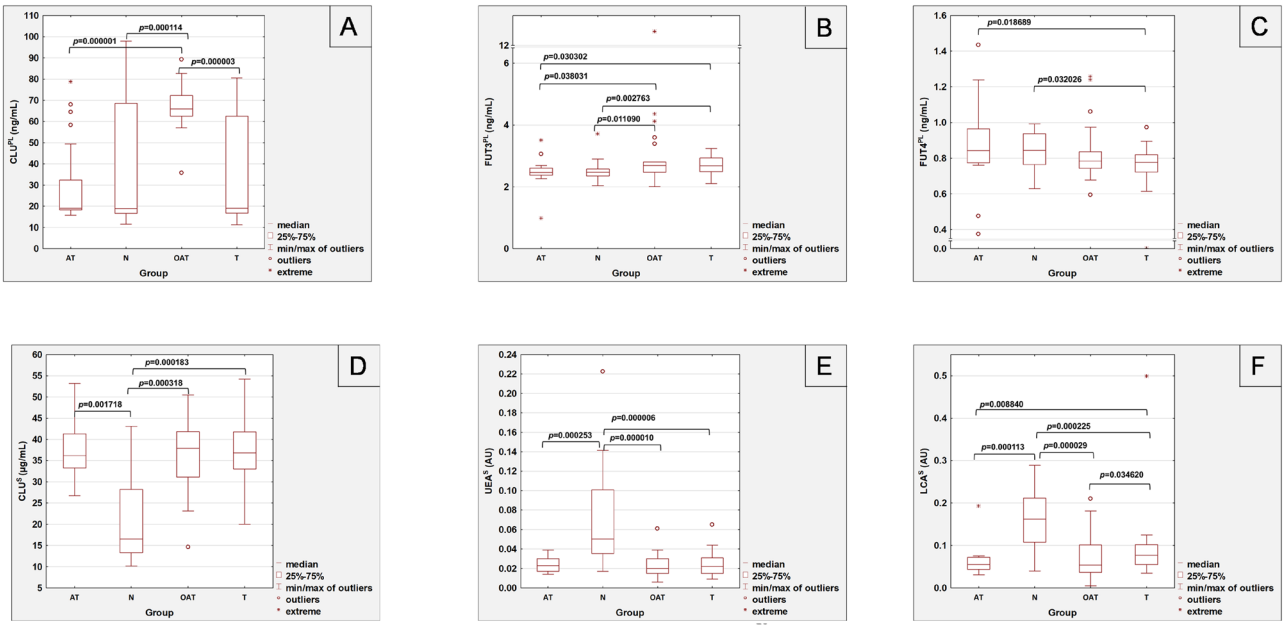

Figure 1. The values of seminal plasma (**A–C**) and serum (**D–F**) parameters measurements. CLU^{PL}—seminal plasma CLU concentration; $FUT3^{PL}$—seminal plasma FUT3 concentration; $FUT4^{PL}$—seminal plasma FUT4 concentration; CLU^{S}—serum CLU concentration; UEA^{S}—relative reactivity of serum CLU glycans with *Ulex europaeus* agglutinin; LCA^{S}- relative reactivity of serum CLU glycans with *Lens culinaris* agglutinin. A two-tailed *p*-Value of less than 0.05 was considered significant.

value: 0.077 AU; $p = 0.000225$) groups. Additionally, the relative reactivity of serum CLU glycans with LCA was significantly higher in the T group compared to the OAT and AT groups, with the significance of $p = 0.03462$ and $p = 0.00884$, respectively (Table 1). The results of correlations between values of analyzed parameters are summarized in Table 2.

Comparison of the fucosylation profile between seminal plasma and serum. The comparison of profile and degree of CLU glycans fucosylation in two biological fluids obtained from the same patient, blood serum and seminal plasma, enabled to demonstrate a weak positive correlation in relative reactivity of CLU glycans with UEA ($r = 0.288$, $p = 0.018$), however, no other associations in fucosylation of seminal plasma and serum clusterin glycans were found. We also found weak positive correlations in concentrations of fucosyltransferases between both examined biological fluids (FUT4: $r = 0.361$, $p = 0.003$; FUT3: $r = 0.352$, $p = 0.003$) (Fig. 2, Table 2).

Correlation analysis between selected semen parameters and seminal plasma parameters. The correlations between selected semen parameters, being a part of standard ejaculate analysis, and seminal plasma parameters analyzed by us are shown in the Supplementary Table 1S. Seminal plasma CLU concentration demonstrated moderate negative correlation with total sperm count ($r = -0.409$; $p = 0.001$) and a weak negative correlation with the percentage of sperm with progressive motility ($r = -0.317$; $p = 0.009$). Seminal plasma FUT3 concentration weakly negatively correlated with the percentage of sperm with normal morphology ($r = -0.300$; $p = 0.014$). A weak positive correlation between relative reactivity of seminal plasma CLU glycans with *Ulex europaeus* agglutinin and the percentage of sperm with normal morphology was also found ($r = 0.270$; $p = 0.027$) (Supplementary Table 1S).

ROC curve analysis. We performed receiver operating characteristic curves for all seminal plasma and serum parameters determined by us (Tables 3, 4, respectively). However, in Figs. 3 and 4, the results of ROC curve analysis were shown for seminal plasma and serum parameters, respectively, for which the area under the curve (AUC) was higher than 0.7. The cut-off points determined using the Youden index, are presented in Table 3 (seminal plasma) and Table 4 (serum).

Cluster analysis. For the cluster analysis were selected parameters that in the ROC curve analysis had moderate or high clinical value (AUC ≥ 0.706 for seminal plasma samples and AUC ≥ 0.710 for serum samples). Three parameters were selected for seminal plasma to perform the cluster analysis: CLU, FUT3, FUT4 concentrations, and four parameters for serum: CLU and FUT4 concentrations, relative reactivities with UEA and LCA. The study was performed for 93 seminal plasmas and 83 serum samples for which all selected parameters were determined. All subjects were divided into groups exclusively based on differences or identities in the values of selected factors.

Parameter	CLU^{PL} (ng/mL)	CLU^{S} (µg/mL)	$FUT3^{PL}$ (ng/mL)	$FUT3^{S}$ (ng/mL)	$FUT4^{PL}$ (ng/mL)	$FUT4^{S}$ (ng/mL)	LTA^{PL} (AU)	LTA^{S} (AU)	UEA^{PL} (AU)	UEA^{S} (AU)	LCA^{PL} (AU)
CLU^{S} (µg/mL)	NS										
$FUT3^{PL}$ (ng/mL)	$r=0.251$ $p=0.039$										
$FUT3^{S}$ (ng/mL)		NS	$r=0.352$ $p=0.003$								
$FUT4^{PL}$ (ng/mL)	NS		NS								
$FUT4^{S}$ (ng/mL)		NS		$r=0.949$ $p<0.001$	$r=0.361$ $p=0.03$						
LTA^{PL} (AU)	NS		NS		NS						
LTA^{S} (AU)		$r=-0.338$ $p=0.005$		$r=0.276$ $p=0.023$		$r=0.300$ $p=0.013$	NS				
UEA^{PL} (AU)	NS		$r=-0.341$ $p=0.004$		NS		$r=0.768$ $p<0.001$				
UEA^{S} (AU)		NS		NS		NS		NS	$r=0.288$ $p=0.017$		
LCA^{PL} (AU)	NS						$r=0.833$ $p<0.001$		$r=0.651$ $p<0.001$		
LCA^{S} (AU)		NS						NS		$r=0.392$ $p=0.001$	NS

Table 2. The correlations between values of analyzed parameters. CLU^{PL}—seminal plasma CLU concentration; CLU^{S}—serum CLU concentration; $FUT3^{PL}$—seminal plasma FUT3 concentration; $FUT3^{S}$—serum FUT3 concentration; $FUT4^{PL}$—seminal plasma FUT4 concentration; $FUT4^{S}$—serum FUT4 concentration; LTA^{PL}—relative reactivity of seminal plasma CLU glycans with *Lotus tetragonolobus* agglutinin; LTA^{S}—relative reactivity of serum CLU glycans with *Lotus tetragonolobus* agglutinin; UEA^{PL}—relative reactivity of seminal plasma CLU glycans with *Ulex europaeus* agglutinin; UEA^{S}—relative reactivity of serum CLU glycans with *Ulex europaeus* agglutinin; LCA^{PL}—relative reactivity of seminal plasma CLU glycans with *Lens culinaris* agglutinin; LCA^{S}—relative reactivity of serum CLU glycans with *Lens culinaris* agglutinin. NS—not significant. A two-tailed *p*-Value of less than 0.05 was considered significant.

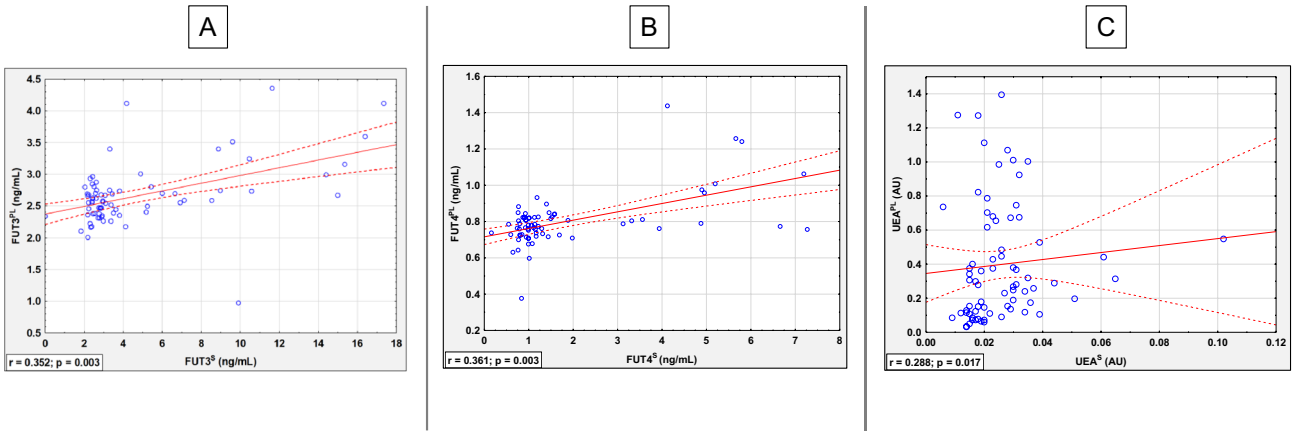

Figure 2. Comparison of glycosylation profile in two biological fluids (seminal plasma and serum) in the study group (**A–C**). $FUT3^{PL}$—seminal plasma FUT3 concentration; $FUT3^{S}$—serum FUT3 concentration; $FUT4^{PL}$—seminal plasma FUT4 concentration; $FUT4^{S}$—serum FUT4 concentration; UEA^{PL}—relative reactivity of CLU glycans with *Ulex europaeus* agglutinin in seminal plasma; UEA^{S}—relative reactivity of CLU glycans with *Ulex europaeus* agglutinin in serum. The correlations were calculated using the Spearman rank test and a two-tailed *p*-Value of less than 0.05 was considered significant. The dashed line points 95% of confidence interval.

In seminal plasma, at 90% distance, all samples could be regarded as homogenous formation. The first cluster was distinguished at 42% distance as a group composed of 42 samples. The second cluster could be distinguished at 21% distance as a group of 44 seminal plasma samples, 25 of which were from the OAT group (89% of all OAT seminal plasma samples). The third cluster could be distinguished at 16% distance and comprised 7 subjects (Supplementary Fig. 2S).

For serum, at 88% distance, all samples were regarded as homogenous formation. The first cluster could be distinguished at 49% distance as a group of 54 subjects. The second cluster could be separated at 26% distance

Parameter	Group		AUC	AUC with 95% confidence interval	Cut off point	Sensitivity	Specificity	*P*
CLU^{PL}	AT	vs. N	0.494	0.352–0.637	68.01	0.955	0.275	0.938
	OAT		**0.774**	**0.658–0.890**	**35.74**	**1.000**	**0.650**	**0.000**
	T		0.495	0.366–0.625	17.71	0.711	0.400	0.945
	AT vs. T		0.502	0.356–0.649	58.41	0.864	0.290	0.975
	OAT vs. T		**0.834**	**0.736–0.932**	**35.74**	**1.000**	**0.684**	**0.000**
	OAT vs. AT		**0.903**	**0.801–1.000**	**56.98**	**0.966**	**0.818**	**0.000**
$FUT3^{PL}$	AT	vs. N	0.541	0.352–0.729	2.38	0.875	0.304	0.672
	OAT		**0.706**	**0.564–0.847**	**2.68**	**0.568**	**0.913**	**0.004**
	T		**0.736**	**0.601–0.871**	**2.65**	**0.618**	**0.913**	**0.001**
	AT vs. T		0.691	0.527–0.855	2.62	0.813	0.618	0.022
	OAT vs. T		0.509	0.363–0.656	3.40	0.233	1.000	0.901
	OAT vs. AT		0.686	0.529–0.844	2.68	0.567	0.813	0.021
$FUT4^{PL}$	AT	vs. N	0.536	0.335–0.738	1.01	0.188	1.000	0.722
	OAT		0.614	0.436–0.792	0.82	0.714	0.667	0.210
	T		0.685	0.515–0.855	0.82	0.813	0.667	0.033
	AT vs. T		**0.710**	**0.539–0.881**	**0.84**	**0.563**	**0.844**	**0.016**
	OAT vs. T		0.574	0.428–0.720	0.81	0.464	0.710	0.323
	OAT vs. AT		0.647	0.468–0.826	0.82	0.714	0.625	0.107
LTA^{PL}	AT	vs. N	0.448	0.268–0.628	1.02	0.875	0.333	0.572
	OAT		0.509	0.347–0.671	0.65	0.517	0.667	0.917
	T		0.559	0.398–0.720	0.12	0.971	0.250	0.474
	AT vs. T		0.517	0.349–0.684	0.34	0.500	0.647	0.847
	OAT vs. T		0.490	0.342–0.638	0.69	0.483	0.677	0.893
	OAT vs. AT		0.500	0.329–0.671	0.72	0.448	0.813	1.000
UEA^{PL}	AT	vs. N	0.583	0.394–0.773	0.79	0.313	0.958	0.389
	OAT		0.510	0.350–0.670	0.67	0.345	0.917	0.902
	T		0.492	0.340–0.644	0.62	0.206	0.917	0.918
	AT vs. T		0.573	0.394–0.751	0.79	0.313	0.941	0.426
	OAT vs. T		0.508	0.355–0.660	0.74	0.276	0.941	0.922
	OAT vs. AT		0.574	0.404–0.745	0.11	0.345	0.875	0.392
LCA^{PL}	AT	vs. N	0.651	0.474–0.827	0.33	0.688	0.682	0.095
	OA		0.567	0.407–0.726	0.32	0.552	0.682	0.414
	T		0.618	0.466–0.770	0.33	0.594	0.682	0.129
	AT vs. T		0.467	0.300–0.633	0.53	0.875	0.344	0.696
	OAT vs. T		0.462	0.315–0.608	0.43	0.448	0.625	0.609
	OAT vs. AT		0.563	0.395–0.730	0.16	0.276	1.000	0.465

Table 3. Summary of receiver operating characteristic (ROC) curves for seminal plasma parameters. CLU^{PL}—seminal plasma CLU concentration; $FUT3^{PL}$—seminal plasma FUT3 concentration; $FUT4^{PL}$—seminal plasma FUT4 concentration; LTA^{PL}—relative reactivity of seminal plasma CLU glycans with *Lotus tetragonolobus* agglutinin; UEA^{PL}—relative reactivity of seminal plasma CLU glycans with *Ulex europaeus* agglutinin; LCA^{PL}—relative reactivity of seminal plasma CLU glycans with *Lens culinaris* agglutinin. Area under the ROC curve (AUC) is given with 95% confidence interval. Data with AUC equal or greater than 0.706 are marked in bold. Based on the AUC, the clinical value of laboratory test can be defined as: 0–0.5—zero, 0.5–0.7—limited, 0.7–0.9—moderate and > 0.9—high.

as 11 samples, containing 8 of 12N samples (67% of all N subjects). The third cluster was distinguished at 16% distance, containing 18 serum samples from AT, OAT and T groups (Supplementary Fig. 3S).

Discussion

Clusterin concentration. Based on the results concerning seminal plasma CLU concentration, we can conclude that the observed significant differences between N, T, and AT groups versus the OAT group may be associated with the increase of CLU production in the male reproductive organs and corresponds with the lowered total number of spermatozoa in the ejaculate. The above finding was additionally confirmed by observed moderate negative correlation between seminal CLU concentration and total sperm count in the semen. Our findings stand in contradiction to the research of Fukuda et al., in which the normozoospermic seminal plasma samples of infertile men had significantly higher CLU concentration (47.9 ± 20.9 ng/mL) in comparison to the oligozoospermic group of patients (28.2 ± 8.6 ng/mL)[31]. On the other hand, Salehi et al. reported higher seminal plasma CLU concentrations in the fertile men (48.3 ± 38.59 ng/mL) in comparison to the infertile group

Parameter	Group		AUC	AUC with 95% confidence interval	Cut off point	Sensitivity	Specificity	*P*
CLU^S	**AT**	**vs. N**	**0.821**	**0.657–0.985**	**26.74**	**1.000**	**0.750**	**0.000**
	OAT		**0.819**	**0.668–0.971**	**23.12**	**0.963**	**0.750**	**0.000**
	T		**0.823**	**0.665–0.981**	**19.97**	**1.000**	**0.750**	**0.000**
	AT vs. T		0.503	0.321–0.685	45.66	0.200	0.903	0.972
	OAT vs. T		0.497	0.345–0.649	37.29	0.623	0.516	0.969
	OAT vs. AT		0.494	0.305–0.683	36.77	0.667	0.533	0.949
$FUT3^S$	AT	vs. N	0.667	0.463–0.870	2.41	1.000	0.308	0.109
	OAT		0.541	0.346–0.737	4.17	0.333	0.923	0.679
	T		0.521	0.333–0.710	4.90	0.290	0.923	0.826
	AT vs. T		0.615	0.452–0.778	2.41	1.000	0.290	0.167
	OAT vs. T		0.555	0.405–0.704	6.65	0.296	0.871	0.471
	OAT vs. AT		0.574	0.399–0.749	3.32	0.667	0.600	0.407
$FUT4^S$	**AT**	vs. N	**0.710**	**0.520–0.899**	**1.13**	**0.733**	**0.643**	**0.030**
	OAT		0.619	0.431–0.808	0.77	1.000	0.357	0.216
	T		0.554	0.364–0.745	0.80	0.862	0.357	0.577
	AT vs. T		0.634	0.456–0.813	1.19	0.667	0.655	0.134
	OAT vs. T		0.591	0.440–0.741	0.92	0.889	0.345	0.238
	OAT vs. AT		0.535	0.348–0.721	1.09	0.556	0.733	0.717
LTA^S	AT	vs. N	0.569	0.362–0.776	0.18	0.733	0.500	0.515
	OAT		0.543	0.364–0.721	0.14	0.741	0.438	0.638
	T		0.537	0.362–0.712	0.16	0.613	0.563	0.676
	AT vs. T		0.485	0.304–0.666	0.16	0.533	0.581	0.871
	OAT vs. T		0.580	0.430–0.730	0.14	0.741	0.484	0.295
	OAT vs. AT		0.584	0.402–0.765	0.14	0.815	0.400	0.365
UEA^S	**AT**	**vs. N**	**0.869**	**0.742–0.995**	**0.04**	**0.867**	**0.750**	**0.000**
	OAT		**0.878**	**0.764–0.993**	**0.03**	**0.926**	**0.813**	**0.000**
	T		**0.879**	**0.771–0.987**	**0.03**	**0.871**	**0.813**	**0.000**
	AT vs. T		0.539	0.366–0.711	0.02	0.933	0.258	0.661
	OAT vs. T		0.520	0.369–0.670	0.03	0.926	0.226	0.797
	OAT vs. AT		0.542	0.359–0.725	0.02	0.259	0.933	0.653
LCA^S	**AT**	**vs. N**	**0.881**	**0.746–1.000**	**0.08**	**0.933**	**0.875**	**0.000**
	OAT		**0.861**	**0.745–0.977**	**0.11**	**0.889**	**0.750**	**0.000**
	T		**0.818**	**0.670–0.965**	**0.11**	**0.903**	**0.750**	**0.000**
	AT vs T		**0.737**	**0.582–0.891**	**0.08**	**0.933**	**0.548**	**0.003**
	OAT vs. T		0.662	0.515–0.810	0.05	0.519	0.839	0.030
	OAT vs. AT		0.493	0.317–0.668	0.08	0.333	0.933	0.934

Table 4. Summary of receiver operating characteristic (ROC) curves for serum parameters. CLU^S—serum CLU concentration; $FUT3^S$—serum FUT3 concentration; $FUT4^S$—serum FUT4 concentration; LTA^S—relatsive reactivity of serum CLU glycans with *Lotus tetragonolobus* agglutinin; UEA^S—relative reactivity of serum CLU glycans with *Ulex europaeus* agglutinin; LCA^S—relative reactivity of serum CLU glycans with *Lens culinaris* agglutinin. Area under the ROC curve (AUC) is given with 95% confidence interval. Data with AUC equal or greater than 0.710 are marked in bold. Based on the AUC, the clinical value of laboratory test can be defined as: 0–0.5—zero, 0.5–0.7—limited, 0.7–0.9—moderate and > 0.9—high.

(14.48 ± 9.74 ng/mL) without particular classification based on the sperm abnormalities[32]. Interestingly, serum CLU concentration values were significantly decreased in the normozoospermic group compared to T, AT, and OAT groups. The observed by us different expression of CLU present in two examined biological fluids is apparently related to the distinct location of its synthesis and the biological role it plays in various parts of male organism.

Fucosyltransferases concentration. A comparison of the seminal plasma FUT3 concentration between T and AT groups suggests that asthenozoospermia may be associated with decreasing FUT3 concentration in this biological fluid. Some authors have demonstrated that seminal plasma FUTs are very important during interactions between sperm surface and zona pellucida. Chiu et al.[33] showed that seminal plasma FUT5 inhibits this process through binding with glycodelin A. The co-immunoprecipitation of FUT3, FUT5, and glycodelin A confirmed the presence of these enzymes in the sperm cells' membrane[33]. Human seminal plasma fucosyltransferases are interesting and need to be investigated, especially in the context of their influence on the expres-

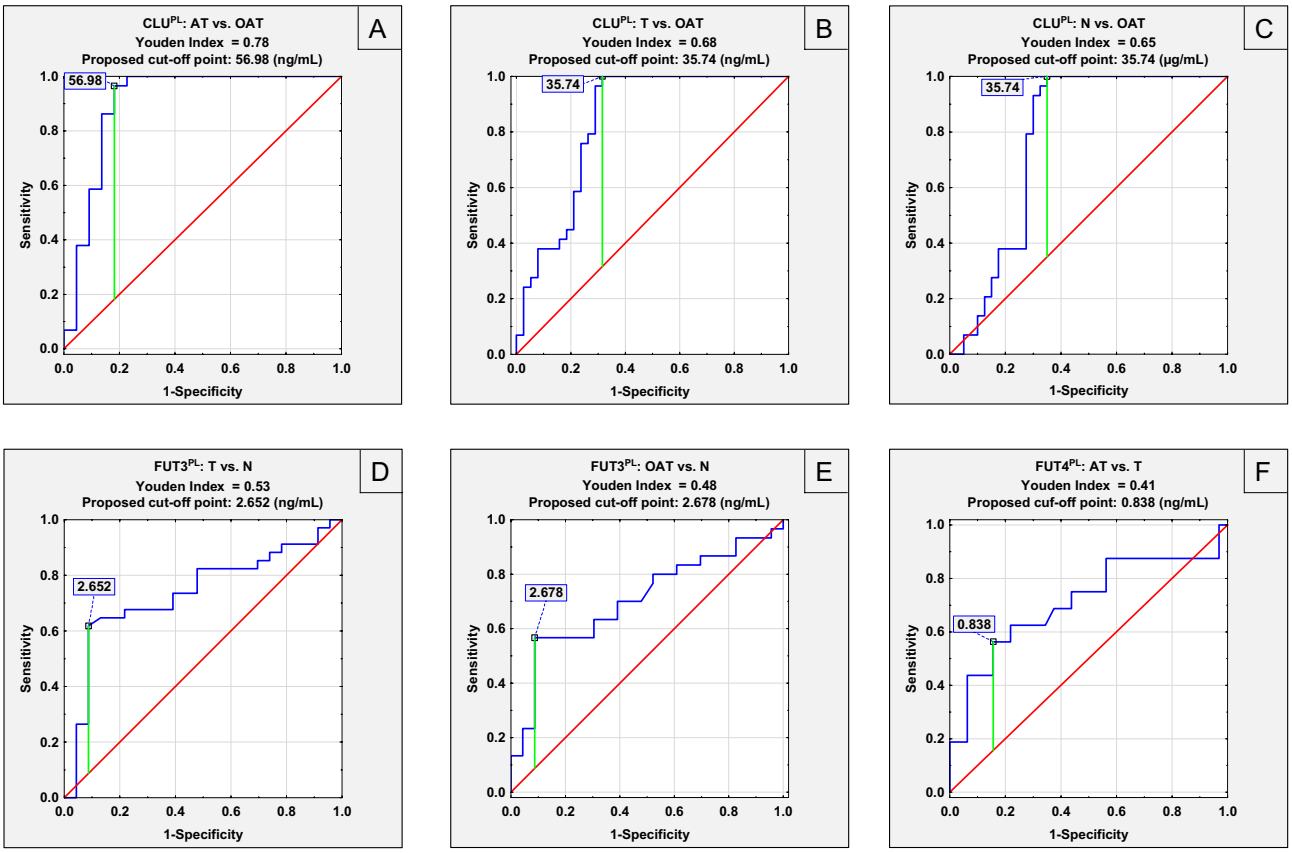

Figure 3. Receiver operating characteristic (ROC) curves for seminal plasma parameters with the area under the curve (AUC) higher than 0.700 (**A–F**). CLU^{PL}—seminal plasma CLU concentration; $FUT3^{PL}$—seminal plasma FUT3 concentration; $FUT4^{PL}$—seminal plasma FUT4 concentration.

sion of Lewis oligosaccharide structures. Decreased total number of spermatozoa in the ejaculate, together with increased expression of sperm malformations, seem to be associated with the elevated expression of wide spectrum of Le oligosaccharide structures in seminal plasma glycoproteins synthesized within the male reproductive tract, which was demonstrated by a significant increase of FUT3 concentration in OAT and T groups versus N, with no differences between N and AT groups; however, this hypothesis should be confirmed in the future study, focused also on analysis of FUTs expression, which specificity of action is not so wide. There is no current literature data concerning FUT3 concentrations in human seminal plasma. Our previous study on seminal plasma AGP (α1-acid glycoprotein) glycosylation showed, that Le^a expression on AGP coincides with a higher degree of glycans branching and a relative increased α4-fucosyltransferase activity[34]. In other study, we reported that the expression of Le^y structures was significantly increased in selected glycoprotein bands detected in lectin-blotting of normozoospermic seminal plasmas compared to the control group of men with proved fertility. However, it should be underlined that in the present study, we examined patients with a variety of sperm abnormalities, and distinct methods were used for the analysis of Le^y oligosaccharide structure expression[35]. In the present study, we also did not have the opportunity to compare the results obtained for infertile patients with those obtained for the normozoospermic group of fertile men. Pang et al.[36] investigated the expression of Le^x and Le^y oligosaccharide structures in sperm cells' N-glycans of normozoospermic infertile men and healthy sperm bank donors. The authors concluded that defective sperm had a high level of Le^y expression, and additionally the localization of these structures was distinct than in the case of the control group[36], what is in accordance with our findings for seminal plasma FUTs levels, which significantly elevated for FUT3 and decreased for FUT4 in T group versus N subjects, if we take into account that FUT3 influence the formation of, among others Le^x and Le^y oligosaccharide structures, and FUT4 the formation of inter alia Le^x structures.

The comparison the seminal plasma FUT4 concentrations between study groups indicates that spermatozoa morphology abnormalities characteristic for teratozoospermia are associated with decreased FUT4 concentration. Moreover, the observed significant differences in seminal plasma FUT4 concentrations between the T and AT group suggest that motility abnormalities of sperm cells together with morphology disorders may be associated with the relative increase in the expression of $Le^{a/x}$ structures in glycans of seminal plasma glycoproteins. It is worth noting that the differences in seminal plasma FUT4 concentration values between the normozoospermic group compared to the OAT, AT, and T groups, are the opposite of the concentration values of FUT3. In contrast, the expression of Le^x structures examined by Kałuża et al. in the asthenozoospermic patients was moderately decreased, compared to the control group for two from five glycoprotein bands. However, the control group investigated by authors was composed of normozoospermic fertile men[35]. Authors found no differences in the

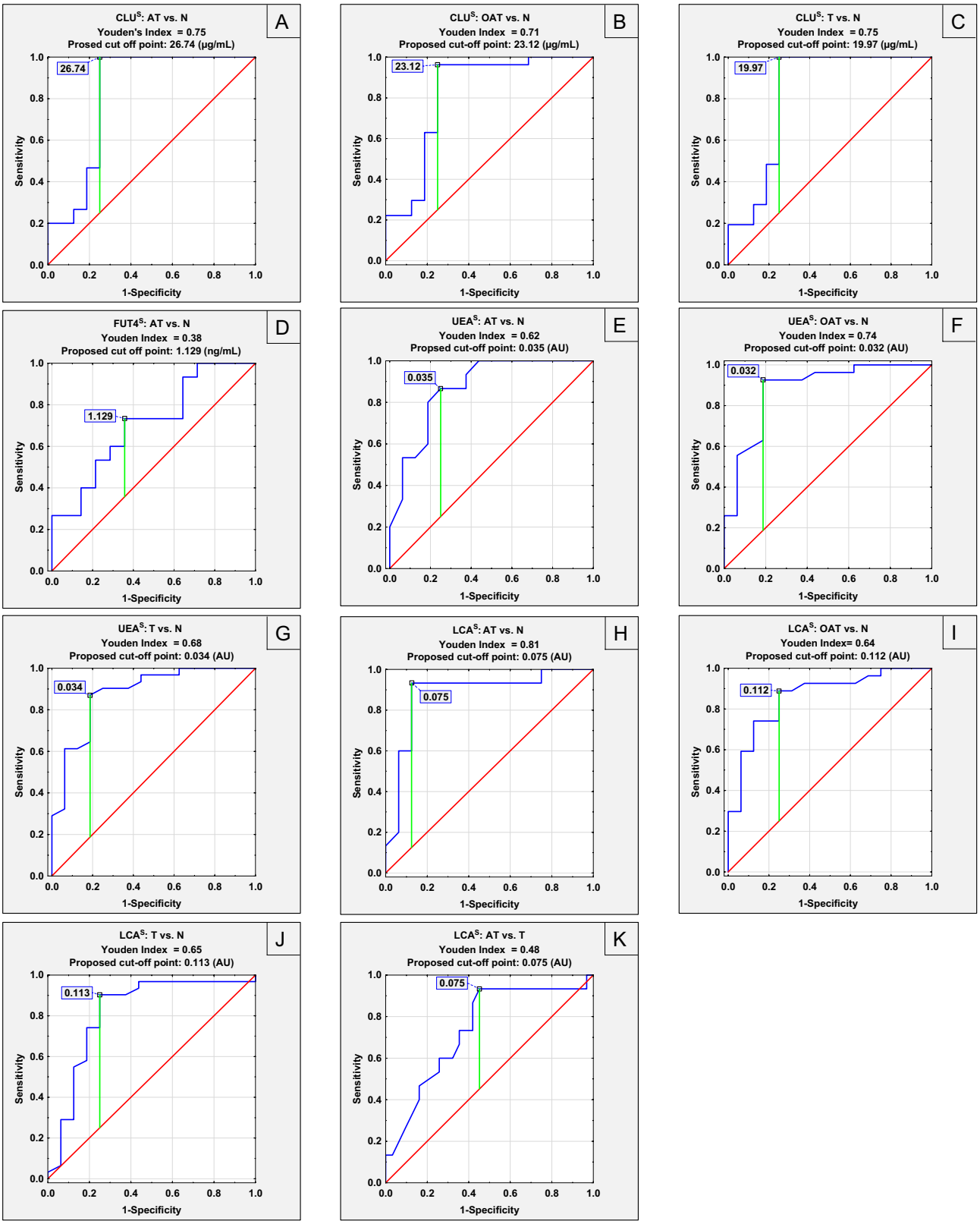

Figure 4. Receiver operating characteristic (ROC) curves for serum parameters with the area under the curve (AUC) more than 0.700 (**A–K**). CLU^S—serum CLU concentration; FUT4^S—serum FUT4 concentration; UEA^S-relative reactivity of serum CLU glycans with *Ulex europaeus* agglutinin; LCA^S-relative reactivity of serum CLU glycans with *Lens culinaris* agglutinin.

expression of Le^x-decorated glycans in the seminal plasma glycoproteins between the asthenozoospermic group and normozoospermic patients with fertility problems[35]. Still, it should be mentioned that lectin-blotting analysis of glycoprotein bands, separated previously in SDS-PAGE, without detailed analysis of specific glycoprotein, significantly differs in its idea from lectin-ELISA in which specific glycoprotein may be analyzed, and thus the comparison of results obtained is difficult and may be problematic. The presence of Le^x and Le^y oligosaccharide structures as the DC-SIGN ligands in human seminal plasma glycome was investigated by Clark et al.[37,38]. The authors showed that one of the three major endogenous DC-SIGN ligands in seminal plasma is clusterin, expressing Le^x and Le^y oligosaccharide structures in the antennary part of its glycans[38]. This conclusion seems to be confirmed by the study conducted by Sabatte et al., who reported that seminal plasma, not serum CLU, is a DC-SIGN ligand[39]. Based on our findings and literature data, we can conclude that any alterations in the expression of Le^x and Le^y oligosaccharide structures, influenced among others by FUT4 and FUT3, may be responsible for disturbances in intermolecular interactions within the male reproductive system.

The relevance of FUT3 and FUT4 concentration values in decreased male fertility was confirmed by correlation analysis. To the best of our knowledge, our study showed for the first time a weak positive correlation between biological fluids examined for FUT3 ($r = 0.352$, $p = 0.003$) and FUT4 ($r = 0.361$, $p = 0.003$) concentrations (Fig. 2). Additionally, serum FUT3 and FUT4 concentrations demonstrated a very strong positive correlation ($r = 0.949$, $p < 0.001$) what may be explained by similarities in mechanisms stimulating of their action. A weak negative correlation between seminal plasma FUT3 concentration and the relative reactivity of seminal plasma CLU glycans with UEA ($r = -0.341$, $p = 0.004$) may suggests lowered expression of α1,2-linked fucose being a part of Le^y oligosaccharide structure which is compensated by the expression of Le^b and/or Le^a oligosaccharide structures. Saraswat et al.[25] confirmed that among branch fucosylated seminal plasma glycoproteins also occur Le^a and less frequently Le^b glyco-motifs, which may be also present in CLU glycans. However, further investigations in this field are needed. In contrast to the above, serum FUT4 concentration weak positively correlated with the relative reactivity of serum CLU glycans with LTA ($r = 0.300$, $p = 0.013$). It is consistent with the specificity of LTA, which detects fucose linked to galactose via α1,3 glycosidic bound, present in the antennary part of N-glycans, forming Le^x oligosaccharide structure, and with the biological function of FUT4, which is responsible for i.a. Le^x glycan structures formation[26,28].

Fucose expression in the glycans of clusterin present in seminal plasma and serum. Lack of differences in relative reactivities of CLU glycans with fucose-specific lectins between infertile normozoospermic seminal plasma group and groups of patients with abnormal semen parameters indicates that CLU fucosylation is independent of sperm count, progression, and morphology. Future studies should check if any differences exist in seminal CLU fucosylation status between normozoospermic infertile patients with idiopathic infertility and a group of normozoospermic subjects with confirmed fertility. The lack of a normozoospermic group of men with proven fertility is a limitation of our study.

On the other hand, the observed relative reactivities of CLU glycans with UEA in sera may led to the conclusion that changes in α1,2-linked fucose expression observed in serum CLU glycans of infertile men are related to the results of routine seminal analyses, because the expression of fucose being a part of Le^y oligosaccharide structures in the CLU glycans was significantly higher in the normozoospermic group. It seems that the expression of UEA-reactive fucose in CLU could be used as a marker of male infertility caused by sperm cells disorders. Based on the results concerning relative reactivity of CLU glycans with LCA in sera, we observed that expression of α1,6-linked core fucose in the CLU glycans in the normozoospermic group is significantly higher than in the other groups characterized by semen abnormalities. Moreover, observed significant differences in LCA reactivity between T and OAT, and between T and AT groups may indicate that complex spermatozoa abnormalities, including more than one disorder, are reflected in the decrease of core fucose expression in CLU glycans. It is also worth noting that, in opposite to serum CLU fucosylation, the expression in glycans of seminal plasma CLU of α1,2-linked fucose, being a part of Le^y oligosaccharide structures, as well as α1,6-linked core fucose, was noticeably lower (insignificant, however) in normozoospermic subjects than in the rest of investigated groups of patients, what may suggest the differences in mechanisms involved in glycosylation pattern formation of CLU in seminal plasma and blood serum.

Based on the presence of negative correlation between the expression in glycans of serum CLU LTA-reactive α1,3-linked fucose and serum CLU concentration, we came to the presumption that high concentration of serum CLU may be accompanied by decreased expression of Le^x oligosaccharide structures; however, it should be taken into account, that the decreased expression of α1,3-linked fucose may be compensated by increased expression of α1,4-linked fucose typical for Le^a oligosaccharide structures and/or by fucose of Le^b structures. The above hypothesis needs to be checked in future studies.

As far as we know, this is the first study based on lectin-ELISA measurements concerning the fucosylation analysis of seminal plasma and serum clusterin in men with fertility problems. Lectin-based ELISA is a good research tool because the reactions between lectins and sugar residues in vitro reflect those that occur between sugar residues of glycoprotein glycans and their corresponding ligands in a living organism. In our previous studies in the leukocytospermic patients we examined the fucosylation profile and degree of some other seminal plasma glycoproteins, fibronectin (FN) and AGP[24]. Like CLU, these glycoproteins influence male fertility, and FN, similarly to CLU, participates in the acrosome reaction[40]. Kałuża et al.[35] evaluated the expression of glycoepitopes that may be important in the context of fetoembryonic defense and confirmed that clusterin is one of the crucial importance. They reported that the relative reactivity of seminal plasma CLU with LTA was significantly lower in infertile patients than in the control group[35]. Olejnik et al., based on their research, suggest that abundant fucosylation of some seminal plasma glycoproteins may bother the normal fertilization process[41]. Further analysis concerning the glycosylation profile of CLU seems to be particularly relevant in the context of

oxidative stress and chaperone activity of CLU. Merlotti et al. confirmed that fucosylated seminal plasma CLU performs its chaperone activity and directs damaged proteins to the dendritic cells through DC-SIGN[20]. It will be also interesting to examine the expression of Le^a and Le^b expression on antennary part of CLU glycans and the comparison of obtained results with the present findings.

Comparison of the glycosylation profile in both biological fluids. Lack of correlations between relative reactivity of seminal plasma and serum CLU glycans with LTA evidently indicate and confirm that seminal plasma and serum CLU is synthesized and glycosylated in different places of the male organism, which results in differences in CLU glycans composition and fucosylation intensity, especially in the Le^x structures formation and core fucosylation, in those two biological fluids.

Our study also showed that exists a strong positive correlation ($r = 0.768$, $p < 0.001$) between LTA and UEA relative reactivities of seminal plasma CLU glycans, probably due to the fact that UEA can detect fucose bounded via α1,2-glycosidic linkage, being a part of bifucosylated structures additionally containing LTA-reactive fucose linked by α1,3-glycosidic bound[42]. Comparison of the glycosylation profile of seminal plasma and serum CLU glycans also showed a weak positive correlation between relative reactivity of CLU glycans with UEA ($r = 0.288$, $p = 0.017$) what may suggest similarity in the relative expression of antennary α1,2-linked fucose in glycans of seminal plasma and serum CLU (Fig. 2).

ROC curve analysis. It should be underlined that, as far as we know, this is the first study in which simultaneously seminal plasma and serum CLU, FUT3, FUT4 concentrations as well as the relative expression of fucose in CLU glycans were analyzed in the context of decreased male fertility. The results of ROC curve analysis showed that the determination of seminal plasma parameters: CLU, FUT3, and FUT4 concentrations may be helpful in the differentiation of infertile groups of patients with both normal and abnormal seminal parameters (Fig. 3, Table 3). In this study, seminal plasma clusterin concentration has a moderate clinical value and enabled differentiation of OAT group from the normozoospermic group with sensitivity and specificity of 100% and 65%, respectively (proposed cut off point: 35.74 ng/mL, AUC = 0.774) and from the teratozoospermic group with sensitivity and specificity of 100% and 68.4%, respectively (proposed cut-off point: 35.74 ng/mL, AUC = 0.834), but CLU concentration has a high clinical value when differentiating OAT patients from asthenoteratozoospermic men with sensitivity and specificity of 96.6% and 81.8%, respectively (proposed cut off point: 56.98 ng/mL, AUC = 0.903). The above differences most probably are the results of decreased sperm count in the OAT group, which may be caused among others by destructive action on spermatogenesis of oxidative stress accompanying high levels of CLU. FUT3 concentration has a moderate clinical value when to distinguish OAT group from the normozoospermic group with sensitivity and specificity of 56.8% and 91.3%, respectively (proposed cut off point: 2.68 ng/mL, AUC = 0.706) and T group from the normozoospermic group with sensitivity and specificity of 61.8% and 91.3%, respectively (proposed cut off point: 2.65 ng/mL, AUC = 0.736), what let us concluding that for observed differences the strongest influence has together decreased sperm count and increased expression of their malformations. FUT4 concentration with moderate clinical value was useful in the differentiation between AT and T groups with sensitivity and specificity of 56.3% and 84.4%, respectively (proposed cut-off point: 0.84 ng/mL, AUC = 0.710), and the decreased motility of sperm cells was crucial here.

ROC curve analysis for examined by us serum parameters revealed that CLU and FUT4 concentrations and relative reactivity of serum CLU glycans with UEA and LCA may be useful in the differentiation of groups of infertile men with abnormal and normal semen parameters (Fig. 4, Table 4). Serum clusterin concentration enabled differentiation with moderate clinical value the normozoospermic group from: AT group with sensitivity and specificity of 100% and 75%, respectively (proposed cut off point: 26.74 µg/mL, AUC = 0.821), OAT group with sensitivity and specificity of 96.3% and 75%, respectively (proposed cut off point: 23.12 µg/mL, AUC = 0.819) and T group with sensitivity and specificity of 100% and 75%, respectively (proposed cut off point: 19.97 µg/mL, AUC = 0.823). FUT4 concentration also has moderate clinical value and can be used to differentiate asthenoteratozoospermic patients from normozoospermic men with sensitivity and specificity of 73.3% and 64.3%, respectively (proposed cut-off point: 1.13 ng/mL, AUC = 0.710). As in the case for serum CLU concentration, relative reactivity of serum CLU glycans with UEA have moderate clinical value and enabled differentiation of normozoospermic patients from: AT patients with sensitivity and specificity of 86.7% and 75%, respectively (proposed cut off point: 0.04 AU, AUC = 0.869), OAT patients with sensitivity and specificity of 92.6% and 81.3%, respectively (proposed cut off point: 0.03 AU, AUC = 0.878) and T patients with sensitivity and specificity of 87.1% and 81.3%, respectively (proposed cut off point: 0.03 AU, AUC = 0.879). Relative reactivity of serum CLU glycans with LCA with moderate clinical value make possible the differentiation of normozoospermic patients from: AT patients with sensitivity and specificity of 93.3% and 87.5%, respectively (proposed cut off point: 0.08 AU, AUC = 0.881), OAT patients with sensitivity and specificity of 88.9% and 75%, respectively (proposed cut off point: 0.11 AU, AUC = 0.861) and T patients with sensitivity and specificity of 90.3% and 75%, respectively (proposed cut off point: 0.11 AU, AUC = 0.818). Moreover, this parameter, which has moderate clinical value, also differentiated teratozoospermic and asthenoteratozoospermic patients with sensitivity and specificity of 93.3% and 54.8%, respectively (proposed cut-off point: 0.08 AU, AUC = 0.737), what is most likely due to reduced sperm cells motility.

Cluster analysis. Based on the results of ROC curves analysis, seminal plasma parameters with AUC equal or greater than 0.706 were selected to perform cluster analysis. Our selection confirmed that in the case of seminal plasma: CLU, FUT3, and FUT4 concentrations may be taken into consideration as a set of additional parameters helpful to distinguish seminal OAT group of patients from men with normal seminal parameters and subjects with decreased sperm motility and abnormal morphology. On the other hand, the cluster analysis made

for selected serum parameters with AUC equal to or greater than 0.710, which were CLU and FUT4 concentrations, together with relative reactivities of serum CLU glycans with UEA and LCA, enabled the determination of an additional panel of parameters helpful in differentiation of normozoospermic patients with fertility problems from those infertile with abnormal sperm morphology, reduced sperm motility, and count.

CLU concentration and the profile and degree of its fucosylation seem to be more useful for differentiating normozoospermic infertile patients from those with abnormal sperm count, motility, and morphology when the material examined is serum, not seminal plasma. The results of our research shed some light on the association between semen characteristics, which is the basis for standard semen analysis, and the expression of clusterin, both in human seminal plasma and serum, and the profile and degree of its fucosylation. In our opinion the present research is a good starting point for further investigations on the role of clusterin glycosylation in male fertility, also in the context of its role in oxidative-antioxidant balance and chaperone activity, and can be used to guide future research directions.

Conclusions

The observed differences in concentration values of serum CLU and FUT4 as well as in the expression of core fucose and fucose α1,2-linked, being a part of Le^y oligosaccharide structures, in CLU glycans between the normozoospermic group of patients with fertility problems, however, and other groups examined, suggest that the disturbances in sperm count, motility and morphology are not the only cause of male fertility problems. Idiopathic infertility may result from many various changes at the molecular level, e.g., in synthesis and/or variability of glycoproteins glycosylation. This is the key to the proper fertilization process and expression of enzymes involved in the glycoproteins fucosylation. On the other hand, the lack of similarities between levels of parameters examined in blood serum and seminal plasma may suggest the differences in mechanisms of glycoproteins synthesis and those responsible for glycoproteins glycosylation. This is confirmed by observed differences in the concentration values of seminal plasma CLU, FUT3 and FUT4 observed between the OAT group and the rest of seminal plasma groups (N, T, AT), indicating that decreased sperm count may be related to these parameters expression. The lack of a representative control group of normozoospermic men with proved fertility makes it impossible to verify if the parameters analyzed in this study may be useful as biomarkers of idiopathic male infertility. This is the limitation of our study and it needs to be examined in further study. It is noteworthy that serum CLU concentration and expression of core fucose and fucose α1,2-linked in glycan structure of CLU seems to be good markers differentiating normozoospermic men from those with abnormal sperm count, motility, and morphology. Still, we cannot say the same about the seminal plasmas we studied, because despite the demonstrated weak correlations between selected semen parameters such as the total number of sperm in the ejaculate, the total number of sperm with progressive movement and the number of sperm with normal morphology versus the glyco-parameters analyzed in the study, no significant differences were found between the studied seminal plasma groups. Another important aspect of our study worth emphasizing is that the glycan-lectin reaction observed in the lectin-based ELISA method mimics the reactions that occur in male organism, showing the availability of sugar residues for endogenous ligands and thus deepening the knowledge about the mechanisms of these interactions. On the other hand, the unanswered questions may be an inspiration to undertake further research by our team and other researchers.

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Author contributions

E.J.: Methodology, Investigation, Data curation, Visualization, Writing—Review and Editing, Funding acquisition. I.K.: Resources. I.G.: Resources. R.F.: Resources. E.M.K.: Conceptualization, Resources, Methodology, Validation, Formal analysis, Writing—Review and Editing, Supervision, Funding acquisition.

Competing interests

The authors declare no competing interests.

Additional information

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The possible association of clusterin fucosylation changes with male fertility disorders

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Supplementary Materials

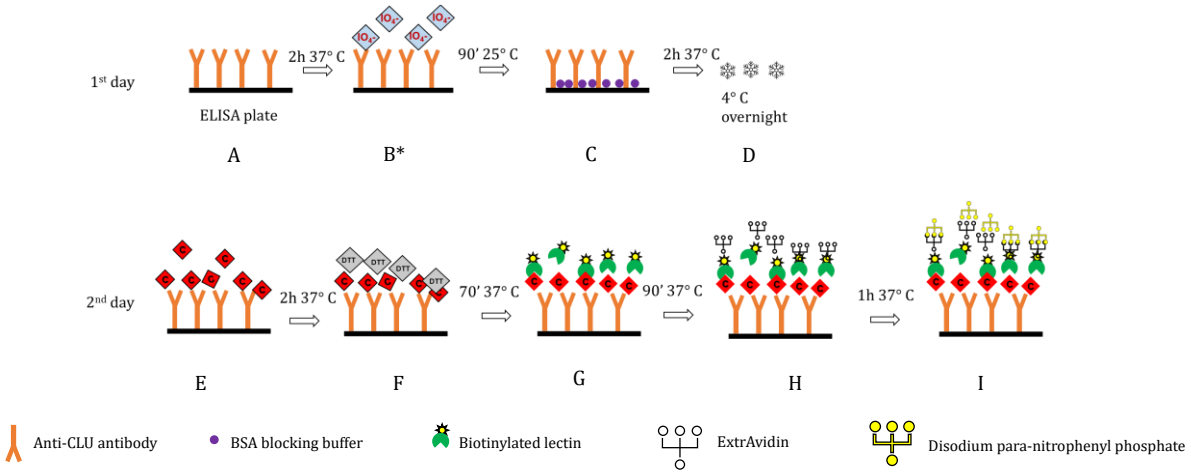
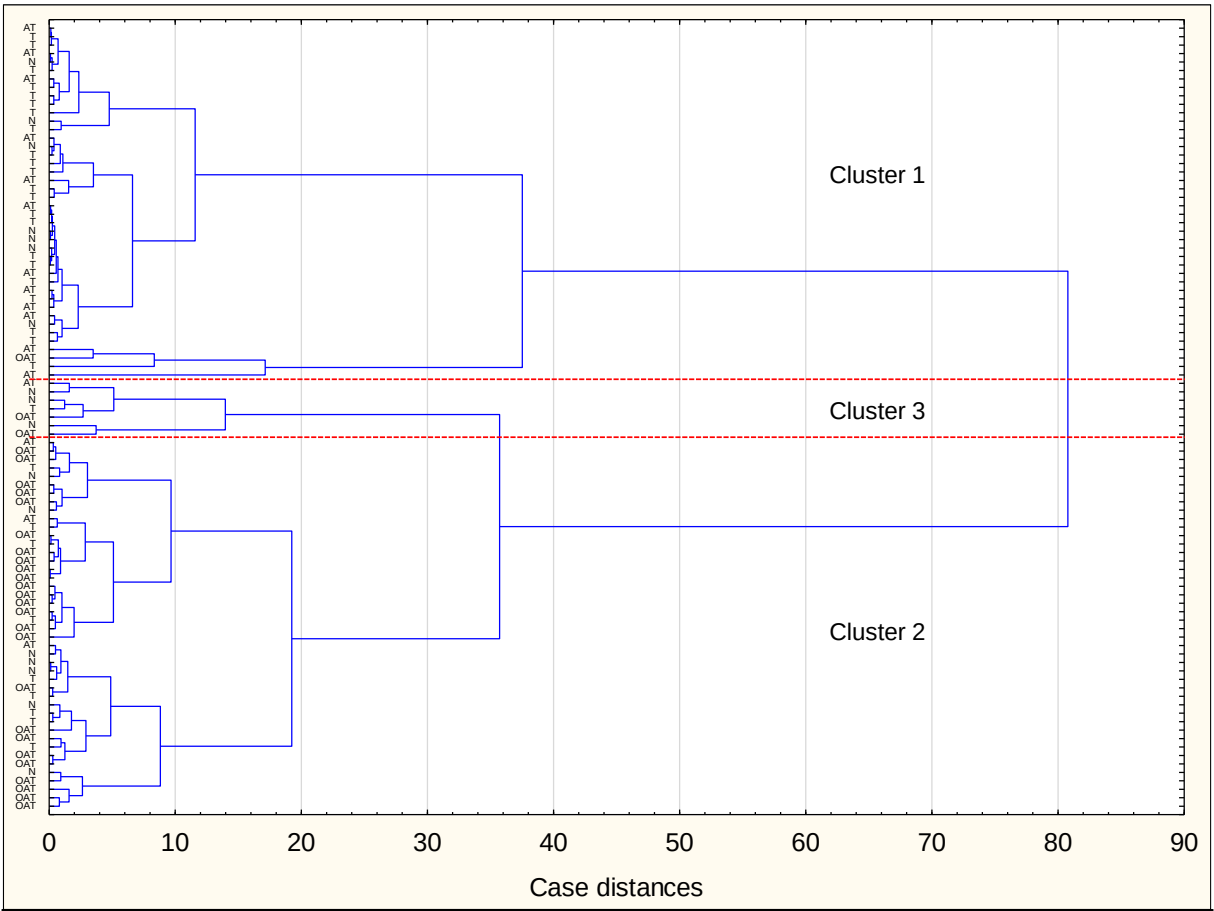
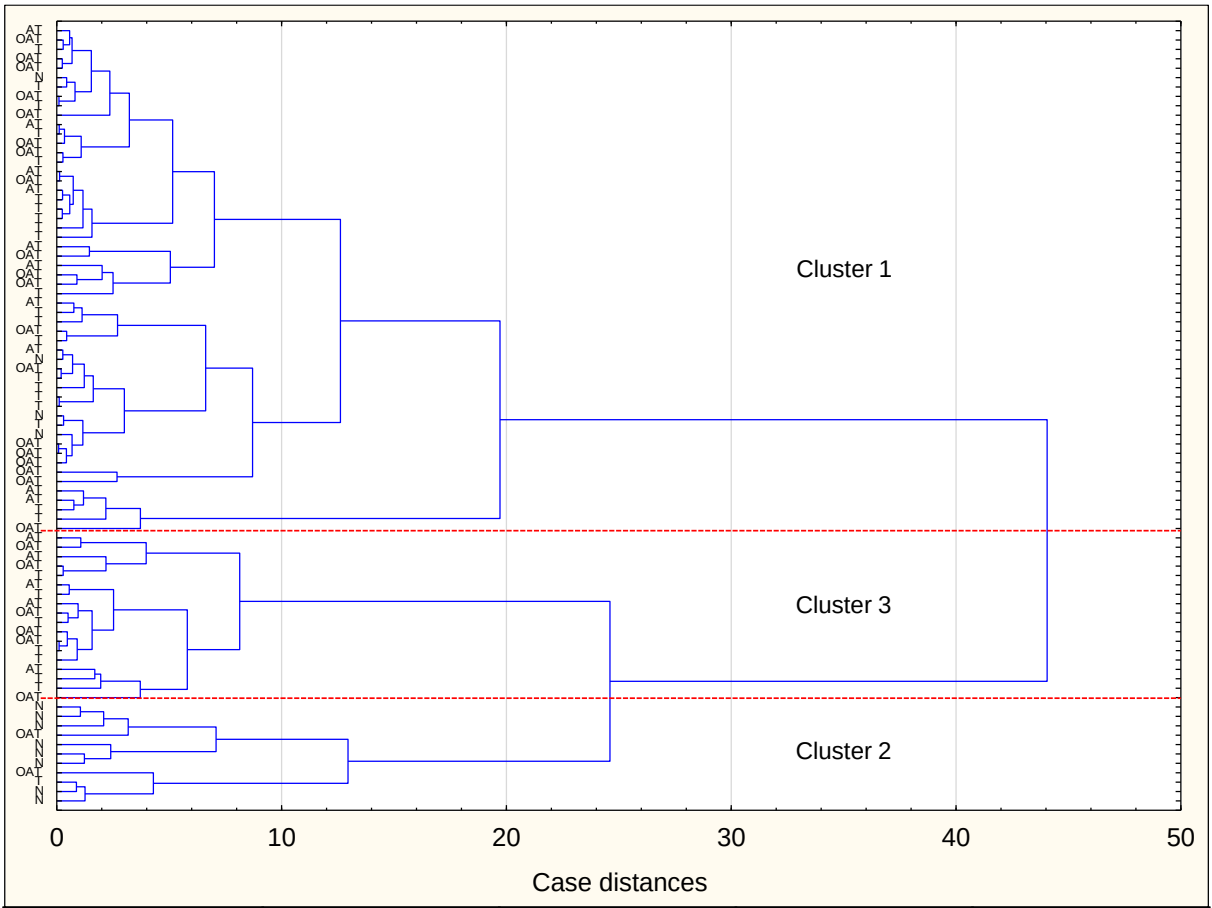


Figure 1S. *Schematic representation of lectin-ELISA procedure.* **A** - ELISA plate with goat anti-human CLU antibody capture; **B** - oxidation of oligosaccharides of anti-human clusterin polyclonal antibody with sodium meta-periodate solution (represented by 'IO_4^-' pictogram); * this IO_4^- step was performed in case of LCA reactivity detection; **C** - blocking of free binding sites using BSA blocking buffer; **D** - overnight incubation of ELISA plate with BSA blocking buffer; **E** - seminal or serum clusterin incubation (marked as 'C' pictogram); **F** - clusterin reduction (using dithiotreitol, DTT); **G** - fucose recognition by specific biotinylated lectins; **H** - fucose-lectin complexes incubation with ExtrAvidin (labeled with alkaline phosphatase); **I** - detection of fucose-lectin complexes using disodium para-nitrophenyl phosphate. Each incubation step was performed in standardized conditions with gentle shaking. Between each lectin-ELISA step three times washing was performed using washing buffer 10 mM TBS 0.1% Tween20, pH=7.5 (except washing after step B and F, where 10 mM TBS, pH=7.5 was used).



Group / Cluster No	N (n=17)	T (n=32)	AT (n=16)	OAT (n=28)
	The number of samples (percentage participation in whole group)			
3 (n=7)	3 (42.9%)	1 (14.3%)	1 (14.3%)	2 (28.5%)
2 (n=44)	7 (15.9%)	9 (20.5%)	3 (6.8%)	25 (56.8%)
1 (n=42)	7 (16.6%)	22 (52.4%)	12 (28.6%)	1 (2.4%)

Figure 2S. *Dendrogram of cluster analysis of seminal plasma samples*. The cluster analysis was performed for parameters which simultaneously comply the following criteria: they allow for the differentiation of study groups and in the ROC curve analysis had moderate or high clinical value (AUC ≥0.706). Each seminal plasma sample is represented by a vector of three parameters CLU, FUT3 and FUT4.



Group / Cluster No	N (n=12)	T (n=29)	AT (n=15)	OAT (n=27)
	The number of samples (percentage participation in whole group)			
3 (n=18)	0 (0%)	7 (38.9%)	5 (27.8%)	6 (33.3%)
2 (n=11)	8 (72.7%)	1 (9.1%)	0 (0%)	2 (18.2%)
1 (n=54)	4 (7.4%)	21 (38.9%)	10 (18.5%)	19 (35.2%)

Figure 3S. Dendrogram of cluster analysis of serum samples. The cluster analysis was performed for parameters which simultaneously comply the following criteria: they allow for the differentiation of study groups and in the ROC curve analysis had moderate or high clinical value (AUC ≥0.710). Each serum sample is represented by a vector of four parameters: CLU, FUT4, UEA and LCA.

Table 1S. The correlations between analyzed seminal plasma parameters and selected criteria of standard semen analysis

Parameter	**total sperm number** (mln per ejaculate)	**sperm progressive motility** (%)	**normal sperm morphology** (%)
CLU (ng/mL)	*r*= -0.409 *p*= 0.001	*r*= -0.317 *p*= 0.009	NS
FUT3 (ng/mL)	NS	NS	*r*= -0.300 *p*= 0.014
FUT4 (ng/mL)	NS	NS	NS
LTA (AU)	NS	NS	NS
UEA (AU)	NS	NS	*r*= 0.270 *p*= 0.027
LCA (AU)	NS	NS	NS

CLU - CLU concentration; FUT3 - FUT3 concentration; FUT4 - FUT4 concentration; LTA - relative reactivity of CLU glycans with *Lotus tetragonolobus* agglutinin; UEA - relative reactivity of CLU glycans with *Ulex europaeus agglutinin*; LCA - relative reactivity of CLU glycans with *Lens culinaris* agglutinin. NS - not significant. A two-tailed p-Value of less than 0.05 was considered significant.

15.3. Załącznik 3 – II artykuł oryginalny:

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The Association between Clusterin Sialylation Degree and Levels of Oxidative–Antioxidant Balance Markers in Seminal Plasmas and Blood Sera of Male Partners with Abnormal Sperm Parameters

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Abstract: Nearly 30% of infertility cases are caused by male factor. This study aimed at checking the associations between the sialylation degree of glycoprotein clusterin (CLU) and levels of oxidative–antioxidant balance markers in infertile men. Using lectin-ELISA with biotinylated lectins specific to α2,6-linked (*Sambucus nigra* agglutinin, SNA) and α2,3-linked (*Maackia amurensis* agglutinin, MAA) sialic acid (SA), the CLU sialylation in 132 seminal plasmas (SP) and 91 blood sera (BS) were analyzed. Oxidative–antioxidant status was measured by determining Sirtuin-3 (SIRT3), Sirtuin-5 (SIRT5), total antioxidant status (TAS), and ferric reducing antioxidant power (FRAP) levels. We indicate that multiple sperm disorders are associated with decreased expression of MAA-reactive SA in SP. Decreased SP SIRT3 concentrations may be associated with teratozoospermia and oligoasthenoteratozoospermia. ROC curve and cluster analysis revealed that SP relative reactivity of CLU glycans with MAA, the value of MAA/SNA ratio, and SIRT3 and SIRT5 concentrations may constitute an additional set of markers differentiating infertile oligoasthenoteratozoospermic patients (OAT) from normozoospermic (N), asthenoteratozoospermic (AT) and teratozoospermic (T). The multinomial logistic regression analysis confirmed the potential utility of SIRT3 determinations for differentiation between N and OAT groups as well as between N and T groups for SIRT3 and SIRT5. For BS, based on ROC curve and cluster analysis, relative reactivities of CLU glycans with SNA, MAA, SIRT3 and FRAP concentrations may be useful in the differentiation of normozoospermic patients from those with sperm disorders. The multinomial logistic regression analysis showed that the SNA relative reactivity with CLU glycans significantly differentiated the N group from AT, OAT and T groups, and FRAP concentrations significantly differed between N and AT groups, which additionally confirms the potential utility of these biomarkers in the differentiation of infertile patients with abnormal sperm parameters. The knowledge about associations between examined parameters may also influence future research aimed at seeking new male infertility therapies.

Keywords: clusterin; clusterin sialylation; markers of oxidative–antioxidant balance; male fertility disorders; male infertility diagnostics

1. Introduction

Infertility is a disease of the reproductive system defined as the failure to achieve a clinical pregnancy after 12 months, or more, of regular unprotected sexual intercourse [1]. It is estimated that globally, nearly 30% of infertility cases are caused by male factor alone [2,3]. Routinely performed semen analysis points toward predominantly spermatozoa parameters (such as total count, motility, morphology, etc.); however, the early and sensitive biomarker of this disorder is still missing [4]. Almost 15% of infertile men are regarded as idiopathic with semen parameters within reference values [5,6]. Further investigations on molecular level, concerning this disease, are needed, and they may shed some new light on the problem of decreased male fertility issue, especially the idiopathic one.

Oxidative stress (OS) is defined as an imbalance between the physiological antioxidant mechanisms and reactive oxygen species (ROS) production [7]. In the male reproductive tract, a certain amount of superoxide anion is essential not only for proper capacitation, hyper-activation processes, and acrosome reaction but also for gamete fusion [8,9]. Nevertheless, many studies proved that ROS are one of the most common causes of decreased male fertility, affecting DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) stability and lipid membrane peroxidation [10,11]. High ROS levels are often associated with decreased male fertility, confirmed also by sperm cells disorders, such as viability, motility and morphology [12,13]. In the case of excess ROS, seminal plasma releases a range of antioxidants that belong both to the non-enzymatic and enzymatic group [14,15].

Human ejaculate consists of abundant ions, lipids and glycoproteins taking part in a range of processes leading to the sperm cells' production and maturation [16,17]. More than 6000 proteins and glycoproteins in human seminal plasma have been identified thus far [18], and clusterin (CLU, also known as Apolipoprotein J, ApoJ) was distinguished by Milardi et al. [19] as one of the main seminal plasma glycoproteins. This glycoprotein contains approximately 30% of carbohydrates and in its secretory isoform plays an important role in the male reproductive tract [20]. CLU is engaged in the semen liquefaction process via contact with eppin (epididymal protease inhibitor) present on the spermatozoa surface [21]. Seminal plasma CLU is liable for immune tolerance for male antigens in the female reproductive tract [22–24]. Taking into account the fact that CLU possesses at least four N-glycosylation sites as well as the importance of glycosylation in the sperm–oocyte interactions, the exploration of CLU glycans expression seems to be an important direction of study concerning male infertility issues [25,26]. Saraswat et al. [25] proposed over 40 complex type glyco-variants of CLU with terminal sialic acid (SA) or galactose. CLU glycans contained fucose, Le^x/Le^a, blood group H or Le^y/Le^b oligosaccharide structures. Sialylation, attaching with sialic acids (group of chemical compounds represented by N-acetylneuraminic acid, Neu5Ac/NANA), is one of the most common posttranslational modifications. NANA affects the physiochemical and biological properties of glycoproteins, protects them against catabolic processes and takes part in immune recognition [27]. Rohne et al. [28] documented that the proteolytic cleavage of clusterin does not impact its chaperone activity, but only the completely glycosylated form of CLU is able to perform its chaperone activity, crucial for cell protection against destroying factors such as ionizing radiation, oxidative agents and others contributing to the oxidative–antioxidant imbalance [28]. The properties of CLU are similar to small heat shock proteins—it can bind unfolded proteins and block their aggregation regardless of the presence of ATP. This feature is particularly important in the DNA oxidation sperm damage context [29].

Sirtuins (SIRTs) are part of group of enzymes with activity of nicotinamide adenine dinucleotide (NAD^+) deacetylases, existing in all cells' compartments, taking part not only in damaged DNA reparation, but also in many processes connected with oxidative–antioxidant balance, cell cycle and metabolism regulation, differentiation, growth and apoptosis [30,31]. SIRT3 and SIRT5 concentrations are strictly associated with oxidative–antioxidant balance. SIRT3 occurs in the nucleus in its full form and is translocated to the mitochondria as a response for stress factors, i.a., mtDNA reparation and assurance of

mitochondria integrity, but also for protecting the cell from apoptosis under conditions of oxidative stress [32]. SIRT5 may be located in the mitochondria, cytoplasm and nucleus, and it plays an important role in the apoptosis pathway, energy production, detoxification, cellular metabolism and oxidative stress regulation [31,33]. The mitochondrial form of this protein takes part in the protein modifications such as demalonylation, deacetylation, and desuccinylation [34,35]. It has been proven that cells transfected with SIRT5 have decreased ROS levels, suggesting that this enzyme suppresses excessive ROS production [36].

Taking into account the multidirectional role of CLU in human seminal plasma, including oxidative stress regulation via its chaperone activity and sperm cell protection against negative effects of oxidative stress, as well as the well-known biological role of glycoprotein glycosylation in a variety of processes in the human body, we decided to investigate the type and expression of sialic acid in seminal plasma and serum CLU glycans together with the expression of selected parameters of oxidative–antioxidant balance, and to check if there are any associations between the profile and degree of CLU sialylation and levels of oxidative stress markers such as seminal plasma and blood serum SIRT3 and SIRT5 concentrations. For antioxidant capacity assessment, total antioxidant status (TAS) and ferric reducing antioxidant power (FRAP) were selected. The potential use of the determinations of the selected parameters of oxidative–antioxidant balance in the diagnostics of male infertility as well as to expand knowledge about associations between examined parameters that may be helpful in developing new therapeutic strategies for male infertility, especially caused by multiple overlapping factors, were also in the sphere of our interests.

2. Results

The values of examined parameters are shown in Table 1.

Table 1. The values of seminal plasma and blood serum parameters analyzed in groups of patients with fertility problems.

Parameter	AT n^{PL} = 22 n^{S} = 15 Median (IQR)	N n^{PL} = 43 n^{S} = 18 Median (IQR)	OAT n^{PL} = 29 n^{S} = 27 Median (IQR)	T n^{PL} = 38 n^{S} = 31 Median (IQR)
SNA^{PL} (AU)	0.203 (0.148–0.244)	0.199 (0.132–0.296)	0.184 (0.133–0.299)	0.202 (0.114–0.266)
MAA^{PL} (AU)	0.933 (0.226–1.680)	0.376 ♦ (0.177–1.348)	0.171 †* (0.098–0.214)	0.740 (0.354–1.476)
MAA^{PL}/SNA^{PL}	4.455 (1.256–7.423)	1.731 (0.460–4.634)	0.756 †* (0.377–1.513)	3.781 (1.436–8.082)
$SIRT3^{PL}$ (ng/mL)	10.90 †♦ (9.23–12.32)	9.35 †♦ (5.68–11.11)	2.11 (1.76–2.42)	2.64 (2.11–3.67)
$SIRT5^{PL}$ (ng/mL)	5.72 (4.72–8.31)	7.28 ♦ (1.67–7.97)	1.34 †* (1.17–1.49)	6.89 (1.38–7.72)
TAS^{PL} (mM)	1.79 (1.59–1.99)	1.71 (1.54–1.97)	1.70 (1.51–1.93)	1.72 (1.55–1.85)
$FRAP^{PL}$ (mM)	3.68 (3.27–4.14)	3.65 (2.85–4.37)	3.04 (2.45–3.81)	3.72 (2.93–4.40)
SNA^{S} (AU)	0.412 (0.366–0.477)	0.881 †*♦ (0.630–1.140)	0.495 (0.368–0.613)	0.428 (0.356–0.525)
MAA^{S} (AU)	0.016 (0.002–0.074)	0.081 (0.054–0.106)	0.020 (0.002–0.086)	0.035 (0.004–0.072)

MAA^{S}/SNA^{S}	0.034 (0.005–0.152)	0.081 (0.064–0.122)	0.036 (0.005–0.167)	0.085 (0.012–0.159)
SIRT3^{S} (ng/mL)	8.94 (6.58–19.15)	2.73 *♦ (1.61–7.35)	6.27 (5.54–15.07)	5.93 (4.29–11.53)
SIRT5^{S} (ng/mL)	2.25 (2.06–3.22)	2.01 (1.39–2.53)	2.02 (1.84–4.11)	2.05 (1.61–3.27)
TAS^{S} (mM)	1.37 (1.26–1.82)	1.54 (1.42–1.70)	1.40 (1.32–1.52)	1.38 (1.29–1.75)
FRAP^{S} (mM)	1.20 (1.00–1.35)	1.49 * (1.43–1.59)	1.33 (1.10–1.55)	1.38 (1.22–1.56)

SNA^{PL}—relative reactivity of seminal plasma CLU glycans with *Sambucus nigra* agglutinin; MAA^{PL}—relative reactivity of seminal plasma CLU glycans with *Maackia amurensis* agglutinin; MAA^{PL}/SNA^{PL}—seminal plasma sialylation ratio; SIRT3^{PL}—seminal plasma SIRT3 concentration; SIRT5^{PL}—seminal plasma SIRT5 concentration; TAS^{PL}—seminal plasma total antioxidant status; FRAP^{PL}—seminal plasma ferric reducing antioxidant power; SNA^{S}—relative reactivity of serum CLU glycans with *Sambucus nigra* agglutinin; MAA^{S}—relative reactivity of serum CLU glycans with *Maackia amurensis* agglutinin; MAA^{S}/SNA^{S}—serum sialylation ratio; SIRT3^{S}—serum SIRT3 concentration; SIRT5^{S}—serum SIRT5 concentration; TAS^{S}—serum total antioxidant status; FRAP^{S}—serum ferric reducing antioxidant power. AT—asthenoteratozoospermia, N—normozoospermia, OAT—oligoasthenoteratozoospermia, T—teratozoospermia. n^{PL} and n^{S}—number of seminal plasma and serum samples, respectively. Significant differences vs.: † T group, * AT group, ♦ OAT group. Taking into account the Bonferroni correction, a two-tailed p value of less than 0.008 was considered significant.

2.1. Sialic Acids Expression in the Glycans of Serum and Seminal Plasma Clusterin

There were no significant differences between seminal plasma groups in relative reactivities of CLU glycans with SNA (median values: AT group: 0.203 AU, N group: 0.199 AU, OAT group: 0.184 AU, T group: 0.202 AU) (Table 1). Relative reactivities of CLU glycans with MAA in seminal plasmas were significantly lower in the OAT group (median value: 0.171 AU) in comparison to the N (median value: 0.376 AU, $p = 0.005120$), T (median value: 0.740 AU, $p < 0.000001$) and AT group (median value: 0.933 AU, $p = 0.000003$; Table 1).

Relative reactivities of serum CLU glycans with SNA of normozoospermic patients were significantly higher (median value: 0.881 AU) in comparison to the other examined groups: teratozoospermic (median value: 0.428 AU, $p = 0.000009$), asthenoteratozoospermic (median value: 0.412 AU, $p = 0.000073$) and oligoasthenoteratozoospermic (median value: 0.495 AU, $p = 0.000123$) (Table 1). There were no significant differences between serum groups in relative reactivities of CLU glycans with MAA (median values: AT group: 0.016 AU, N group: 0.081 AU, OAT group: 0.020 AU, T group: 0.035 AU) (Table 1).

The values of sialylation ratio (MAA/SNA) for seminal plasma samples were significantly lower in the OAT group (median value: 0.756) in comparison to the T (median value: 3.781, $p = 0.000005$) and AT group (median value: 4.455, $p = 0.000054$; Table 1). No significant differences between serum groups in the values of sialylation ratio were found, and the median values were following: 0.034 AU in AT group, 0.081 AU in N group, 0.036 AU in OAT group and 0.085 AU in T group (Table 1).

The results of the correlation analysis between parameters investigated in seminal plasmas and sera are summarized in Figure 1A–L, which present only significant correlations. The comparison between seminal plasma MAA/SNA ratio and SNA relative reactivity with CLU glycans showed the presence of significant negative correlation between both parameters ($r = -0.5556$; $p < 0.001$; Figure 1A). The significant positive correlations between values of sialylation ratio and MAA relative reactivities with CLU glycans were found for seminal plasma and blood serum ($r = 0.8698$; $p < 0.001$ and $r = 0.9529$; $p < 0.001$, respectively; Figure 1B,C).

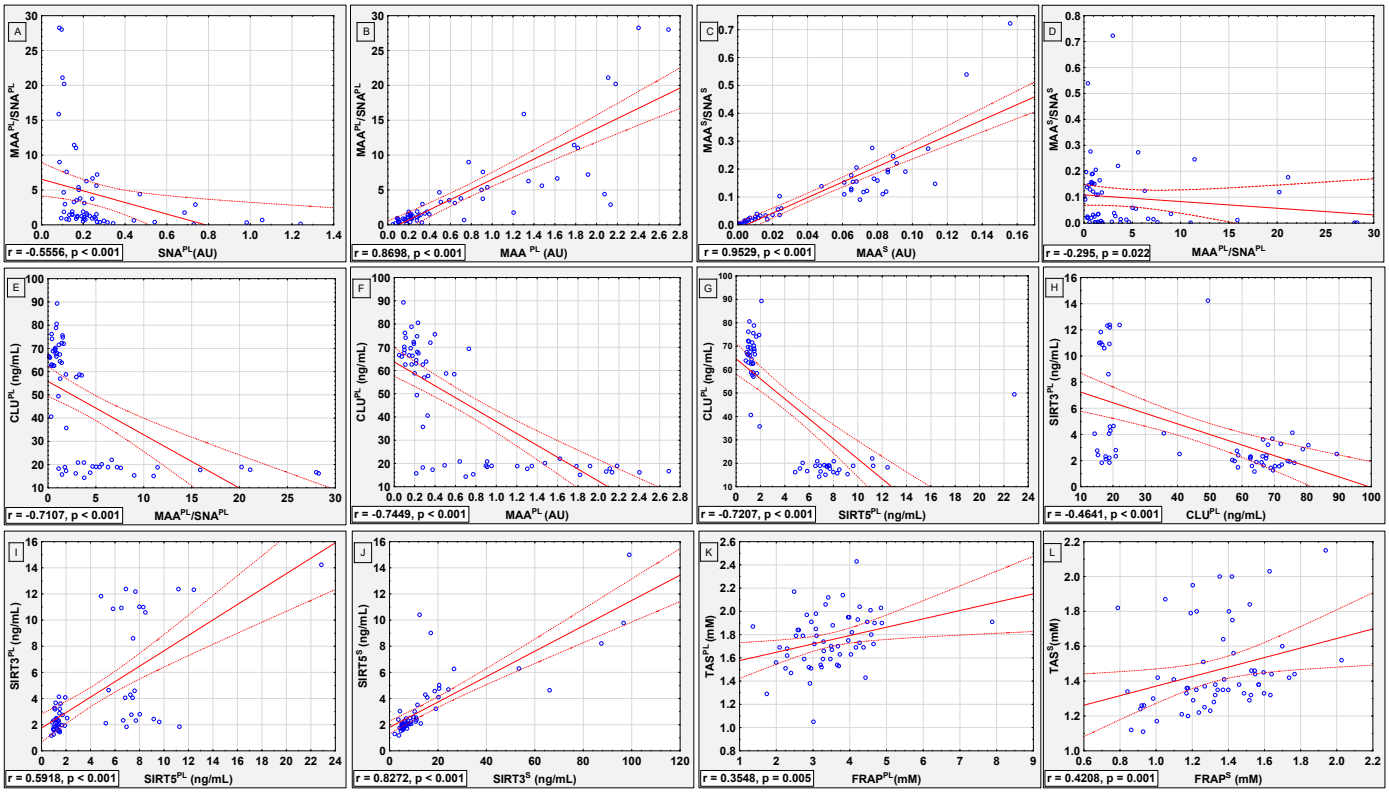

Figure 1. The correlations between selected parameters in seminal plasma (**A**,**B**,**E**–**I**,**K**) and blood serum (**C**,**J**,**L**) and comparison of MAA/SNA sialylation ratio between both biological fluids (**D**). MAA^{PL}/SNA^{PL}-seminal plasma sialylation ratio; SNA^{PL}-relative reactivity of seminal plasma CLU glycans with *Sambucus nigra* agglutinin; MAA^{PL}-relative reactivity of seminal plasma CLU glycans with *Maackia amurensis* agglutinin; MAA^{S}/SNA^{S}-serum sialylation ratio; MAA^{S}-relative reactivity of serum CLU glycans with *Maackia amurensis* agglutinin; CLU^{PL}-seminal plasma CLU concentration; SIRT5^{PL}-seminal plasma SIRT5 concentration; SIRT3^{PL}-seminal plasma SIRT3 concentration; SIRT5^{S}-serum SIRT5 concentration; SIRT3^{S}-serum SIRT3 concentration; TAS^{PL}-seminal plasma total antioxidant status; FRAP^{PL}-seminal plasma ferric reducing antioxidant power; TAS^{S}-serum total antioxidant status; FRAP^{S}-serum ferric reducing antioxidant power. The red dashed line points 95% of confidence interval. A two-tailed *p* value of less than 0.05 was considered significant.

2.2. The Comparison of Sialylation Profile in the Seminal Plasma and Blood Serum

There were no significant correlations between both biological fluids analyzed by us in the relative expression of MAA- and SNA-reactive sialic acid in CLU glycans; however, a weak negative correlation between values of sialylation ratio (MAA/SNA) was found (r= −0.2950; $p = 0.022$, Figure 1D).

2.3. SIRTs Concentrations

Seminal plasma SIRT3 concentrations were significantly higher in the N group (median value: 9.35 ng/mL) in comparison to the T (median value: 2.64 ng/mL; $p < 0.000001$) and OAT group (median value: 2.11 ng/mL; $p < 0.000001$). Similar differences were observed between the AT group (median value: 10.90 ng/mL) vs. the T and OAT groups with significances of $p = 0.000001$ and $p < 0.000001$, respectively (Table 1). SIRT3 concentrations in sera were significantly lower in the N group (median value: 2.73 ng/mL) in comparison to the asthenoteratozoospermic (median value: 8.94 ng/mL; $p = 0.000859$) and oligoasthenoteratozoospermic (median value: 6.27 ng/mL; $p = 0.002728$) groups. The median value of serum SIRT3 concentrations in the teratozoospermic group was 5.93 ng/mL (Table 1).

Seminal plasma SIRT5 concentrations were significantly lower in the OAT group (median value: 1.34 ng/mL) in comparison to the N (median value: 7.28 ng/mL, $p =$

0.000019), T (median value: 6.89 ng/mL, $p = 0.000423$) and AT group (median value: 5.72 ng/mL, $p = 0.000001$; Table 1). No significant differences for serum SIRT5 concentrations were found. Median values of serum SIRT5 concentrations were following: 2.25 ng/mL in AT group, 2.01 ng/mL in N group, 2.02 ng/mL in OAT group and 2.05 ng/mL in T group (Table 1).

2.4. TAS Measurement

No significant differences for concentrations of seminal plasma and serum TAS were found (Table 1). Median values for seminal plasma TAS levels were the following: 1.79 mM in AT group, 1.71 mM in N group, 1.70 mM in OAT group and 1.72 mM in T group. Median values of serum TAS concentrations were: 1.37 mM in AT group, 1.54 mM in N group, 1.40 mM in OAT group and 1.38 mM in T group. (Table 1).

2.5. FRAP Determination

No significant differences in values of seminal plasma ferric reducing antioxidant capacity (FRAP) between analyzed groups were found (Table 1). Median seminal plasma FRAP values were as follow: 3.68 mM in AT group, 3.65 mM in N group, 3.04 mM in OAT group and 3.72 mM in T group. FRAP values in sera were significantly higher in the N group (median value: 1.49 mM) in comparison to the AT group (median value: 1.20 mM) with significancy of $p = 0.005187$. The median values of serum FRAP level in OAT and T groups were 1.33 and 1.38 mM, respectively (Table 1).

2.6. ROC Curves Analysis

The receiver operating characteristic curves were performed for all determined seminal plasma and serum parameters. Two ways of arranging the obtained results for ROC curves analysis were used, namely, the levels of each parameter were compared between: (1) two groups of patients distinguished on the basis of standard semen analysis (Table 2 and Table 3 for seminal plasma and blood serum, respectively); (2) each group of patients distinguished on the basis of seminogram analysis versus all other patients treated as one group (Supplementary Materials, Tables S2 and S3 for seminal plasma and blood serum, respectively). Based on the AUC, the clinical value of laboratory test can be defined as: 0–0.5—zero, 0.5–0.7—limited, 0.7–0.9—moderate and >0.9—high [37]. We used an AUC of ≥0.7, $p < 0.05$, as the criterion demonstrating the moderate clinical value of an examined parameter.

Table 2. Summary of the results of receiver operating characteristic (ROC) curves analysis for seminal plasma parameters.

Parameter	Compared Groups		AUC	AUC with 95% Confidence Interval	Cut Off Point	Sensitivity	Specificity	*p*
SNA^{PL}	AT	vs. N	0.489	0.319–0.658	0.140	0.833	0.310	0.894
	OAT	vs. N	0.512	0.361–0.662	0.155	0.690	0.310	0.877
	T	vs. N	0.479	0.333–0.624	0.165	0.676	0.414	0.774
	AT	vs. T	0.504	0.342–0.667	0.12	0.889	0.265	0.961
	OAT	vs. T	0.487	0.341–0.633	0.155	0.414	0.735	0.859
	OAT	vs. AT	0.503	0.333–0.673	0.147	0.379	0.722	0.974
MAA^{PL}	AT	vs. N	0.611	0.446–0.776	0.911	0.556	0.690	0.186
	OAT	vs. N	0.715	0.571–0.858	0.224	0.793	0.724	0.003
	T	vs. N	0.375	0.229–0.521	2.111	1.000	0.103	0.094
	AT	vs. T	0.484	0.303–0.666	0.226	0.278	0.912	0.867
	OAT	vs. T	0.890	0.802–0.979	0.224	0.793	0.912	0.000
	OAT	vs AT	0.883	0.786–0.980	0.214	0.759	0.944	0.000
MAA^{PL}/SNA^{PL}	AT	vs. N	0.632	0.473–0.792	0.837	1.000	0.345	0.104
	OAT	vs. N	0.671	0.531–0.811	1.545	0.793	0.552	0.017
	T	vs. N	0.639	0.495–0.782	3.285	0.588	0.724	0.058
	AT	vs. T	0.513	0.348–0.679	6.000	0.444	0.706	0.877

	OAT	vs. T	0.836	0.738–0.934	2.033	0.897	0.676	0.000
	OAT	vs. AT	0.837	0.724–0.950	0.824	0.552	1.000	0.000
SIRT3^{PL}	AT	vs. N	0.624	0.472–0.776	10.859	0.636	0.649	0.110
	OAT	vs. N	0.951	0.905–0.996	2.500	0.821	0.946	0.000
	T	vs. N	0.887	0.810–0.965	4.645	1.000	0.757	0.000
	AT	vs. T	0.906	0.813–0.999	8.604	0.818	1.000	0.000
	OAT	vs. T	0.646	0.500–0.791	2.500	0.821	0.533	0.050
	OAT	vs. AT	0.948	0.882–1.000	7.985	1.000	0.818	0.000
SIRT5^{PL}	AT	vs. N	0.491	0.330–0.653	5.840	0.324	0.455	0.917
	OAT	vs. N	0.812	0.698–0.926	2.084	0.964	0.730	0.000
	T	vs. N	0.543	0.409–0.677	7.728	0.778	0.351	0.527
	AT	vs. T	0.460	0.302–0.617	5.840	0.545	0.639	0.616
	OAT	vs. T	0.759	0.631–0.887	2.084	0.964	0.694	0.000
	OAT	vs. AT	0.916	0.829–1.000	2.084	0.964	0.818	0.000
TAS^{PL}	AT	vs. N	0.559	0.409–0.708	1.56	0.909	0.324	0.440
	OAT	vs. N	0.523	0.370–0.676	1.98	0.875	0.235	0.765
	T	vs. N	0.455	0.316–0.594	1.62	0.694	0.382	0.525
	AT	vs. T	0.618	0.468–0.768	1.53	1.000	0.250	0.124
	OAT	vs. T	0.466	0.308–0.624	1.38	0.167	0.972	0.671
	OAT	vs. AT	0.580	0.412–0.747	1.52	0.292	1.000	0.352
FRAP^{PL}	AT	vs. N	0.509	0.365–0.653	3.393	0.727	0.442	0.902
	OAT	vs. N	0.629	0.498–0.759	3.141	0.586	0.698	0.054
	T	vs. N	0.497	0.369–0.626	2.251	0.972	0.116	0.969
	AT	vs. T	0.495	0.344–0.646	3.393	0.727	0.417	0.948
	OAT	vs. T	0.641	0.505–0.778	3.678	0.724	0.528	0.043
	OAT	vs. AT	0.345	0.192–0.497	4.585	0.103	0.955	0.046

SNA^{PL}—relative reactivity of seminal plasma CLU glycans with *Sambucus nigra* agglutinin; MAA^{PL}—relative reactivity of seminal plasma CLU glycans with *Maackia amurensis* agglutinin; MAA^{PL}/SNA^{PL}—seminal plasma CLU sialylation ratio; SIRT3^{PL}—seminal plasma SIRT3 concentration; SIRT5^{PL}—seminal plasma SIRT5 concentration; TAS^{PL}—seminal plasma total antioxidant status; FRAP^{PL}—seminal plasma ferric reducing antioxidant power. AT—asthenoteratozoospermia, N—normozoospermia, OAT—oligoasthenoteratozoospermia, T—teratozoospermia. Area under the ROC curve (AUC) is given with 95% confidence interval. Data with AUC equal or greater than 0.715 are marked in grey. Based on the AUC, the clinical value of the laboratory test can be defined as: 0–0.5—zero, 0.5–0.7—limited, 0.7–0.9—moderate and >0.9—high. An AUC of ≥0.7, $p < 0.05$, was used as the criterion demonstrating the moderate clinical value of an examined parameter.

Table 3. Summary of receiver operating characteristic (ROC) curves for serum parameters.

Parameter	Compared Groups		AUC	AUC with 95% Confidence Interval	Cut Off Point	Sensitivity	Specificity	*p*
SNA^{S}	AT	vs. N	0.892	0.774–1.000	0.609	0.867	0.813	0.000
	OAT	vs. N	0.837	0.703–0.971	0.694	0.889	0.688	0.000
	T	vs. N	0.872	0.748–0.996	0.591	0.871	0.813	0.000
	AT	vs. T	0.549	0.367–0.732	0.477	0.800	0.419	0.600
	OAT	vs. T	0.575	0.423–0.728	0.532	0.444	0.774	0.334
	OAT	vs. AT	0.615	0.436–0.794	0.437	0.667	0.667	0.208
MAA^{S}	AT	vs. N	0.756	0.582–0.930	0.023	0.600	0.875	0.004
	OAT	vs. N	0.650	0.480–0.821	0.024	0.556	0.875	0.083
	T	vs. N	0.716	0.551–0.880	0.035	0.516	0.875	0.010
	AT	vs. T	0.544	0.364–0.724	0.023	0.600	0.613	0.632
	OAT	vs. T	0.481	0.327–0.636	0.024	0.556	0.581	0.814
	OAT	vs. AT	0.556	0.379–0.732	0.105	0.222	1.000	0.537
MAA^{S}/SNA^{S}	AT	vs. N	0.577	0.355–0.799	0.055	0.600	0.813	0.496
	OAT	vs. N	0.534	0.356–0.711	0.036	0.519	0.875	0.711
	T	vs. N	0.510	0.341–0.679	0.138	0.355	0.938	0.907
	AT	vs. T	0.546	0.372–0.720	0.055	0.600	0.548	0.602
	OAT	vs. T	0.514	0.363–0.666	0.036	0.519	0.581	0.853
	OAT	vs. AT	0.522	0.343–0.701	0.02	0.741	0.400	0.808
SIRT3^{S}	AT	vs. N	0.830	0.690–0.969	4.979	1.000	0.611	0.000
	OAT	vs. N	0.761	0.605–0.918	5.000	1.000	0.611	0.001

	T	vs. N	0.699	0.535–0.863	3.647	0.871	0.611	0.018
	AT	vs. T	0.740	0.595–0.884	6.044	0.933	0.516	0.001
	OAT	vs. T	0.621	0.477–0.765	5.000	1.000	0.290	0.010
	OAT	vs. AT	0.607	0.433–0.782	6.010	0.444	0.933	0.229
SIRT5^S	AT	vs. N	0.663	0.476–0.850	2.035	0.867	0.500	0.088
	OAT	vs. N	0.382	0.207–0.556	2.063	0.593	0.500	0.185
	T	vs. N	0.525	0.352–0.698	1.414	0.871	0.278	0.776
	AT	vs. T	0.647	0.492–0.803	1.798	1.000	0.387	0.064
	OAT	vs. T	0.418	0.269–0.566	2.019	0.556	0.548	0.277
	OAT	vs. AT	0.607	0.435–0.780	2.019	0.556	0.867	0.221
TAS^S	AT	vs. N	0.637	0.427–0.847	1.38	0.600	0.833	0.200
	OAT	vs. N	0.687	0.527–0.848	1.42	0.630	0.722	0.022
	T	vs. N	0.639	0.477–0.801	1.38	0.581	0.833	0.094
	AT	vs. T	0.514	0.322–0.706	1.28	0.333	0.806	0.886
	OAT	vs. T	0.501	0.349–0.653	1.64	0.926	0.290	0.988
	OAT	vs. AT	0.516	0.312–0.720	1.30	0.852	0.333	0.878
FRAP^S	AT	vs. N	0.780	0.606–0.953	1.352	0.800	0.778	0.002
	OAT	vs. N	0.644	0.477–0.811	1.429	0.667	0.778	0.090
	T	vs. N	0.618	0.453–0.784	1.422	0.581	0.778	0.162
	AT	vs. T	0.701	0.531–0.871	1.208	0.600	0.774	0.021
	OAT	vs. T	0.566	0.410–0.721	1.168	0.370	0.903	0.408
	OAT	vs. AT	0.620	0.444–0.795	1.372	0.481	0.800	0.181

SNA^S—relative reactivity of serum CLU glycans with *Sambucus nigra* agglutinin; MAA^S—relative reactivity of serum CLU glycans with *Maackia amurensis* agglutinin; MAA^S/SNA^S—serum sialylation ratio; SIRT3^S—serum SIRT3 concentration; SIRT5^S−serum SIRT5 concentration; TAS^S—serum total antioxidant status; FRAP^S—serum ferric reducing antioxidant power. AT—asthenoteratozoospermia, N—normozoospermia, OAT—oligoasthenoteratozoospermia, T—teratozoospermia. Area under the ROC curve (AUC) is given with 95% confidence interval. Data with AUC equal or greater than 0.701 are marked in grey. Based on the AUC, the clinical value of the laboratory test can be defined as: 0–0.5—zero, 0.5–0.7—limited, 0.7–0.9—moderate and >0.9—high. An AUC of ≥0.7, $p < 0.05$, was used as the criterion demonstrating the moderate clinical value of an examined parameter.

2.7. Cluster Analysis

During the preliminary cluster analyses of seminal plasma and blood serum data, we performed the analyses for all parameters in many variants, starting from the complete data (results not shown), which gave us the possibility to check for whether the parameters selected for the cluster analysis were a good choice. Finally, we decided that seminal plasma parameters chosen for the cluster analysis should comply with the following criteria: they differentiated the study groups with a two-tailed p value of less than 0.05 considered as significant (Table S1) and in the ROC curve analysis had moderate or high clinical value (AUC ≥ 0.700, $p < 0.05$) when the levels of given parameter were compared between two groups of patients distinguished on the basis of standard semen analysis (AUC ≥ 0.715, Table 2). To perform cluster analysis for seminal plasma, four parameters were selected: CLU relative reactivity with MAA, sialylation ratio (MAA/SNA) as well as SIRT3 and SIRT5 concentrations. Selected parameters also met the assumed selection criteria in the ROC curve analysis, in which each group of patients distinguished on the basis of seminogram analysis was compared with all other patients treated as one group (Table S2). The analysis was performed for 100 seminal plasma samples. The whole distance (100%) was considered at 35 value on the x-axis. At 91.6% distance, all samples could be regarded as homogenous formation (the value 32.1 on the x-axis). The first cluster was distinguished at 53.2% distance (the value 18.6 on the x-axis) and constitutes 11 samples. The second cluster could be distinguished at 52.8% distance (the value 18.5 on the x-axis) and is composed of two AT samples. The third cluster could be distinguished at 29.6% distance (the value 10.4 on the x-axis) and comprised 50% asthenoteratozoospermic samples (9 from 18 chosen for the analysis) and 40% normozoospermic samples (10 from 25 chosen for the analysis). Cluster no. 4 and 5 were

distinguished at 29% and 26.4% distance, respectively (values on the *x*-axis: 10.2 and 9.2 on the *x*-axis, respectively). In cluster 4, there were 17 samples, and all of them were teratozoospermic (56.7% of T samples chosen for the analysis). Cluster 5 comprised 100% of analyzed OAT samples (Figure S1, Table S4).

In the case of serum, cluster analysis was performed for four parameters that differentiated the study groups with a two-tailed *p* value of less than 0.05 considered as significant (Table S1) and in the ROC curve analysis had moderate or high clinical value with AUC ≥0.701 when the levels of the given parameter were compared between two groups of patients distinguished on the basis of standard semen analysis: CLU relative reactivities with SNA and MAA, SIRT3 and FRAP concentrations (Table 3). Three out of four selected parameters (CLU relative reactivities with SNA and MAA, and SIRT3 concentrations) also met the assumed selection criteria when in the ROC curve analysis. Each group of patients distinguished on the basis of seminogram analysis was compared with all other patients treated as one group. Although this way of analyzing the ROC curves resulted in AUC for the FRAP concentration values being below 0.7 (Table S2), we decided to include this parameter in the cluster analysis. The analysis was performed for 89 serum samples. The whole distance (100%) was considered at 140 values on the *x*-axis. At 98.8% distance, all samples could be regarded as homogenous formation (the value 138.3 on the *x*-axis). The first cluster could be distinguished at 59.5% distance (the value 83.3 on the *x*-axis) and constitutes six samples. The second cluster was distinguished at 12.1% distance (the value 16.9 on the *x*-axis) and comprised 22 serum samples. The third cluster, distinguished at 4.6% distance (the value 6.4 on the *x*-axis), contained 60.7% of the OAT samples chosen for analysis (17 from 28 OAT samples) and 66.7% of T samples (20 from 30 T samples). The fourth cluster could be distinguished at 2.4% (the value 3.4 on the *x*-axis) distance and comprised 50% normozoospermic serum samples (8 form 16 N samples) (Figure S2, Table S5).

2.8. Relationships between the Group Classification of Patients vs. Seminal Plasma and Serum Parameters

Seminal plasma parameters: MAA^{PL}, MAA/SNA^{PL}, $SIRT3^{PL}$ and $SIRT5^{PL}$, taken into consideration during cluster analysis were also chosen as predictors in the multinomial logistic regression model. Due to the lack of representative fertile normozoospermic group of men, which is a limitation of our study, the infertile normozoospermic group was chosen as the reference group. In the case of seminal plasma, the Spearman's rank correlation coefficient in correlation between MAA^{PL} and MAA^{PL}/SNA^{PL} was 0.87, which means that the predictor collinearity assumption was not met here, and the results should be treated with caution. The model showed a significant effect of $SIRT3^{PL}$ (OR = 0.236, p = 0.003 on the affiliation to the OAT or to the N group as well as the effect of $SIRT3^{PL}$ (OR = 0.234, $p < 0.001$) and $SIRT5^{PL}$ (OR = 2.008, $p = 0.006$; Table 4) on the affiliation to the T or N group.

Table 4. The results of multinomial logistic regression analysis of selected seminal plasma parameters.

Predictor (Parameter)	Group	OR	Low 95% CI	High 95% CI	Wald Statistics	*p*
MAA^{PL} (AU)	AT	1.597	0.616	4.139	0.927	0.336
MAA^{PL}/SNA^{PL}		0.947	0.838	1.070	0.773	0.379
$SIRT3^{PL}$ (ng/mL)		1.091	0.883	1.348	0.651	0.420
$SIRT5^{PL}$ (ng/mL)		1.019	0.811	1.279	0.026	0.873
MAA^{PL} (AU)	OAT	1.966	0.093	41.407	0.189	0.664
MAA^{PL}/SNA^{PL}		1.106	0.620	1.971	0.116	0.733
$SIRT3^{PL}$ (ng/mL)		0.236	0.093	0.603	9.104	0.003
$SIRT5^{PL}$ (ng/mL)		0.908	0.386	2.138	0.049	0.826
MAA^{PL} (AU)	T	3.565	0.335	37.896	1.111	0.292
MAA^{PL}/SNA^{PL}		1.038	0.703	1.532	0.035	0.851
$SIRT3^{PL}$ (ng/mL)		0.234	0.104	0.529	12.211	<0.001
$SIRT5^{PL}$ (ng/mL)		2.008	1.226	3.287	7.681	0.006

MAA^{PL}—relative reactivity of seminal plasma CLU glycans with *Maackia amurensis* agglutinin; MAA^{PL}/SNA^{PL}—seminal plasma CLU sialylation ratio; $SIRT3^{PL}$—seminal plasma SIRT3 concentration; $SIRT5^{PL}$—seminal plasma SIRT5 concentration. AT—asthenoteratozoospermia, OAT—oligoasthenoteratozoospermia, T—teratozoospermia. OR—odds ratio, CI—confidence interval. *p* value of less than 0.05 was considered significant.

Serum parameters: SNA^{S}, MAA^{S}, $SIRT3^{S}$ and $FRAP^{S}$ were chosen for the multinomial logistic regression model. Serum predictors, which were also chosen for the cluster analysis, did not demonstrate the collinearity (the pair-wise Spearman's rank correlation coefficients were lower than 0.35). The model showed a significant effect of SNA^{S} (OR = 0.000, $p = 0.003$) on the affiliation to the AT or N group as well as on the affiliation to the OAT or N group (OR = 0.001, $p = 0.007$) and on the affiliation to the T or N group (OR = 0.000, $p = 0.002$). The model also showed a significant effect of $FRAP^{S}$ on the affiliation to the AT or N group (OR = 0.016, $p = 0.040$; Table 5).

Table 5. The results of multinomial logistic regression analysis of selected serum parameters.

Predictor (Parameter)	Group	OR	Low 95% CI	High 95% CI	Wald Statistics	*p*
SNA^{S} (AU)	AT	0.000	0.000	0.048	9.088	0.003
MAA^{S} (AU)		2.225	0.000	6.175×10^{10}	0.004	0.948
$SIRT3^{S}$ (ng/mL)		1.084	0.894	1.316	0.677	0.411
$FRAP^{S}$ (mM)		0.016	0.000	0.822	4.237	0.040
SNA^{S} (AU)	OAT	0.001	0.000	0.171	7.239	0.007
MAA^{S} (AU)		155.638	0.000	2.225×10^{11}	0.220	0.639
$SIRT3^{S}$ (ng/mL)		1.101	0.909	1.334	0.967	0.326
$FRAP^{S}$ (mM)		0.205	0.007	6.049	0.843	0.359
SNA^{S} (AU)	T	0.000	0.000	0.051	10.019	0.002
MAA^{S} (AU)		3.247	0.000	3.655×10^{9}	0.012	0.912
$SIRT3^{S}$ (ng/mL)		1.036	0.853	1.259	0.129	0.719
$FRAP^{S}$ (mM)		0.212	0.007	6.068	0.821	0.365

SNA^{S}—relative reactivity of serum CLU glycans with *Sambucus nigra* agglutinin; MAA^{S}—relative reactivity of serum CLU glycans with *Maackia amurensis* agglutinin; $SIRT3^{S}$—serum SIRT3 concentration; $FRAP^{S}$—serum ferric reducing antioxidant power. AT—asthenoteratozoospermia, OAT—oligoasthenoteratozoospermia, T—teratozoospermia. OR—odds ratio, CI—confidence interval. *p* value of less than 0.05 was considered significant.

3. Discussion

3.1. Clusterin Concentration

Seminal plasma CLU concentration as well as serum levels of this glycoprotein were discussed in our previous study [38] in which we reported that the concentrations of seminal plasma clusterin were significantly higher in the OAT group in comparison to the normozoospermic, asthenoteratozoospermic and teratozoospermic groups ($p = 0.000114$, $p = 0.000001$ and $p = 0.000003$, respectively). Furthermore, in sera, the CLU concentrations were significantly lower in the normozoospermic group in comparison to the asthenoteratozoospermic ($p = 0.001718$), oligoasthenoteratozoospermic ($p = 0.000318$) and teratozoospermic ($p = 0.000183$) groups [38]. We concluded that the differences in CLU expression in seminal plasma and blood sera may be caused by distinct location of CLU synthesis and by the biological role it plays depending on the part of the male organism [38]. To the best of our knowledge, there is lack of more recent data concerning this issue.

3.2. Sialic Acids Expression in the Glycans of Seminal Plasma and Blood Serum Clusterin

As far as we know, this is the first study assessing the semi-quantitative analysis of sialic acids expression in the glycans of seminal plasma and blood serum clusterin using lectin-ELISA assay. Lack of significant differences in relative reactivities of seminal plasma CLU glycans with SNA between studied groups may indicate that the expression of α2,6-linked SA is independent of sperm parameters assessed during routine semen analysis according to the WHO criteria used by us [39]. In our previous study on seminal plasma AGP (α1-acid glycoprotein) glycosylation [40], we also observed a lack of differences in AGP sialylation between seminal plasmas obtained from men with sperm abnormalities (astheno-, azoo-, oligozoo-, teratozoospermia, and mixed AT, OT, OAT) and normozoospermic infertile patients. As both CLU and AGP are known as acute phase proteins having anti-inflammatory properties, and they are synthesized locally within the male reproductive organs, the similarities in trend of their sialylation degree in human seminal plasma are not particularly surprising. However, these findings stand in contrast with our previous study, where the relative reactivities of seminal plasma glycoproteins with SNA were significantly lower in the asthenozoospermic group in comparison to the normozoospermic fertile and infertile group as well as to the oligozoospermic group; however, it should be taken into account that, now, we analyzed the sialylation of CLU, not the sialylation of the whole panel of seminal plasma glycoproteins [41].

Based on the results obtained, we can presume that multiple sperm disorders (containing more than one anomaly) may reflect the decrease in expression of α2,3-linked MAA-reactive SA in seminal plasma CLU glycans. Moreover, a strong negative correlation between seminal plasma CLU concentration and relative reactivity of CLU glycans with MAA (Figure 1F) suggest that high concentrations of seminal CLU are accompanied by lower expression of sialic acid α2,3-linked. We have found a similar relationship in our previous studies [40] focused on the analysis of AGP sialylation degree in infertile men with abnormal values of standard semen analysis parameters, in which it was observed that together with increasing AGP concentration, the expression of MAA-reactive sialic acid decreases, regardless of the presence or absence of sperm abnormalities. This may provide additional evidence that there are certain patterns of variability in the expression of glycans of acute phase glycoproteins.

The intravascular survival of most mammalian blood plasma glycoproteins is dependent upon the integrity of carbohydrate chains. Removal of the terminal sialic acid residues from glycoproteins results in their rapid transfer from circulation into the liver. The exposition of terminal galactose results shows that these proteins are rapidly transferred from the circulation into the hepatocellular lysosomes where they are catabolized [42]. The same mechanism of glycoprotein elimination applies to the serum CLU, but whether as well to the human semen CLU is an open question. To date, the role

of sialic acids in CLU glycans has not been fully explored yet. There are no literature data concerning the specific role of SA in CLU glycans in cellular interactions, apoptosis and immune tolerance.

Analyzing the obtained results, we must also take into account other aspects that we are not able to verify and which may affect the degree of CLU sialylation. It has been proven that during permanent alcohol overdose, the CLU molecule is desialylated. Conversely, abstinence results in an increase in SA present on CLU glycans [43,44]. In 1995, Ghosh et al. [45] showed that chronic ethanol exposure can lead to the modification of Golgi apparatus membranes, which is a key cell compartment for clusterin sialylation. Loss of sialic acid by the clusterin molecule may result in a change in its molecular conformation, which may in turn affect its stability, antigenic expression or its receptors recognition. Hale et al. [46] in their study reported that long-term ethanol exposure significantly impairs sialylation, which is a key step in clusterin biosynthesis. Taking into account the above information, although we do not have information on the addictions of patients covered by our research, including increased alcohol consumption, we cannot exclude this factor as affecting the degree of CLU sialylation.

Kałuża et al. [47] determined the content of immunomodulatory glycoepitopes in seminal plasma glycoproteins. Mass spectrometry enabled for distinguishing several glycan types within CLU. Their results revealed significantly decreased relative reactivity with MAA of glycoprotein glycans in the band within the most matches for CLU in the oligoasthenozoospermic group in comparison to the normozoospermic, oligozoospermic as well as to the control group [47]. Our previous study concerning, i.a., sialylation profile of seminal plasma proteins, showed clearly that the relative reactivities of seminal plasma proteins with MAA were significantly lower in the oligozoospermic group compared to the infertile normozoospermic and asthenoteratozoospermic groups [41]. Quite apart from the different study groups, the discrepancies between our previous and present results are most probably caused by the fact that in the previous study, a mixture of seminal plasma glycoproteins was examined, and thus, its sialylation profile differs from those obtained in case of clusterin.

Current research also included the analysis of sialic acid expression in glycans of serum CLU, examined in context of decreased male fertility. The obtained results showed that the reactivity of serum CLU glycans with SNA increased in the infertile normozoospermic men in comparison to the other analyzed groups of patients. Hence, we can presume that any sperm disorders confirmed in the routine seminal analysis reflect the decrease in α2,6-linked sialic acid expression in the serum CLU glycans. Analyzing the profile and degree of CLU sialylation, both in the seminal plasma and in the serum, and the observed significant differences between the analyzed groups, the question arises of how the pattern and degree of blood serum CLU sialylation (and whether at all) is related to seminal plasma CLU sialic acid expression and if the analysis of serum CLU sialylation may be usable as an additional parameter associated with disorders of male gametes. There are no current literature data associated with particular CLU synthesis places. Rosenior et al. [48] in their study on rats proved that CLU is synthetized de novo in epithelial cells; however, there is no research concerning the issue of human seminal plasma CLU synthesis: is it derived only from male accessory glands or is the blood–testis barrier permeable for blood CLU synthetized in liver? Answers for such questions remain unknown.

Lack of significant correlations in sialic acids expression in CLU glycans between biological fluids compared by us may suggest the differences in mechanisms of seminal plasma and serum CLU sialylation, indicating organ-specific mechanisms and place of CLU synthesis associated with the biological functions it plays in each of body fluids analyzed in the present study; however, further research in this field is needed. Furthermore, a weak negative correlation between seminal plasma and serum CLU sialylation ratio MAA/SNA was found (Figure 1E). However, the relative reactivities of seminal plasma and serum CLU glycans with sialo-specific lectins are not associated with

each other, but the proportion between sialic acids attached via α2,3 and α2,6 glycoside bound may have an impact on CLU structure and thus on its biological function, but it should be confirmed in an another study. It seems that although the intensity of processes of seminal plasma and blood serum CLU sialylation differ from each other, the proportions in the expression of MAA- and SNA-reactive sialic acids are somewhat similar. How important it could be from the point of view of CLU biological function and its utility in the diagnostics of male fertility is difficult to determine at this time, because additional tests are needed for deeper analysis of this issue.

3.3. SIRTs Concentrations

Our results concerning seminal plasma SIRT3 concentrations indicate that teratozoospermia and oligoasthenoteratozoospermia are associated with decreased levels of this enzyme. As far as we know, the literature data concerning seminal plasma SIRTs concentration are not so abundant. Nasiri et al. [49] assessed seminal plasma SIRTs concentrations within SIRT3 and other oxidative–antioxidant balance parameters in normozoospermic and asthenoteratozoospermic men. Results of the above study are in contrast to our findings because the authors reported that seminal plasma SIRT3 levels were significantly lower in the AT group in comparison to the N group [49]; however, none of the subjects from N and AT group analyzed by Nasiri et al. [49] declared proven fertility and/or fertility problems as it was for subjects from our groups of patients. Bello et al. [50] investigated the expression of mitochondrial sirtuins gene expression in semen of fertile and infertile (normozoospermic, asthenoteratozoospermic and oligosthenoteratozoospermic) men. The authors observed that SIRT3 gene expression was significantly reduced in infertile patients in comparison to fertile men; moreover, normozoospermic patients had significantly higher relative expression of SIRT3 mRNA in comparison to the other infertile groups [50], which allowed us to find some analogies with our findings for SIRT3 concentrations.

Based on the results regarding serum SIRT3 concentrations, we can presume that sperm abnormalities such as morphology, total sperm count and motility are associated with elevated serum SIRT3 levels. Although the observed lack of correlations in SIRT3 concentrations between studied body fluids, seminal plasmas and blood sera indicate that serum sirtuin-3 expression is independent on its seminal plasma expression, it is possible that systemic oxidative–antioxidant imbalance also affects male fertility disorders, which are manifested by abnormalities in the number, morphology and movement of sperm. As far as we know, there are no current literature data concerning SIRT3 concentrations analyzed in context of decreased male fertility, and thus, we cannot refer to the results obtained by other authors. Rato et al. [51] hypothesized that SIRT3 together with peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) are associated with pre-diabetic state and oxidative stress. Results of their study proved that SIRT3 levels are significantly decreased in the testes of pre-diabetic rats, accompanied by decreased antioxidant capacity measured as FRAP levels [51]. Moreover, decreased SIRT3 concentrations promoted glycolysis in testes [51,52]. Taking into account the multidirectional role of testes in male fertility disorders, this information seems to be significant. Further investigations concerning the impact of testes glucose metabolism on sperm cells properties, together with SIRT3 concentrations and levels of other oxidative stress parameters, may shed some new light on the cause of male fertility problems.

Di Emidio et al. [53] suggests that despite that SIRT5 is located mainly in mitochondria, it probably serves as a regulator of redox homeostasis by initiating a multiple antioxidant response that activates not only mitochondria, but also other redox-active organelles. Taking into account this fact, lowered seminal plasma SIRT5 concentration in the OAT group in comparison to the other groups suggests that sperm abnormalities involving sperm count, motility and morphology are related to the increase in ROS formation, reflecting in the inadequate seminal plasma SIRT5 concentration. Bello et al. [50] also found that relative SIRT5 mRNA expression is significantly reduced in

oligoasthenospermic and asthenospermic men compared to the fertile group. Lack of significant differences between analyzed groups in sera SIRT5 concentrations may indicate that oxidative–antioxidative balance disorders associated with the role of SIRT5 are confined to the male reproductive system; however, our hypothesis requires further studies.

Very strong and strong positive correlation between concentrations of SIRTs in serum (Figure 1I) as well as in seminal plasma analyzed by us (Figure 1J), respectively, may indicate their synergistic action in both analyzed body fluids. Furthermore, observed by us, lack of correlation in SIRT3 and SIRT5 levels between both examined biological fluids was most probably caused by the differences in mechanisms and intensity of their action in seminal plasma and blood serum. Taking into account that many enzymes, including sirtuins, can act both synergistically and antagonistically toward each other as well as toward other biologically active molecules, it is not surprising that in our study, a negative correlation between concentrations of seminal plasma CLU vs. SIRT3 and SIRT5 was found (Figure 1G,H). One of the possible explanations for the obtained results is the fact that both molecules, however, are associated with oxidative–antioxidant balance, but their modes of action are different, not excluding an antagonistic effect.

3.4. TAS Measurement

We found no significant differences between analyzed groups of patients in seminal plasma TAS levels that are in accordance with the results of our previous study concerning silent inflammatory markers examined in the context of oxidative stress [54]. The observed lack of significant differences between seminal plasma groups in TAS concentrations may be associated with group size, and/or, most likely, with the lack of normozoospermic control group composed from men with proven fertility, which is a limitation of our study. In addition, the information on patient BMI (body mass index), type of infertility, duration of infertility and diseases related to reduced fertility may be helpful in the interpretation of the obtained results. Moreover, a factor that may affect the level of the measured values of oxidative stress parameters is the intake of widely available antioxidant dietary supplements by infertile patients, which, unfortunately, we were not able to verify. Furthermore, exposure to pro-oxidative factors (e.g., in the work environment) can significantly affect the level of oxidative stress markers. Information on this subject would undoubtedly be helpful in interpreting the results we obtained. Khosrowbeygi and Zarghami [55] measured TAS levels in seminal plasmas of infertile asthenoteratozoospermic, asthenozoospermic, oligoasthenozoospermic patients and compared the obtained results with those for fertile normozoospermic men. The authors reported that seminal TAS concentrations were significantly lower in all infertile groups in comparison to fertile normozoospermic men [55]. Hosen et al. [56] investigated, i.a., seminal plasma TAS levels in fertile and infertile men and proved that seminal plasma TAS was significantly lower in the infertile patients. Similar trend was observed by Ozer et al. [57] who measured seminal plasma TAS levels in infertile teratozoospermic patients and fertile normozoospermic men and showed that TAS concentrations were significantly lower in the infertile group of patients. Fatima et al. [58] also investigated seminal plasma TAS concentrations in the seminal plasma of fertile normozoospermic and infertile asthenozoospermic and oligoasthenozoospermic patients. The authors also found lowered TAS concentrations in all infertile groups of men when compared with fertile subjects. It seems that seminal plasma total antioxidant status is decreased in infertile male patients, independent of the type of sperm disorders, which was also proven by Emokpae et al. [59] who revealed that seminal plasma TAS was significantly lower in the infertile group in comparison to the control group of fertile men.

Observed by us, a lack of significant correlations between TAS concentrations and sperm parameters such as total sperm count, motility and morphology confirm the abovementioned findings of other authors that TAS levels are independent of the type of sperm disorders (preliminary experiments, not published). Gumus et al. [60] investigated

antioxidant parameters in the infertile azoospermic men in comparison to the fertile control group and concluded that seminal plasma TAS concentrations were significantly lower in the azoospermic group. Fingerova et al. [61] conducted the study using the same method as in our research, assessing both seminal plasma and serum TAS concentrations in two groups: a study group that was composed of infertile normozoospermic men and patients with combined sperm disorders, and control group of fertile men. Their findings were consistent with those mentioned before: seminal plasma TAS concentrations were significantly lower in the group of infertile patients when compared with normozoospermic fertile men [61].

There is a little literature data concerning serum TAS concentrations analyzed in the context of decreased male fertility. Our study revealed no significant differences in serum TAS concentrations between groups of infertile men. Results obtained by Fingerova et al. [61] showed a lack of significant differences in serum TAS levels between fertile and infertile men; however, the authors found strong positive correlations between seminal plasma and serum TAS concentrations [61], which stands in contrast to our results of correlations analysis (preliminary experiments, not published). Furthermore, Gumus et al. [60] found increased serum TAS levels in the azoospermic group of patients in comparison to the control group of fertile men. Further investigations are needed, concerning not only seminal plasma and serum TAS concentrations, but also the expression of other parameters being markers of systemic and/or local inflammatory condition, which may directly or indirectly influence TAS level.

3.5. FRAP Determination

To the best of our knowledge, this is the first study assessing and comparing FRAP levels in biological fluids such as seminal plasma and serum. Lack of significant differences in seminal plasma FRAP concentrations between studied groups may be associated with a relative small number of subjects in each of group. The study of Aktan et al. [62] assessing some oxidative stress parameters, i.a., FRAP levels in seminal plasmas of idiopathic infertile men and control group of fertile donors, also revealed no significant differences between these groups. Our previous study revealed that seminal plasma FRAP concentrations in azoospermic samples were significantly higher in comparison to the teratozoospermic and normozoospermic subjects [63]. Moreover, in our another study, we reported that seminal plasma FRAP levels were significantly higher in infertile normozoospermic and oligozoospermic patients in comparison to the fertile normozoospermic men [12]. The results obtained by Abdulrahman et al. [64] also documented that seminal plasma FRAP concentrations were significantly higher in the infertile normozoospermic group in comparison to the asthenozoospermic and oligoasthenozoospermic patients. Lack of a group of fertile normozoospermic men make such comparisons impossible in the present study. Pahune et al. [65] examined FRAP levels in seminal plasmas of asthenoteratozoospermic, oligoasthenoteratozoospermic and azoospermic patients and compared the obtained results with those of the fertile normozoospermic group, showing that seminal plasma FRAP concentrations were significantly lower in all infertile groups in comparison to the normozoospermic group and positively correlated with sperm concentration, motility and morphology [65]. These findings are similar to the research of Colagar et al. [66] who proved that seminal plasma FRAP was significantly lower in the AT group and OAT group in comparison to the fertile normozoospermic group; moreover, positive correlation between seminal plasma FRAP and sperm count, motility and morphology was found. In addition, Gholinezhad et al. [67] showed that seminal plasma FRAP was significantly lower in the infertile men and positively correlated with the sperm count and motility. In the present study, we did not find any significant correlations between semen parameters and the concentration of FRAP in seminal plasma (preliminary experiments, not published). Fazeli et al. [68] investigated, i.a., seminal plasma FRAP levels in the men with idiopathic infertility and compared the obtained results to those of fertile men, also proving that seminal plasma

FRAP in the study group was significantly lower in comparison to fertile men. Nasiri et al. [49] found that seminal plasma FRAP concentrations in asthenoteratozoospermic group were significantly lower in comparison to the normozoospermic group of healthy men.

Based on the results of the current study concerning serum FRAP concentrations, we can presume that sperm motility abnormalities together with their morphology disorders reflect in the decrease in antioxidant capacity measured as FRAP concentration. Donatus et al. [69] assessed serum oxidative stress parameters including FRAP in infertile men (oligozoospermic, oligoasthenozoospermic and asthenozoospermic groups) and compared the obtained results to the control group of fertile men. They revealed that serum FRAP concentrations were significantly lower in all infertile groups [69].

Weak positive correlations were found between TAS and FRAP levels in both examined biological fluids (Figure 1K,L); however, Rao et al. [70] who also determined and compared these two oxidative stress parameters found no correlations between them in blood serum. Furthermore, when TAS was corrected for proteins, positive correlation was observed with FRAP [70].

3.6. ROC Curve Analysis

Based on the results of the ROC curve analysis carried out for each of the selected seminal plasma and blood serum parameters, we at first can propose the level of sensitivity and specificity as well as the value of cut-off point for each of them, simultaneously analyzing the potential clinical value each of the discussed parameters. A detailed presentation deserves the results of the ROC curve analysis for which the AUC value was equal to or higher than 0.700 ($p < 0.05$), which proves their moderate or high clinical value. We used two ways of arranging the obtained results for ROC curve analysis: (1) the values for a given parameter were compared between two groups of patients separated on the basis of standard semen analysis (Tables 2 and 3 for seminal plasma and blood serum, respectively), and (2) between a given group of patients distinguished on the basis of standard semen analysis and other study groups gathered together and treated as one study group (Supplementary Materials, Tables S2 and S3 for seminal plasma and blood serum, respectively).

According to the first way of ROC curve analysis, we can presume that seminal plasma parameters such as relative reactivity of CLU glycans with MAA, MAA/SNA sialylation ratio, SIRT3 and SIRT5 concentrations may constitute additional markers differentiating patients with sperm disorders from those with normal sperm parameters (Table 2).

In this study, relative reactivities of seminal plasma CLU glycans with MAA have a moderate clinical value and enabled differentiation of the OAT group from (1) normozoospermic men with sensitivity and specificity of 79.3% and 72.4%, respectively (proposed cut off point: 0.224 AU, AUC = 0.715), (2) teratozoospermic group with sensitivity and specificity of 79.3% and 91.2%, respectively (proposed cut off point: 0.224 AU, AUC = 0.890) and (3) asthenoteratozoospermic group with sensitivity and specificity of 75.9% and 94.4%, respectively (proposed cut off point: 0.214 AU, AUC = 0.883). Based on this, we can conclude that altered expression of α2,3-linked sialic acid in seminal plasma CLU glycans is strictly associated with mixed sperm disorders such as decreased total sperm count, motility and sperm morphology abnormalities. The above observations were also confirmed by the results of the ROC curve analysis, in which CLU reactivity with MAA also has a moderate clinical value in differentiation of the OAT group from the other groups of patients treated as one group (proposed cut off point: 0.214 AU, AUC = 0.826, sensitivity 75.9%, specificity 85.2%).

The sialylation ratio has a moderate clinical value and enabled differentiation of the OAT group from the teratozoospermic patients with sensitivity and specificity of 89.7% and 67.6%, respectively (proposed cut off point: 2.033, AUC = 0.836) and from the asthenoteratozoospermic patients with sensitivity and specificity of 55.2% and 100%,

respectively (proposed cut off point: 0.824, AUC = 0.837). The MAA/SNA ratio also has a moderate clinical value when the OAT group was compared with the other patients treated as one group (the proposed cut off point was 2.033, AUC = 0.777, sensitivity 89.7%, specificity 55.6%).

The observed differences between clinical values of relative reactivities of CLU glycans with the MAA and MAA/SNA ratio may suggest that the expression of α2,3-linked SA seems to be a more suitable marker for seminal plasma group differentiation than the ratio of α2,3-linked to α2,6-linked sialic acid. Furthermore, taking into account the shown differences in MAA relative reactivity with CLU glycans as well as in the level of MAA/SNA ratio between OAT patients vs. other analyzed groups taken together, both parameters seem to be usable for differentiation of the OAT group from AT, N and T groups, but the values of relative reactivities of CLU glycans with MAA seems to be more strongly associated with lowered sperm count.

Seminal plasma SIRT3 concentrations, when compared between two groups of patients distinguished on the basis of standard semen analysis, have a high clinical value and enabled differentiation of the OAT group from the normozoospermic group with sensitivity and specificity of 82.1% and 94.6%, respectively (proposed cut off point: 2.5 ng/mL, AUC = 0.951) and from the asthenoteratozoospermic group with sensitivity and specificity of 100% and 81.8%, respectively (proposed cut off point: 7.985 ng/mL, AUC = 0.948). This parameter also enabled the differentiation of the AT group from the teratozoospermic group with sensitivity and specificity of 81.8% and 100%, respectively (proposed cut of point: 8.604 ng/mL, AUC = 0.906). Seminal plasma SIRT3 has a moderate clinical value in the differentiation of the T group from the normozoospermic group with sensitivity and specificity of 100% and 75.7%, respectively (proposed cut off point: 4.645 ng/mL, AUC = 0.887). Based on the obtained results, we can presume that seminal plasma SIRT3 concentration may be used as an additional marker helpful in the discrimination between patients with one or two sperm disorders as well as with two and three sperm disorders found in routine semen analysis. The above observation was confirmed by the results of ROC curve analysis in which each of the seminal plasma groups (AT, N, OAT and T) was compared with the rest of the groups together. SIRT3 concentration has a moderate clinical value (AUC: 0.720–0.847) with sensitivity 75.7–100% and specificity of 54–80.9%. SIRT3 is the only seminal plasma parameter for which the mean value of AUC has a moderate clinical value (AUC = 0.786).

Seminal plasma SIRT5 has a moderate clinical value and enabled differentiation of the OAT group from normozoospermic men with sensitivity and specificity of 96.4% and 73%, respectively (proposed cut off point: 2.084 ng/mL, AUC = 0.812) and from the teratozoospermic group with sensitivity and specificity of 96.4% and 69.4%, respectively (proposed cut off point: 2.084 ng/mL, AUC = 0.759), but seminal plasma SIRT5 concentration has a high clinical value when distinguishing the OAT group from the asthenoteratozoospermic group with sensitivity and specificity of 96.4% and 81.8%, respectively (proposed cut off point: 2.084 ng/mL, AUC = 0.916). Moreover, seminal plasma SIRT5 concentration has a moderate clinical value and enabled differentiation of the OAT group from other samples treated as one group, with sensitivity and specificity of 96.4% and 73.7%, respectively (proposed cut off point: 2.084 ng/mL, AUC = 0.816).

ROC curve analysis of serum parameters showed that relative reactivities of CLU glycans with SNA and MAA, together with SIRT3 and FRAP concentrations, may be useful in the differentiation of infertile men with abnormal as well as normal semen parameters (Table 3).

Relative reactivity of serum CLU glycans with SNA, when compared between two groups of patients separated on the basis of standard semen analysis, has a moderate clinical value and enabled differentiation of N patients from: (1) asthenoteratozoospermic subjects with sensitivity and specificity of 86.7% and 81.3%, respectively (proposed cut off point: 0.609 AU, AUC = 0.892), (2) oligoasthenoteratozoospermic patients with sensitivity and specificity of 88.9% and 68.8%, respectively (proposed cut off point: 0.694 AU, AUC =

0.837), and (3) teratozoospermic patients with sensitivity and specificity of 87.1% and 81.3%, respectively (proposed cut off point: 0.591 AU, AUC = 0.872). Relative reactivity of serum CLU glycans with MAA has a moderate clinical value and enabled differentiation of the N group from the asthenoteratozoospermic group with sensitivity and specificity of 60% and 87.5%, respectively (proposed cut off point: 0.023 AU, AUC = 0.756) and from the teratozoospermic group with sensitivity and specificity of 51.6% and 87.5%, respectively (proposed cut off point: 0.035 AU, AUC = 0.716). The moderate clinical values were also observed in the ROC curve analysis, when CLU reactivity with SNA and MAA was compared between the N group and the other groups of patients gathered together (for SNA: proposed cut off point: 0.612 AU, AUC = 0.863, sensitivity 83.1%, specificity 82.2%; for MAA: proposed cut off point: 0.036 AU, AUC = 0.700, sensitivity 87.5%, specificity 54.8%).

Serum SIRT3 concentration enabled differentiation of the AT group from the normozoospermic patients with sensitivity and specificity of 100% and 61.1%, respectively (proposed cut off point: 4.979 ng/mL, AUC = 0.830) and from teratozoospermic patients with sensitivity and specificity of 93.3% and 51.6%, respectively (proposed cut off point: 6.044 ng/mL, AUC = 0.740). This parameter also enabled differentiation of the OAT group from normozoospermic patients with sensitivity and specificity of 100% and 61.1%, respectively (proposed cut off point: 5 ng/mL, AUC = 0.761). Serum SIRT3 concentration also has a moderate clinical value in differentiation of asthenoteratozoospermic and normozoospermic groups from other samples treated as one group, with sensitivity and specificity of 93.3% and 52.6%; 61.1% and 94.5%, respectively (proposed cut off points: 6.044 ng/mL, AUC = 0.714 and 3.608 ng/mL, AUC = 0.749, respectively).

Serum FRAP concentration enabled, with a moderate clinical value, differentiation of the AT group from the normozoospermic group with sensitivity and specificity of 80% and 77.8%, respectively (proposed cut off point: 1.352 mM, AUC = 0.780) and from the teratozoospermic group with sensitivity and specificity of 60% and 77.4%, respectively (proposed cut off point: 1.208 mM, AUC = 0.701). For this serum parameter, the results of the ROC curve analysis in which one group of patients is distinguished on the basis of standard semen analysis was compared with other study groups gathered together and treated as one study group, which did not confirm its moderate or high clinical value.

The results of the ROC curves analysis discussed by us indicate those of the proposed parameters that may be usable in the diagnosis of certain types of male infertility and may constitute the basis for further research, extended with additional parameters of the oxidative–antioxidant balance. It would also be advisable to include additional data on patients in future studies (e.g., BMI, comorbidities, duration of infertility) and to relate the results obtained for patients with fertility problems, especially for those with idiopathic infertility, to the group of healthy, normozoospermic men with proven fertility.

3.7. Cluster Analysis

On the basis of ROC curve analysis results, seminal plasma and serum parameters with AUC equal to or greater than 0.715 and 0.701, respectively, and simultaneously differentiated studied groups of patients, were selected for cluster analysis. Seminal plasma cluster analysis showed that the following parameters: relative reactivities of CLU glycans with MAA, MAA/SNA sialylation ratio, SIRT3 and SIRT5 concentrations, may be useful in the differentiation of the OAT group from patients with normal semen parameters, as well as from subjects with decreased sperm motility and/or abnormal morphology. Furthermore, serum parameters selected for cluster analysis such as relative reactivities of CLU glycans with SNA and MAA together with SIRT3 and FRAP concentrations may be taken into account as a promising additional set of markers helpful in the differentiation of normozoospermic patients from those with sperm disorders manifested as teratozoospermia, asthenoteratozoospermia and oligoasthenoteratozoospermia. The results of cluster analysis were in accordance with the

results of the Wilcoxon test and ROC curve analysis, indicating that seminal plasma and blood serum parameters selected by us as a set of additional biomarkers of male infertility associated with sperm disorders are worth taking into consideration as a base for construction the diagnostic algorithms of male infertility associated with sperm abnormalities.

3.8. The Relationships between the Selected Markers and Classifying Patients into Study Groups

To assess the diagnostic utility the set of parameters proposed by us as the additional markers of male infertility linked with sperm disorders, multinomial logistic regression analysis was performed. The normozoospermic group was defined as a reference group. A p value of less than 0.05 was considered significant. In the case of seminal plasma, the results of multinomial regression analysis indicated the significant differences between the N and OAT groups for SIRT3 only, as well as between the N and T groups for SIRT3 and SIRT5 (Table 4). For serum parameters selected for the multinomial regression model, the SNA relative reactivity with CLU glycans was the parameter that significantly differentiated the normozoospermic group from the AT, OAT and T groups. Additionally, FRAP concentrations significantly differed between the N and AT groups (Table 5). The results obtained in this analysis additionally confirmed the potential diagnostic utility of some of the biomarkers we have selected for differentiation between the infertile normozoospermic group of patients and other infertile men.

4. Materials and Methods

4.1. Patient Samples

The seminal plasma and serum samples were collected between 2019 and 2020 from 132 infertile male patients visiting the Clinical Center of Gynecology, Obstetrics and Neonatology in Opole (Poland) and Fertility Clinics InviMed in Warsaw (Poland). Each patient gave informed consent for this study. Our study was conducted according to the guidelines of the Helsinki II declaration, and the protocol was approved by the Bioethics Human Research Committee of Wroclaw Medical University (no. KB 549/2019 and no. KB 707/2019).

The ejaculates were collected via masturbation into sterile containers after 3–5 days of sexual abstinence. After liquefaction (maximum 60 min at 37 °C), standard semen analysis was carried out according to WHO 2010 directives [39] (i.a., semen volume, pH and sperm viability), and supplemented by using computer-assisted sperm analysis (total sperm count in ejaculate, sperm concentration, total motility, progressive motility, and morphology), SCA Motility and Concentration, software version 6.5.0.5. (Microptic SL, Barcelona, Spain). All input data in this method were consistent with current WHO semen analysis recommendations [39]. Next, the ejaculates were centrifuged at 3500× g for 10 min at room temperature. Serum samples were obtained by peripheral blood collection and after coagulation centrifuged at 2000× g for 10 min at room temperature. All samples were then aliquoted and frozen at −86 °C until use.

Based on the results of standard semen analysis (sperm concentration, progressive motility, morphology of spermatozoa), seminal samples (n = 132) were divided into groups: asthenoteratozoospermic (AT, n = 22; <32% of sperm demonstrated progressive motility and lower than 4% of spermatozoa had normal morphology, the median age: 34 years (IQR 31–36)), normozoospermic (N, n = 43, normal values of ejaculate parameters, the median age: 32 years (IQR 24–49)), oligoasthenoteratozoospermic (OAT, n = 29; sperm count lower than 15 × 106 mL^{-1}, <32% of sperm demonstrated progressive motility and lower than 4% of spermatozoa had normal morphology, the median age: 32 years (IQR 30–35)) and teratozoospermic (T, n = 38; lower than 4% of spermatozoa had normal morphology, the median age: 33 years (IQR 28–36)). Corresponding blood serum samples (n = 91) were divided into asthenoteratozoospermic (AT, n = 15), normozoospermic (N, n = 18), oligoasthenoteratozoospermic (OAT, n = 27) and teratozoospermic (T, n = 31)

groups. In the normozoospermic ejaculates, the concentration of spermatozoa was higher than 15×10^6 mL^{-1}, and >4% of sperm exhibited normal morphology, with a total motility of ≥40% or progressive motility ≥32% (0.5 h after ejaculation). None of the seminal samples were leukospermic and/or infected by bacteria, and none of the serum samples were hemolyzed.

4.2. Methods

4.2.1. Clusterin Concentration

Seminal plasma and blood serum CLU concentrations were determined using commercial enzyme-linked immunosorbent assay (ELISA): Human Clusterin ELISA kit from Bioassay Technology Laboratory (catalog no. E1189Hu; Shanghai, China) and Human Clusterin Elisa Kit from Invitrogen (Thermo Fisher Scientific, catalog no. EHCLU; Frederick, MD, USA), respectively, as described by us previously [38]. The determination of the CLU concentration was the basis for calculation of the constant amount of glycoprotein in 100 µL of solution that was applied to the well of ELISA plate for lectin-ELISA analysis of CLU sialylation (see "Sample dilution" in Section 4.2.3). All tests were performed without any modifications, according to the manufacturer instructions. The intra-assay and inter-assay coefficients of variations (CV%) for both tests were defined by the manufacturers and were also mentioned in our previous article [38].

4.2.2. Determination of Sialic Acid Expression in the Seminal Plasma and Blood Serum Clusterin Glycans

Two biotinylated SA-specific lectins: *Sambucus nigra* agglutinin (SNA, catalog number B-1305, Vector Laboratories Inc., Burlingame, CA, USA) and *Maackia amurensis* agglutinin (MAA, catalog number B-1265, Vector Laboratories Inc., Burlingame, CA, USA) were used to determine sialic acid expression in the lectin-ELISA procedure according to Kratz et al. [26] with modifications described below. The specificity of lectins is following: SNA detects terminal sialic acids α2,6-linked to galactose (Gal) of antennary part of glycan, whereas MAA is specific to SA α2,3-linked to antennary Gal [71].

4.2.3. Lectin-ELISA Procedure

ELISA-Plate Coating

The wells of ELISA plate (Nunc MaxiSorp, Thermo Fisher Scientific, Glostrup, Denmark) were coated by goat anti-human clusterin polyclonal antibodies (Invitrogen, Thermo Fisher Scientific, catalog no. PA1-26903; Rockford, IL, USA). The antibodies were diluted in 10 mM TBS, pH = 8.5. For SNA and MAA, the dilutions of 1:10,000 and 1:5000, respectively, were established. After 2 h incubation at 37 °C, the plate was washed three times by the same buffer. Free binding sites were blocked by 10 mM TBS, 0.1% Tween20, 1% BSA, pH = 7.5 (blocking buffer); next, the plate was incubated for 2 h at 37 °C and then stored at 4 °C overnight.

Sample Dilution

Seminal plasma and sera samples were diluted in 10 mM TBS 0.1% Tween20 buffer was used to obtain proper CLU amount in each well: 1 ng CLU/100 µL for seminal plasma and 50 ng CLU/100 µL form serum samples. The amounts of seminal plasma and serum CLU for lectin-ELISA were calculated basing on the CLU concentrations determined by us previously [38]. After application of the proper CLU amount in each well, the ELISA plate was incubated at 37 °C for two hours with gentle shaking. All samples were analyzed in duplicate to minimize imprecision. To each lectin–ELISA experiment, two pairs of blank were added. They contained all reagents, but instead of patients' samples, 10 mM TBS, 0.1% Tween20, pH = 7.5 (washing buffer) was used. After each next step of lectin–ELISA, the wells were washed using washing buffer.

Lectin-SA Interactions

In the next step, the biotinylated lectins SNA and MAA, specific to α2,6-linked and α2,3-linked sialic acid, respectively, were used. The dilutions of lectin used were established in the series of initial experiments using 10 mM TBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2 \times 6H_2O$, 1 mM $MnCl_2 \times 4H_2O$, 1% BSA and 0.1% Tween20, pH = 7.5. *Sambucus nigra* agglutinin was diluted 1:2000, whereas *Maackia amurensis* agglutinin was diluted 1:250. Then, plates were incubated one hour at 37 °C with gentle shaking.

The Detection Clusterin–Lectin Complexes

To detect the clusterin–lectin complexes, ExtrAvidin alkaline phosphatase labeled (Sigma-Aldrich, catalog no. E2636; Saint Louis, MO, USA), diluted 1:10,000 in the washing buffer, was used. Next, plates were incubated for one hour at 37 °C, and then, the color reaction with disodium para-nitrophenyl phosphate was induced. The absorbances were measured with Mindray MR-96A Microplate Reader (Mindray Bio-Medical Electronics, Shenzen, China) at 405 nm with a reference filter 630 nm. The relative reactivities of sialic acid with lectins were expressed in absorbance units (AU), after subtracting the absorbances of the blank samples. Sialylation ratio (MAA/SNA) was calculated as the ratio between SA MAA reactivity to SNA reactivity.

4.2.4. SIRTs Concentration

SIRT3 and SIRT5 concentrations were estimated using Human Sirtuin3 ELISA Kit (catalog number: E2559Hu, Bioassay Technology Laboratory, Shanghai, China) and Human Sirtuin-5 ELISA Kit (catalog number: E2561Hu, Bioassay Technology Laboratory, Shanghai, China), respectively. All tests were performed following manufacturer instructions, without any modifications. The intra- and inter-assay precisions, expressed as CV%, were determined by manufacturer, and for both tests, the intra-assay CV ranged <8% and the inter-assay was <10%.

4.2.5. Total Antioxidant Capacity Assessment

Total antioxidant capacity (TAC) was expressed as measurement of total antioxidant status (TAS) and ferric reducing antioxidant power (FRAP), and their measurement procedures are described below.

Total Antioxidant Status Measurement

The levels of total antioxidant status for total antioxidant capacity of patient samples were performed on the autoanalyzer Konelab20i (Thermo Scientific, Vantaa, Finland) using TAS Randox reagents (TAS, catalog number NX2332, Crumlin, UK) according to manufacturer instructions, as described by us previously [72]. In short, the stable blue-green radical cation is a product of incubation (2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate] with a peroxidase (metmyoglobin) and H_2O_2. Antioxidants from the patient's sample suppress the color production proportionally to their concentration. The measurements were performed at 600 nm. The calibration curve with linearity up to 2.50 mM of Trolox standard was constructed, and thus, results were expressed as Trolox equivalents in mM.

Ferric Reducing Antioxidant Power Assessment

Ferric reducing antioxidant power (FRAP) was measured using spectrophotometric method according to the Benzie et al. [73]. Reduction of Fe^{3+} to Fe^{2+} at low pH leads to formation of a Fe^{2+}–tripyridyltriazine complex. For these reactions, 500 µL freshly prepared FRAP reagent (25 mL of 300 mM acetate buffer (pH 3.6)), 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ; Sigma-Aldrich, St. Louis, MO, USA) in 40 mM HCl and 2.5 mL of 20 mM aqueous solution of $FeCl_3 \times 6H_2O$ (CHEMPUR, Piekary Śląskie, Poland) was mixed with 100 µL of a 1:20 diluted seminal plasma sample and 1:10 diluted serum sample

(diluted in distilled water). The reaction mixture was incubated at 37 °C for 5 min and then centrifuged for 10 min at 4000 rpm. The absorbance was measured at 593 nm in a VWR, UV-6300PC UV-VIS Spectrophotometer (Avantor, Radnor Township, PA, USA). The absorbance of the samples was read against blank samples containing the FRAP reagent instead of biological material. A calibration curve was prepared basing on the aqueous $FeSO_4$ solution, within concentration ranges from 0.025 to 0.05 mM Fe^{2+}. The seminal plasma and serum samples with a known FRAP concentration were used as our internal controls. All measurements were performed in duplicate.

4.2.6. Statistical Analysis

Statistica 13.3PL software (StatSoft Inc., Tulsa, OK, USA) was used to perform the statistical analysis. To analyze the normality of distribution for values of all parameters investigated, the Shapiro–Wilks' test was used. Values obtained for relative reactivities with lectins were presented as median with interquartile range (Q1–Q3). As the values of examined parameters were not drawn with normal distribution, to compare the relative reactivities of CLU glycans with sialo-specific lectins SNA and MAA, as well as the concentrations of oxidative stress parameters (SIRTs, TAS, FRAP) between examined groups, the nonparametric Mann–Whitney U test with Bonferroni correction was used. Taking into account the significance of pairwise differences, a p value of less than 0.008 was accepted as the level of significance. The relationships between values of determined parameters in seminal plasma and in sera, as well as between these both biological fluids, were checked by Spearman's rank correlation. The receiver operating characteristic (ROC) curves were used to determine the diagnostic significance of relative reactivities of CLU glycans with sialo-specific lectins, and levels of SIRT3, SIRT5, TAS and FRAP, which were aimed at selecting parameters of the examined biological fluids that will have moderate or high clinical value for differentiation of patients with various variants of sperm disorders, and then, they were used to cluster analysis. Cluster analysis was performed for seminal plasma and serum samples, separately for both these body fluids, in which all parameters selected on the basis of the results of the ROC curve analysis were determined: the results were presented as a dendrogram that represents complete linkage clustering, in agglomerative hierarchical clustering. The data were not normalized before analysis, and all obtained values for each of the parameters tested in this analysis were absolute numbers. First, all examined subjects were gathered in one cluster, and then, the patients were clustered into the next clusters in which the subjects were more similar to each other. Patients that performed parallel in terms of the values of all the analyzed traits were grouped together, and a separate cluster was formed for those with different values. In conclusion, the smaller distance of separation means greater similarities in subject characteristics. The similarities between samples were calculated using a Euclidean metric on the original data points, with no reference to the clinical status of the subjects. The selection criteria were based on the results of Mann–Whitney U test with p values less than 0.05 considered as significant (Supplementary Materials, Table S1).

To check the diagnostic utility of the set of male infertility biomarkers proposed by us, multinomial logistic regression analysis was performed, in which the N group was defined as a reference group. The collinearity of the predictors was checked with the use of pair-wise Spearman's rank correlation coefficients. A p value less than 0.05 was considered as significant. Results of the multinomial logistic regression for seminal plasma and blood serum are shown in Tables 4 and 5, respectively.

5. Conclusions

The differences observed between groups of patients with abnormal sperm parameters and normozoospermic infertile men in relative reactivities of seminal plasma and serum CLU glycans with sialo-specific lectins MAA and SNA, respectively, may indicate that the alterations in CLU sialylation degree may be linked with improper sperm production and maturation, which as a consequence may lead to decreased fertility of

men at the reproductive age. Lack of significant differences between analyzed groups of patients in relative reactivities of seminal plasma CLU glycans with SNA suggests that the expression of α2,6-linked SA in CLU is independent of sperm parameters. Furthermore, our results showed that any sperm disorders reflect the decrease in α2,6-linked SA in serum CLU glycans. Based on the results of our study, this serum parameter differentiates normozoospermic infertile men from patients with abnormal sperm parameters with high sensitivity and specificity and thus may be taken into account as an additional male infertility biomarker. However, additional confirmation of these hypotheses is needed in further studies carried out in groups with a larger number of patients, including the group of normozoospermic men of proven fertility. There is a possibility of different mechanisms of clusterin sialylation in both biological fluids analyzed by us, which may be indicated by the lack of significant correlations in relative reactivities of CLU glycans with SNA and MAA between blood serum and seminal plasma samples. We also observed that the degree of expression of MAA-reactive α2,3-linked sialic acid in the seminal plasma CLU differentiate the OAT group of men with reduced sperm count, abnormal motility and morphology of sperm from the other examined groups of patients, and this parameter has moderate clinical value. Taking into consideration the above observations, the level of seminal plasma CLU reactivity with MAA seems to be associated with sperm count disorders. Moreover, the values of seminal plasma MAA/SNA ratio also significantly decreased in patients with lowered sperm count when compared to patients with other sperm abnormalities.

The results of our study support the thesis that oxidative stress plays an important role in the male infertility issue. Sperm cells' alterations in their morphology, motility or total count may reflect in the increase in serum SIRT3, one of the relevant oxidative–antioxidant balance parameters, suggesting that systemic antioxidant imbalance may affect male reproductive potential. Significant positive correlations between concentrations of SIRT3 and SIRT5 in both analyzed body fluids suggest synergistic action of these enzymes. Furthermore, the negative correlations between seminal plasma CLU vs. SIRT3 and SIRT5 concentrations suggest different modes of their action in the maintenance of the oxidative–antioxidant balance.

ROC curve and cluster analysis revealed that seminal plasma relative reactivity of CLU glycans with MAA and the value of MAA/SNA ratio, together with SIRT3 and SIRT5 concentrations, may constitute an additional set of markers differentiating infertile oligoasthenoteratozoospermic patients from normozoospermic, asthenoteratozoospermic and teratozoospermic men. However, considering the fact that in multinomial logistic regression analysis of both CLU reactivity with MAA and the values of MAA/SNA ratio, the value of Spearman's rank correlation coefficient in the correlation between these two parameters indicated that the predictor collinearity assumption was not met here; thus, the potential diagnostic utility of these seminal plasma biomarkers should be treated with caution. The results of multinomial regression analysis indicated the significant differences between the N and OAT group for SIRT3 only as well as between the N and T group for SIRT3 and SIRT5, which additionally confirmed the potential diagnostic utility of these seminal plasma parameters. For blood serum, based on the results of the ROC curve and cluster analysis, relative reactivities of CLU glycans with SNA, and MAA, SIRT3 and FRAP concentrations may be useful in the differentiation of normozoospermic patients from those with sperm abnormalities. The results of multinomial logistic regression analysis showed that the SNA relative reactivity with CLU glycans was the parameter that significantly differentiated the normozoospermic group from AT, OAT and T groups, and FRAP concentrations significantly differed between N and AT groups, indicating the potential utility of these blood serum biomarkers for differentiation of infertile patients with sperm parameters disorders.

In this study, we examined and compared two biological fluids, looking for an additional male infertility biomarkers and exploring the associations between the expression of sialic acid on CLU glycans as well as selected oxidative stress parameters.

Lack of a representative control group of men at reproductive age with proven fertility as well as lack of information on patient BMI, duration of infertility and diseases related to reduced fertility are the limitations of our study that make it impossible to verify whether the analyzed parameters may constitute as useful biomarkers of idiopathic male infertility. Nevertheless, according to the ROC curve and cluster analyses, several seminal plasma and blood serum parameters may be proposed by us as useful additional male infertility indicators. It seems to be surprising that some serum parameters were associated with disorders of sperm parameters. However, one should not draw hasty conclusions, and the observed dependencies should be checked in subsequent studies covering not only a larger number of patients, but also extending the spectrum of the analyzed oxidative stress parameters. To the best of our knowledge, this is the first study extensively concerning CLU sialylation alterations as well as changes in oxidative stress parameter expression in both human biological fluids: seminal plasma and blood serum. Lectin–ELISA used in this study enables to mimic the interactions between glycans and their endogenic ligands, including glycans availability, and therefore, the observed glycan–lectin reactions correspond to the in vivo processes, showing also the accessibility of sugar residues for ligands present in the human organism. Further studies based on lectin–glycan interaction, especially when such interactions will be analyzed simultaneously for multiple lectins and/or multiple glycoproteins, may shed new light on the molecular processes accompanying changes in glycoproteins glycosylation, analyzed in the context of male fertility disorders. An additional benefit from our research was to expand knowledge about potential associations between the parameters we examined, which may guide further research aimed at developing new therapeutic strategies of male infertility, especially caused by multiple overlapping factors.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms231810598/s1.

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Data Availability Statement: All data needed to evaluate the conclusions in the article are present in the article. Additional data related to this study are available upon reasonable request from the corresponding author or first author.

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Abbreviations

AGP	α1-acid glycoprotein
ApoJ	apolipoprotein J
AT	asthenoteratozoospermia

ATP	adenosine triphosphate
AUC	area under the curve
BMI	body mass index
BS	blood serum
BSA	bovine serum albumin
CAT	catalase
$CaCl_2$	calcium dichloride
CLU	clusterin
CV	coefficients of variations
DNA	deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
Fe^{2+}	ferrous iron
Fe^{3+}	ferric ion
$FeCl_3 \times 6H_2O$	ferric chloride hexahydrate
$FeSO_4$	ferrous sulfate
FRAP	ferric reducing antioxidant power
$FRAP^{PL}$	seminal plasma ferric reducing antioxidant power
$FRAP^{S}$	serum ferric reducing antioxidant power
Gal	galactose
GPX	glutathione peroxidase
HCl	hydrochloric acid
IQR	interquartile range
Le^{a}	Lewis^{a} oligosaccharide structure
Le^{b}	Lewis^{b} oligosaccharide structure
Le^{x}	Lewis^{x} oligosaccharide structure
Le^{y}	Lewis^{y} oligosaccharide structure
MAA	*Maackia amurensis* agglutinin
MAA^{PL}	relative reactivity of seminal plasma CLU glycans with *Maackia amurensis* agglutinin
MAA^{PL}/SNA^{PL}	seminal plasma sialylation ratio
MAA^{S}	relative reactivity of serum CLU glycans with *Maackia amurensis* agglutinin
MAA^{S}/SNA^{S}	serum sialylation ratio
$MgCl_2 \times 6H_2O$	magnesium dichloride hexahydrate
$MnCl_2 \times 4H_2O$	manganese(II) chloride tetrahydrate
mRNA	messenger RNA
mtDNA	mitochondrial DNA
N	normozoospermia
NAD+	nicotinamide adenine dinucleotide
Neu5Ac/NANA	N-acetylneuraminic acid
OAT	oligoasthenoteratozoospermia
OS	oxidative stress
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1α
RNA	ribonucleic acid
ROC	receiver operating characteristics
ROS	reactive oxygen species
SA	sialic acid
SIRT3	sirtuin-3
$SIRT3^{PL}$	seminal plasma SIRT3 concentration
$SIRT3^{S}$	serum SIRT3 concentration
SIRT5	sirtuin-5
$SIRT5^{PL}$	seminal plasma SIRT5 concentration
$SIRT5^{S}$	serum SIRT5 concentration
SIRTs	sirtuins
SNA	*Sambucus nigra* agglutinin
SNA^{PL}	relative reactivity of seminal plasma CLU glycans with *Sambucus nigra* agglutinin
SNA^{S}	relative reactivity of serum CLU glycans with *Sambucus nigra* agglutinin
SOD	superoxidase dismutase

SP	seminal plasma
T	teratozoospermia
TAC	total antioxidant capacity
TAS	total antioxidant status
TAS^{PL}	seminal plasma total antioxidant status
TAS^{S}	serum total antioxidant status
TBS	Tris-buffered saline
TPTZ	2,4,6-tripyridyl-s-triazine
WHO	World Health Organization

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Table S1. The differences in the values of seminal plasma and blood serum parameters analyzed between groups of patients with fertility problems without Bonferroni correction.

Parameter	AT n^{PL}=22 n^{S}=15 median (IQR)	N n^{PL}=43 n^{S}=18 median (IQR)	OAT n^{PL}=29 n^{S}=27 median (IQR)	T n^{PL}=38 n^{S}=31 median (IQR)
SNA^{PL} (AU)	0.203 (0.148-0.244)	0.199 (0.132-0.296)	0.184 (0.133-0.299)	0.202 (0.114-0.266)
MAA^{PL} (AU)	0.933 (0.226-1.680)	0.376$^{♦}$ (0.177-1.348)	0.171†* (0.098-0.214)	0.740 (0.354-1.476)
MAA^{PL}/SNA^{PL}	4.455 (1.256-7.423)	1.731$^{♦}$ (0.460-4.634)	0.756†* (0.377-1.513)	3.781 (1.436-8.082)
$SIRT3^{PL}$ (ng/mL)	10.90$^{†♦}$ (9.23-12.32)	9.35$^{†♦}$ (5.68-11.11)	2.11 (1.76-2.42)	2.64 (2.11-3.67)
$SIRT5^{PL}$ (ng/mL)	5.72 (4.72-8.31)	7.28$^{♦}$ (1.67-7.97)	1.34†* (1.17-1.49)	6.89 (1.38-7.72)
TAS^{PL} (mM)	1.79 (1.59-1.99)	1.71 (1.54-1.97)	1.70 (1.51-1.93)	1.72 (1.55-1.85)
$FRAP^{PL}$ (mM)	3.68 (3.27-4.14)	3.65 (2.85-4.37)	3.04 (2.45-3.81)	3.72 (2.93-4.40)
SNA^{S} (AU)	0.412 (0.366-0.477)	0.881$^{†*♦}$ (0.630-1.140)	0.495 (0.368-0.613)	0.428 (0.356-0.525)
MAA^{S} (AU)	0.016 (0.002-0.074)	0.081†* (0.054-0.106)	0.020 (0.002-0.086)	0.035 (0.004-0.072)
MAA^{S}/SNA^{S}	0.034 (0.005-0.152)	0.081 (0.064-0.122)	0.036 (0.005-0.167)	0.085 (0.012-0.159)
$SIRT3^{S}$ (ng/mL)	8.94† (6.58-19.15)	2.73$^{†*♦}$ (1.61-7.35)	6.27 (5.54-15.07)	5.93 (4.29-11.53)
$SIRT5^{S}$ (ng/mL)	2.25 (2.06-3.22)	2.01 (1.39-2.53)	2.02 (1.84-4.11)	2.05 (1.61-3.27)
TAS^{S} (mM)	1.37 (1.26-1.82)	1.54$^{♦}$ (1.42-1.70)	1.40 (1.32-1.52)	1.38 (1.29-1.75)
$FRAP^{S}$	1.20†	1.49*	1.33	1.38

(mM)	(1.00-1.35)	(1.43-1.59)	(1.10-1.55)	(1.22-1.56)

SNA^{PL} - relative reactivity of seminal plasma CLU glycans with *Sambucus nigra* agglutinin; MAA^{PL} - relative reactivity of seminal plasma CLU glycans with *Maackia amurensis* agglutinin; MAA^{PL}/SNA^{PL} - seminal plasma sialylation ratio; $SIRT3^{PL}$ - seminal plasma SIRT3 concentration; $SIRT5^{PL}$ - seminal plasma SIRT5 concentration; TAS^{PL} - seminal plasma total antioxidant status; $FRAP^{PL}$ - seminal plasma ferric reducing antioxidant power; SNA^{S} - relative reactivity of serum CLU glycans with *Sambucus nigra* agglutinin; MAA^{S} - relative reactivity of serum CLU glycans with *Maackia amurensis* agglutinin; MAA^{S}/SNA^{S} - serum sialylation ratio; $SIRT3^{S}$ - serum SIRT3 concentration; $SIRT5^{S}$ - serum SIRT5 concentration; TAS^{S} - serum total antioxidant status; $FRAP^{S}$- serum ferric reducing antioxidant power. AT – asthenoteratozoospermia, N - normozoospermia, OAT - oligoasthenoteratozoospermia, T - teratozoospermia. n^{PL} and n^{S} – number of seminal plasma and serum samples, respectively. Significant differences versus: †T group, *AT group, $^{\blacklozenge}$OAT group. The two-tailed *p*-Value of less than 0.05 was considered significant.

Table S2. The results of receiver operating characteristic (ROC) curves analysis for seminal plasma parameters.

Parameter	Compared group (vs. the rest of the samples taken together)	AUC	mean AUC	AUC with 95% confidence interval	Cut off point	Sensitivity	Specificity	*p*
SNA^{PL}	AT	0.499	0.492	0.364-0.633	0.120	0.889	0.239	0.986
	N	0.484		0.357-0.611	0.164	0.414	0.654	0.808
	OAT	0.499		0.376-0.623	0.155	0.414	0.716	0.992
	T	0.486		0.367-0.605	0.179	0.618	0.461	0.816
MAA^{PL}	AT	0.662	0.670	0.532-0.791	0.911	0.556	0.750	0.015
	N	0.500		0.370-0.630	0.261	0.724	0.407	0.997
	OAT	0.826		0.739-0.913	0.214	0.759	0.852	0.000
	T	0.693		0.594-0.792	0.313	0.824	0.592	0.000
MAA/SNA^{PL}	AT	0.653	0.692	0.533-0.773	0.837	1.00	0.337	0.012
	N	0.527		0.396-0.657	0.631	0.345	0.802	0.690
	OAT	0.777		0.686-0.868	2.033	0.897	0.556	0.000
	T	0.678		0.574-0.782	2.301	0.676	0.671	0.001
SIRT3^{PL}	AT	0.809	0.786	0.709-0.908	9.230	0.773	0.800	0.000
	N	0.769		0.684-0.854	5.677	0.757	0.763	0.000
	OAT	0.847		0.775-0.919	2.500	0.821	0.809	0.000
	T	0.720		0.630-0.811	4.645	1.000	0.540	0.000
SIRT5^{PL}	AT	0.633	0.655	0.516-0.749	1.466	0.955	0.376	0.026
	N	0.617		0.510-0.725	5.594	0.730	0.581	0.033
	OAT	0.816		0.730-0.902	2.084	0.964	0.737	0.000
	T	0.555		0.440-0.670	5.249	0.694	0.540	0.352
TAS^{PL}	AT	0.587	0.538	0.465-0.708	1.53	1.000	0.245	0.161
	N	0.489		0.365-0.613	1.55	0.324	0.780	0.865
	OAT	0.514		0.376-0.652	1.50	0.250	0.891	0.840
	T	0.562		0.454-0.669	1.93	0.889	0.288	0.260
FRAP^{PL}	AT	0.544	0.567	0.424-0.663	3.393	0.727	0.481	0.473
	N	0.542		0.433-0.650	4.713	0.209	0.920	0.453
	OAT	0.639		0.524-0.754	3.141	0.586	0.693	0.018
	T	0.544		0.434-0.653	4.219	0.333	0.787	0.435

SNA^{PL} - relative reactivity of seminal plasma CLU glycans with *Sambucus nigra* agglutinin; MAA^{PL} - relative reactivity of seminal plasma CLU glycans with *Maackia amurensis* agglutinin; MAA^{PL}/SNA^{PL} - seminal plasma CLU sialylation ratio; SIRT3^{PL} - seminal plasma SIRT3 concentration; SIRT5^{PL} - seminal plasma SIRT5 concentration; TAS^{PL} - seminal plasma total antioxidant status; FRAP^{PL} - seminal plasma ferric reducing antioxidant power. AT – asthenoteratozoospermia, N – normozoospermia, OAT – oligoasthenoteratozoospermia, T – teratozoospermia. Area under the ROC curve (AUC) is given with 95% confidence interval. Data with AUC greater than 0.700 are marked in grey. Based on the AUC, the clinical value of laboratory test can be defined as: 0-0.5 - zero, 0.5-0.7 - limited, 0.7-0.9 - moderate and >0.9 - high. An AUC of ≥0.7, $p<0.05$, was used as the criterion demonstrating the moderate clinical value of an examined parameter.

Table S3. The results of receiver operating characteristic (ROC) curves analysis for serum parameters.

Parameter	Compared group (vs. the rest of the samples taken together)	AUC	mean AUC	AUC with 95% confidence interval	Cut off point	Sensitivity	Specificity	*p*
SNA^S	AT	0.647	0.653	0.506-0.789	0.477	0.800	0.554	0.041
	N	0.863		0.745-0.981	0.612	0.813	0.822	0.000
	OAT	0.478		0.349-0.608	0.437	0.667	0.435	0.745
	T	0.625		0.510-0.740	0.569	0.839	0.448	0.034
MAA^S	AT	0.594	0.592	0.448-0.739	0.023	0.600	0.622	0.203
	N	0.700		0.553-0.847	0.036	0.875	0.548	0.008
	OAT	0.516		0.377-0.655	0.024	0.556	0.613	0.821
	T	0.557		0.436-0.677	0.080	0.871	0.310	0.357
MAA/SNA^S	AT	0.544	0.514	0.388-0.701	0.055	0.600	0.581	0.581
	N	0.524		0.391-0.657	0.055	0.875	0.493	0.724
	OAT	0.510		0.371-0.650	0.036	0.519	0.629	0.883
	T	0.479		0.348-0.609	0.015	0.355	0.759	0.748
SIRT3^S	AT	0.714	0.656	0.596-0.832	6.044	0.933	0.526	0.000
	N	0.749		0.605-0.892	3.608	0.611	0.945	0.001
	OAT	0.607		0.484-0.730	5.000	1.000	0.328	0.088
	T	0.555		0.432-0.677	6.630	0.645	0.500	0.381
SIRT5^S	AT	0.637	0.561	0.516-0.758	2.035	0.867	0.513	0.028
	N	0.588		0.431-0.745	1.388	0.278	0.945	0.273
	OAT	0.452		0.323-0.581	2.019	0.556	0.609	0.467
	T	0.566		0.437-0.695	1.795	0.387	0.817	0.313
TAS^S	AT	0.544	0.572	0.359-0.729	1.28	0.333	0.842	0.642
	N	0.656		0.525-0.787	1.39	0.833	0.534	0.019
	OAT	0.549		0.427-0.672	1.64	0.926	0.297	0.423
	T	0.538		0.408-0.668	1.38	0.581	0.600	0.570
FRAP^S	AT	0.691	0.610	0.542-0.840	1.352	0.800	0.579	0.012
	N	0.661		0.530-0.792	1.431	0.778	0.658	0.016
	OAT	0.544		0.404-0.685	1.176	0.407	0.797	0.537
	T	0.544		0.425-0.664	1.170	0.903	0.300	0.468

SNA^S - relative reactivity of serum CLU glycans with *Sambucus nigra* agglutinin; MAA^S - relative reactivity of serum CLU glycans with *Maackia amurensis* agglutinin; MAA^S/SNA^S - serum sialylation ratio; SIRT3^S - serum SIRT3 concentration; SIRT5^S - serum SIRT5 concentration; TAS^S - serum total antioxidant status; FRAP^S - serum ferric reducing antioxidant power. AT – asthenoteratozoospermia, N – normozoospermia, OAT – oligoasthenoteratozoospermia, T – teratozoospermia. Area under the ROC curve (AUC) is given with 95% confidence interval. Data with AUC equal or greater than 0.700 are marked in grey. Based on the AUC, the clinical value of laboratory test can be defined as: 0–0.5 - zero, 0.5–0.7 - limited, 0.7–0.9 - moderate and >0.9 - high. An AUC of ≥0.7, $p<0.05$, was used as the criterion demonstrating the moderate clinical value of an examined parameter.

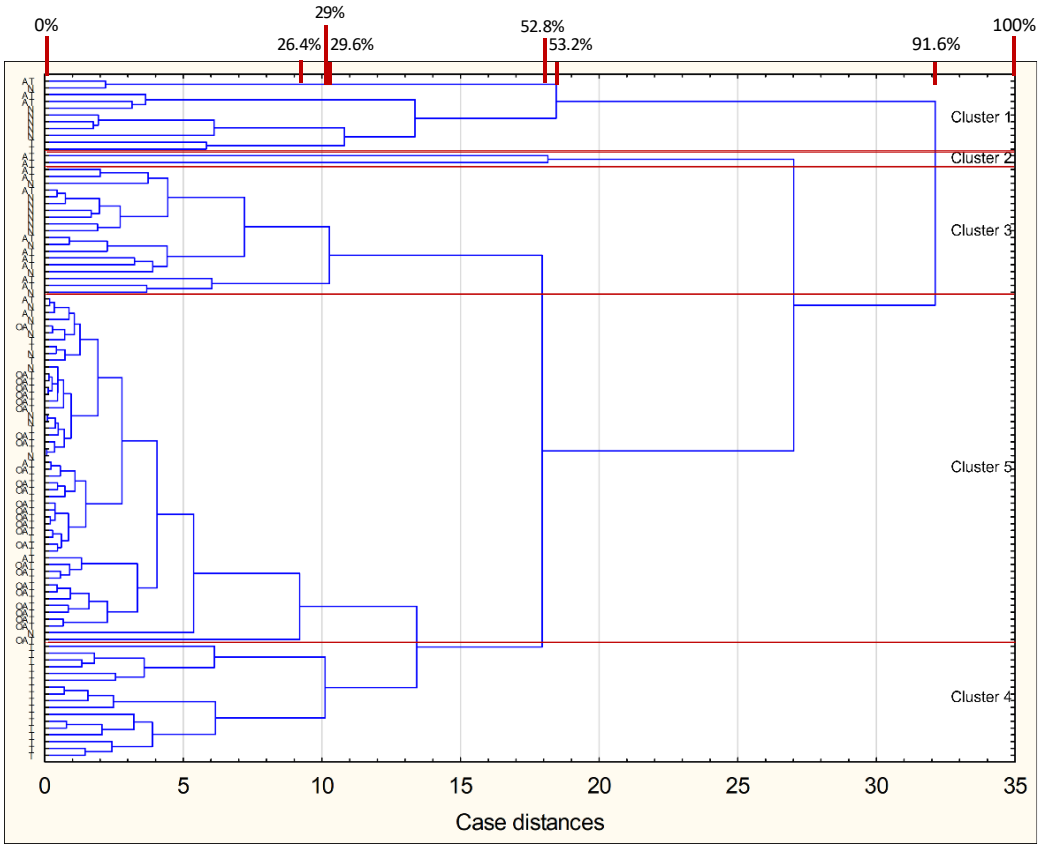

Figure S1. Dendrogram of cluster analysis of selected seminal plasma parameters. The cluster analysis was performed for parameters that simultaneously comply the following criteria: they allow for the differentiation of study groups and in the ROC curve analysis had moderate or high clinical value (AUC ≥0.715). Each seminal plasma sample is represented by a combination of four parameters: CLU relative reactivity with MAA, MAA/SNA ratio, SIRT3 and SIRT5 concentrations. AT – asthenoteratozoospermia, N – normozoospermia, OAT – oligoasthenoteratozoospermia, T – teratozoospermia. Case distances for each cluster were expressed in % of total distance (on x-axis 35=100%): cluster 1 – 53.2%, cluster 2 – 52.8%, cluster 3 – 29.6%, cluster 4 – 29%, cluster 5 – 26.4% (for details see Results section).

Table S4. The results of cluster analysis performed for seminal plasma samples.

Cluster No.	**AT (n=18)**	**N (n=25)**	**OAT (n=27)**	**T (n=30)**
	The number of samples (percentage participation in whole group)			
5 (n=51)	4 (22.2%)	9 (36%)	27 (100%)	11 (36.7%)
4 (n=17)	0 (0%)	0 (0%)	0 (0%)	17 (56.7%)
3 (n=19)	9 (50%)	10 (40%)	0 (0%)	0 (0%)
2 (n=2)	2 (11.1%)	0 (0%)	0 (0%)	0 (0%)
1 (n=11)	3 (16.7%)	6 (24%)	0 (0%)	2 (6.6%)

AT - asthenoteratozoospermia, N - normozoospermia, OAT - oligoasthenoteratozoospermia, T - teratozoospermia. Each group is presented as a vector of four parameters: relative reactivity of seminal plasma CLU glycans with *Maackia amurensis* agglutinin, CLU sialylation ratio (MAA^{PL}/SNA^{PL}), seminal plasma SIRT3 and SIRT5 concentrations.

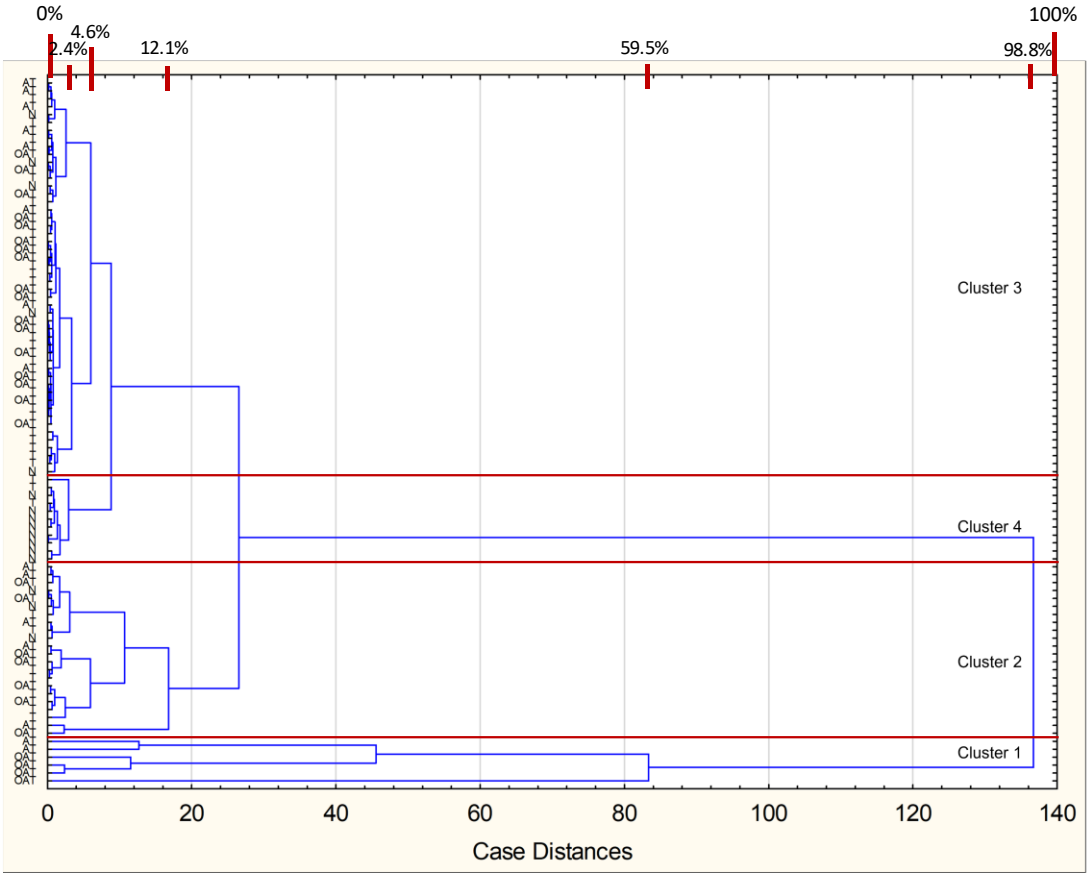

Figure S2. Dendrogram of cluster analysis of selected blood serum parameters. The cluster analysis was performed for parameters that simultaneously comply the following criteria: they allow for the differentiation of study groups and in the ROC curve analysis had moderate or high clinical value (AUC ≥0.701). Each seminal plasma sample is represented by a combination of four parameters: CLU relative reactivity with SNA and MAA, SIRT3 and FRAP concentrations. AT – asthenoteratozoospermia, N – normozoospermia, OAT – oligoasthenoteratozoospermia, T – teratozoospermia. Case distances for each cluster were expressed in % of total distance (on x-axis 140=100%): cluster 1 – 59.5%, cluster 2 – 12.1%, cluster 3 – 4.6%, cluster 4 – 2.3% (for details see Results section).

Table S5. The results of cluster analysis performed for blood serum samples.

Cluster No.	**AT (n=15)**	**N (n=16)**	**OAT (n=28)**	**T (n=30)**
	The number of samples (percentage participation in whole group)			
4 (n=11)	0 (0%)	8 (50%)	0 (0%)	3 (10%)
3 (n=50)	8 (53.4%)	5 (31.2%)	17 (60.7%)	20 (66.7%)
2 (n=22)	5 (33.3%)	3 (18.8%)	7 (25%)	7 (23.3%)
1 (n=6)	2 (13.3%)	0 (0%)	4 (14.3%)	0 (0%)

AT - asthenoteratozoospermia, N - normozoospermia, OAT - oligoasthenoteratozoospermia, T - teratozoospermia. Each group is presented as a vector of four parameters: relative reactivity of serum CLU glycans with *Sambucus nigra* agglutinin, relative reactivity of serum CLU glycans with *Maackia amurensis* agglutinin, serum SIRT3 and FRAP concentrations.

15.4. Załącznik 4 – III artykuł oryginalny:

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Are There Associations between Seminal Plasma Advanced Oxidation Protein Products and Selected Redox-Associated Biochemical Parameters in Infertile Male Patients? A Preliminary Report

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Abstract: Oxidative stress (OS) is one of the reasons for male infertility. Seminal plasma contains a multitude of enzymes and ions which influence OS and thus may affect male fertility. The aim of the study was to check for associations between seminal plasma advanced oxidation protein products (AOPP) concentrations and levels of selected biochemical parameters (total protein, iron, uric acid, magnesium, calcium) in infertile men, and establish whether they are associated with sperm disorders. Seminal plasma AOPP, as well as total protein, iron, uric acid, calcium, and magnesium concentrations, were determined for the following patient groups: normozoospermic (N; n = 33), teratozoospermic (T; n = 30), asthenoteratozoospermic (AT; n = 18), and oligoasthenoteratozoospermic (OAT; n = 28). AOPP concentrations were significantly higher in N and T groups in comparison to AT and OAT groups. Total protein concentrations were significantly lower in the T group in comparison to the AT and OAT groups, whereas iron concentrations significantly decreased in the OAT group in comparison to the T and N patients. AOPP differentiates AT patients from men with other sperm disorders. Our results suggest that asthenozoospermia may be connected with total protein levels. Insufficient iron levels may reflect a decrease in sperm count.

Keywords: male infertility; seminal plasma; oxidative stress parameters; oxidative-antioxidant balance; seminal plasma biochemical parameters

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1. Introduction

Male infertility alone constitutes approximately 40% of infertility cases [1] and has become a growing problem worldwide [2]. The World Health Organization (WHO) recommends semen analysis, which applies to semen and spermatozoa properties [3], but seminal plasma contains a multitude of proteins, glycoproteins, lipids, enzymes, ions, and other chemical compounds that may influence the proper spermatozoa maturation process, as well as gamete fusion [4,5]. Among possible reasons for male infertility, oxidative stress (OS) is one of the main causes of idiopathic male infertility [6,7]. It has been established that a lack of oxidative-antioxidant balance affects the quality of sperm parameters, such as morphology, motility and viability [8,9].

Oxidative stress is defined as an imbalance between the generation of reactive oxygen species (ROS) and the protective action of antioxidant systems responsible for their neutralization and removal [10]. Imbalance between the production and utilization of ROS leads

to the damage of many cell structures, especially the phospholipids of cellular membranes. In turn, lipid peroxidation triggers signaling cascades of the inflammatory processes which promote the peroxidation of lipids, resulting in intracellular oxidative burden. The sequence of events involves lipid peroxidation, loss of membrane integrity with increased permeability, reduced sperm motility, structural DNA damage, and, finally, apoptosis [11]. Sperm cells are mitochondria-rich structures, and these cell organs enable proper sperm motility. In the case of oxidative stress, as a product of nicotinamide adenine dinucleotide (NAD)-dependent redox reactions, ROS damage mitochondria, leading to sperm motility disorders [12–14]. The effect of oxidative-antioxidative imbalance is also reflected in the sperm cells' DNA damage, which affects both single- and double-stranded DNA molecules. These damages have been proven to lead to lowered total sperm count [7,15,16]. Although the antioxidant defense system is active in semen, its activity is limited, as the amount of cytoplasm in the sperm cell is low [17]. Spermatozoa are extremely vulnerable to oxidative stress because they lack the necessary repair systems and are unable to restore oxidative damages. Spermatozoa membranes are rich in polyunsaturated fatty acids, which makes them highly susceptible to lipid peroxidation. Oxidative stress results in axonemal damage, decreased sperm viability, and increased midpiece sperm morphological defects. These dysfunctions may contribute to decreased sperm motility [18].

Human seminal plasma constitutes an antioxidant system including both enzymatic and non-enzymatic components. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) belong to the main enzymatic antioxidant defense. Low molecular weight non-enzymatic antioxidants such as vitamins (A, E, C, B complex), ions (calcium, iron, zinc, selenium, copper, chrome), glutathione, pantothenic acid, carnitine, and coenzyme Q10 support the enzymatic group of an antioxidant system [19–21].

Seminal plasma contains a multitude of protein compounds that may take part in the inactivation of reactive species generated during oxidative stress; as a result, they are oxidized themselves. One of the parameters enabling the measurements of such compounds are advanced oxidation protein products (AOPP) [22]. Other important factors associated with the proper oxidative-antioxidant balance are, i.a., iron (Fe), uric acid (UA), magnesium (Mg), and calcium (Ca), which have been chosen for this study. It has been described that, in the case of cellular metabolism altered by oxidative stress, iron takes part in the lipid membranes' peroxidation [23]. It is also considered a potent pro-oxidant when not stored in transferrin or ferritin [24]. Uric acid is a product of purine metabolism and belongs to the main low molecular weight antioxidants. UA is a scavenger of peroxyl radicals, hydroxyl radicals, and singlet oxygen generated during OS [25]. Magnesium is the fourth most abundant cation in the human body, and acts as a cofactor of 300 enzymes, i.a., those engaged in protein synthesis, glycolysis, and the transmembrane transport of ions [26]. It is also responsible for reactions with ATP, as well as competing with calcium for binding sites on proteins and membranes [27]. It has been reported that magnesium may be a marker of the prostate gland, and reduced seminal magnesium levels may be associated with premature ejaculation [28]. It has also been reported that hypomagnesemia is associated with elevated lipoprotein oxidation and, via calcium overload, also contributes to an increase in protein oxidation [29]. Calcium plays a crucial role in the acrosome reaction during the fertilization process [4], as well as in determining proper sperm motility [28].

The literature data concerning the basic biochemical laboratory parameters in the oxidative stress context performed in human seminal plasma are insufficient. Therefore, we decided to assess seminal plasma advanced oxidation protein products (AOPP) as well as levels of selected biochemical parameters (total protein (TP), iron (Fe), uric acid (UA), magnesium (Mg), and calcium (Ca)) which may have an impact on the oxidative-antioxidant balance. We were interested in whether they correlated with each other and additionally whether they were associated with disorders of sperm parameters.

2. Materials and Methods

2.1. Patient Samples

Seminal plasma samples were collected from infertile male patients who attended the Clinical Center of Gynecology, Obstetrics and Neonatology in Opole (Poland) and Fertility Clinics InviMed in Warsaw (Poland). The informed consent was signed by all patients that participated in this study. Our study was conducted according to the guidelines of the Helsinki II declaration, and the protocol was approved by the Bioethics Human Research Committee of Wroclaw Medical University (No. KB 549/2019 and No. KB 707/2019).

Seminal samples were collected into sterile containers after 3–5 days of sexual abstinence (inclusion criterium) through masturbation. After liquefaction (maximum 60 min at 37 °C), standard semen analysis was performed according to WHO 2010 directives (WHO 2010). Semen volume, pH, and sperm viability were assessed using manual techniques, whereas total sperm count in the ejaculate, sperm concentration, total motility, progressive motility, and morphology were carried out using computer-assisted sperm analysis (SCA Motility and Concentration, software version 6.5.0.5, Microptic SL, Barcelona, Spain). All input data in this method were consistent with current WHO recommendations for semen analysis. Next, the ejaculates were centrifuged at 3500× *g* for 10 min at room temperature, and the supernatants were aliquoted and stored at −86 °C in the Wroclaw Medical University Biobank until use.

Based on standard semen analysis (sperm concentration, progressive motility and morphology of spermatozoa), seminal plasma samples (n = 109) were divided into groups: normozoospermic (N, n = 33; normal values of ejaculate parameters; median age: 32 years [IQR 24–49]), teratozoospermic (T, n = 30; <4% of spermatozoa had normal morphology; median age: 33 years [IQR 28–36]), asthenoteratozoospermic (AT, n = 18; <32% of sperm demonstrated progressive motility and <4% of spermatozoa had normal morphology; median age: 34 years [IQR 31–36]) and oligoasthenoteratozoospermic (OAT, n = 28; sperm count $<15 \times 10^6$ mL^{-1}, <32% of sperm demonstrated progressive motility and <4% of spermatozoa had normal morphology; median age: 32 years [IQR 30–35]). None of the seminal samples were infected by bacteria and/or leukospermic. Active inflammation manifested by elevated serum C-reactive protein levels was also an exclusion criterion.

2.2. AOPP Determination

AOPP determination, based on the redox reaction, was carried out according to Witko-Sarsat et al. [30], with the modifications described below. Advanced Oxidation Protein Products present in seminal plasma react with potassium iodide solution (KI) in the presence of acetic acid solution, producing the reduced form of oxidation protein products and iodate ions [21,31]. Briefly, 10 µL of KI was added directly into the well of ELISA plate to 200 µL of twenty-fold diluted in phosphate-buffered saline (PBS) seminal plasma sample, incubated for 2 min at room temperature, and mixed with 20 µL of glacial acetic acid. Absorbance was measured immediately at 340 nm against a blank sample without biological material but containing all other reagents, using the Multiskan Go ELISA plate reader (Thermo Fischer Scientific, Roskilde, Denmark). The obtained results were expressed in chloramine T concentrations which served as a standard, containing parallel chemical moiety as proteins present in seminal plasma. A calibration curve was constructed for chloramine T concentrations ranging from 0 to 80 µmol/L. Blood serum samples with known AOPP concentrations were used as measurement controls for each experiment. All determinations were performed in duplicate to minimize measurement imprecision, using ELISA plates (Nunc MaxiSorp, Thermo Fisher Scientific, Roskilde, Denmark) to reduce the volume of samples used for analysis.

2.3. Biochemical Parameters Measurement

The concentrations of total protein, iron, uric acid, calcium, and magnesium were measured using the biochemical autoanalyzer Konelab20i® (Thermo Scientific, Vantaa, Finland). To determine the seminal plasma total protein concentrations, the biuret method

was used (Total Protein Plus, Thermo Scientific, catalog No. 981826, Vantaa, Finland) according to the manufacturer's instruction. Briefly, protein and copper ions in alkaline solutions formed a colored complex, and the absorbance of the formed complex was subsequently measured at 540 nm. The method used EDTA as a chelating and stabilizing agent for copper ions. Iron concentrations were measured using the colorimetric method with Ferene-S (Iron, Thermo Scientific, catalog No. 981236, Vantaa, Finland), following the manufacturer's instruction. Briefly, in the first step, the iron bonded with proteins was released by guanidine buffer. Then, the total iron (free iron ions as well as iron released from the proteins) reacted with Ferene-S, forming a complex chemical compound, the absorbance of which was read at 600 nm. To measure the uric acid concentrations, a commercial Uric Acid AOX (catalog No. 981391, Thermo Scientific, Vantaa, Finland) reagent was used following the manufacturer's instruction. The methodology of UA determination used in our study was based on UA oxidation by uricase to allantoin. The generated hydrogen peroxide reacted with 4-aminoantipyrine (4-AAP) and N-ethyl-N-(hydroxy-3-sulfopropyl)-m-toluidine (TOOS), forming a blue–violet product. The absorbance of the formed colored reaction product was measured at 540 nm. Magnesium concentration was measured using xylidyl blue (Magnesium XL FS, DiaSys, catalog No. 146109910021, Holzheim, Germany) according to the manufacturer's protocol. The absorbance of the purple complex, formed as a product of the reaction, was measured at 540 nm. To determine the calcium concentration, a colorimetric method using arsenazo III was used (Calcium AS FS, DiaSys, catalog No. 11309910021, Holzheim, Germany), following the manufacturer's instruction. The intensity of the reaction product, the blue complex, was measured at 600 nm.

2.4. Statistical Analysis

All statistical calculations were performed using Statistica 13.3 PL software (StatSoft Inc., Tulsa, OK, USA). The normality of distribution for values of all parameters investigated was analyzed with the Shapiro–Wilk test. The obtained values of AOPP and other biochemical parameters' concentrations were presented as mean ± SD (SD—standard deviation), as well as on the graphs as median with interquartile range (Q1–Q3). As the values of examined parameters did not reach normal distribution, the nonparametric Mann–Whitney U-test was used to compare the levels of determined parameters between examined groups. The associations between values of all parameters were checked by Spearman's rank correlation. The diagnostic significance of the tested parameters was analyzed using receiver operating characteristic (ROC) curves. The p-Values < 0.05 were considered significant.

3. Results

The levels of parameters analyzed are presented in Table 1. Significant differences between studied groups are shown in Figure 1.

3.1. Seminal Plasma AOPP Levels

Seminal plasma AOPP concentrations were significantly higher in normozoospermic patients (median value: 483.19 µmol/L) when compared to the AT group (median value: 163.51 µmol/L) and OAT (median value: 134.26 µmol/L) groups, with significances of $p = 0.000035$ and $p = 0.000002$, respectively. Moreover, in teratozoospermic patients, seminal plasma AOPP concentrations were also significantly higher (median value: 505.00 µM) in comparison to the AT group (median value: 163.51 µmol/L) and OAT men (median value: 134.26 µmol/L), with significances of $p = 0.000043$ and $p = 0.000002$, respectively (Table 1, Figure 1A).

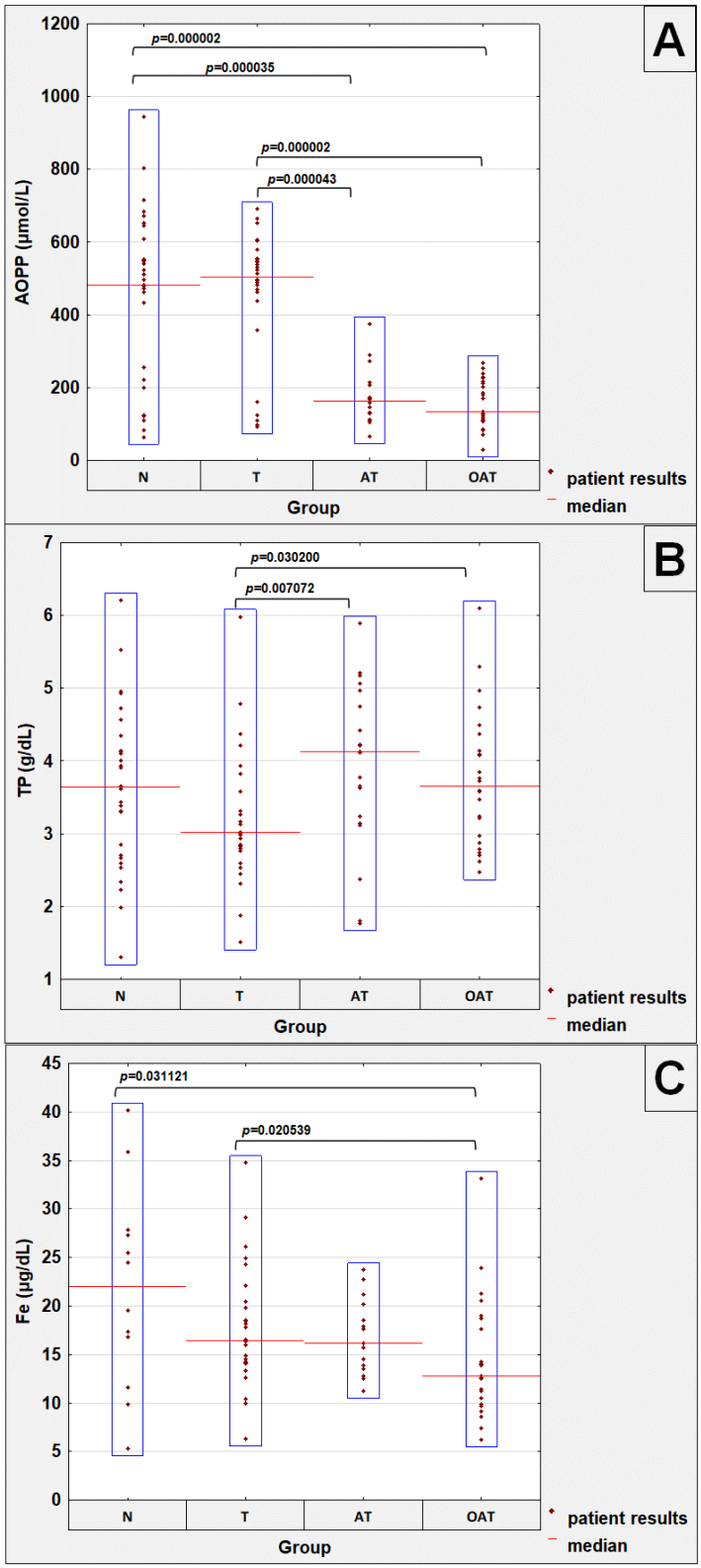

Figure 1. Concentration values of selected seminal plasma parameters which significantly differentiated examined groups of patients: AOPP—advanced oxidation protein products (**A**), TP—total protein (**B**), Fe—iron (**C**). N—normozoospermia, T—teratozoospermia, AT—asthenoteratozoospermia, OAT—oligoasthenoteratozoospermia. All patient results in a given group are gathered in the blue boxes. A two-tailed *p*-Value < 0.05 was considered significant.

Table 1. Concentrations of seminal plasma parameters in male patients with fertility disorders.

Parameter \ Group	N *n* = 33	T *n* = 30	AT *n* = 18	OAT *n* = 28
	MEAN ± SD Median (Range)	**MEAN ± SD Median (Range)**	**MEAN ± SD Median (Range)**	**MEAN ± SD Median (Range)**
AOPP (µmol/L)	451.89 ± 222.02 483.19 (255.53–552.34) p = 0.000035 [a] p = 0.000002 [b]	452.70 ± 183.11 505.00 (439.57–554.47) p = 0.000043 [a] p = 0.000002 [b]	173.79 ± 76.06 163.51 (128.94–206.60)	154.05 ± 61.52 134.26 (110.85–207.13)
TP (g/dL)	3.62 ± 1.12 3.64 (2.69–4.25)	3.18 ± 0.86 3.02 (2.80–3.32) p = 0.007072 [a] p = 0.030200 [b]	3.94 ± 1.13 4.13 (3.19–4.86)	3.75 ± 0.93 3.66 (2.92–4.26)
Fe (µg/dL)	21.82 ± 10.40 22.01 (14.24–27.57) p = 0.031121 [b]	17.75 ± 6.14 16.44 (14.16–20.47) p = 0.020539 [b]	16.80 ± 3.88 16.15 (13.53–20.14)	14.43 ± 6.18 12.80 (9.82–18.69)
UA (mg/dL)	6.44 ± 2.36 6.58 (4.48–7.34)	5.85 ± 1.26 5.84 (4.91–6.44)	6.28 ± 1.75 5.84 (4.83–7.14)	6.48 ± 1.97 6.47 (4.98–7.42)
Mg (mg/dL)	7.40 ± 2.58 8.25 (5.82–9.41)	6.88 ± 1.97 7.28 (5.01–8.59)	6.68 ± 1.73 6.87 (5.00–7.85)	6.35 ± 2.27 5.80 (4.65–8.54)
Ca (mg/dL)	25.16 ± 13.27 26.79 (12.56–32.86)	21.73 ± 9.66 23.58 (13.03–27.55)	19.38 ± 7.61 18.37 (13.76–23.21)	17.03 ± 10.12 13.95 (9.14–27.81)

AOPP—advanced oxidation protein products, TP—total protein, Fe—iron, UA—uric acid, Mg—magnesium, Ca—calcium, N—normozoospermia, T—teratozoospermia, AT—asthenoteratozoospermia, OAT—oligoasthenoteratozoospermia. Significant differences versus: [a] AT group, [b] OAT group. A two-tailed p-Value < 0.05 was considered significant.

3.2. Seminal Plasma Biochemical Parameters Concentrations

Seminal plasma total protein concentrations were significantly lower in the teratozoospermic group (median value: 3.02 g/dL) in comparison to the AT (median value: 4.13 g/dL) and OAT (median value: 3.66 g/dL) groups, with significances of p = 0.007072 and p = 0.030200, respectively. No significant differences between the normozoospermic group (median value: 3.64 g/dL) and the other analyzed groups were found (Table 1, Figure 1B).

Seminal plasma iron concentrations were significantly lower in the OAT group (median value: 12.80 µg/dL) when compared to the teratozoospermic (median value: 16.44 µg/dL) and normozoospermic men (median value: 22.01 µg/dL), with significance of p = 0.020539 and p = 0.031121, respectively. No significant differences between the AT group (median value: 16.15 µg/dL) and the other analyzed groups were found (Table 1, Figure 1C).

No significant differences were found between the groups of men examined for seminal plasma uric acid, magnesium, and calcium concentrations (Table 1). The median value of seminal plasma uric acid concentration was 6.58 mg/dL in normozoospermic patients, 5.84 mg/dL in both teratozoospermic and asthenoteratozoospermic patients, and 6.47 mg/dL in oligoasthenoteratozoospermic patients (Table 1). Median seminal plasma magnesium concentrations in N, T, AT and OAT groups were as follows: 8.25 mg/dL, 7.28 mg/dL, 6.87 mg/dL and 5.80 mg/dL (Table 1). The median value of seminal plasma calcium concentrations was 26.79 mg/dL in the normozoospermic group, 23.58 mg/dL in teratozoospermic men, 18.37 mg/dL in asthenoteratozoospermic patients, and 13.95 mg/dL in the oligoasthenoteratozoospermic group (Table 1).

Significant correlations between the analyzed parameters are shown in Table 2 and Figure 2.

Table 2. Significant correlations between concentrations of analyzed parameters.

Compared Parameters	*R*	*p*
Ca & Mg	0.8714	<0.001
Ca & Fe	0.8120	<0.001
Mg & Fe	0.7885	<0.001
AOPP & Fe	0.5393	<0.001
AOPP & Ca	0.5262	<0.001
AOPP & Mg	0.4511	<0.001
AOPP & TP	−0.2650	0.022

Ca—calcium, Mg—magnesium, Fe—iron, AOPP—advanced oxidation protein products, TP—total protein, R—Spearman's rank coefficient. A two-tailed *p*-value < 0.05 was considered significant.

Very strong positive correlations were observed between seminal plasma calcium and magnesium concentrations (R = 0.8714, $p < 0.001$; Table 2, Figure 2A), as well as between calcium and iron levels (R = 0.8120, $p < 0.001$; Table 2, Figure 2B). Strong positive correlations between seminal plasma magnesium and iron concentrations (R = 0.7885, $p < 0.001$; Table 2, Figure 2C) were found. Moderate correlations between AOPP levels and iron (R = 0.5393, $p < 0.001$; Table 2, Figure 2D), calcium (R = 0.5262, $p < 0.001$; Table 2, Figure 2E), and magnesium (R = 0.4511, $p < 0.001$; Table 2, Figure 2F) concentrations were found. Moreover, there were weak negative correlations between AOPP and total protein levels (R = −0.2650, p = 0.022; Table 2, Figure 2G).

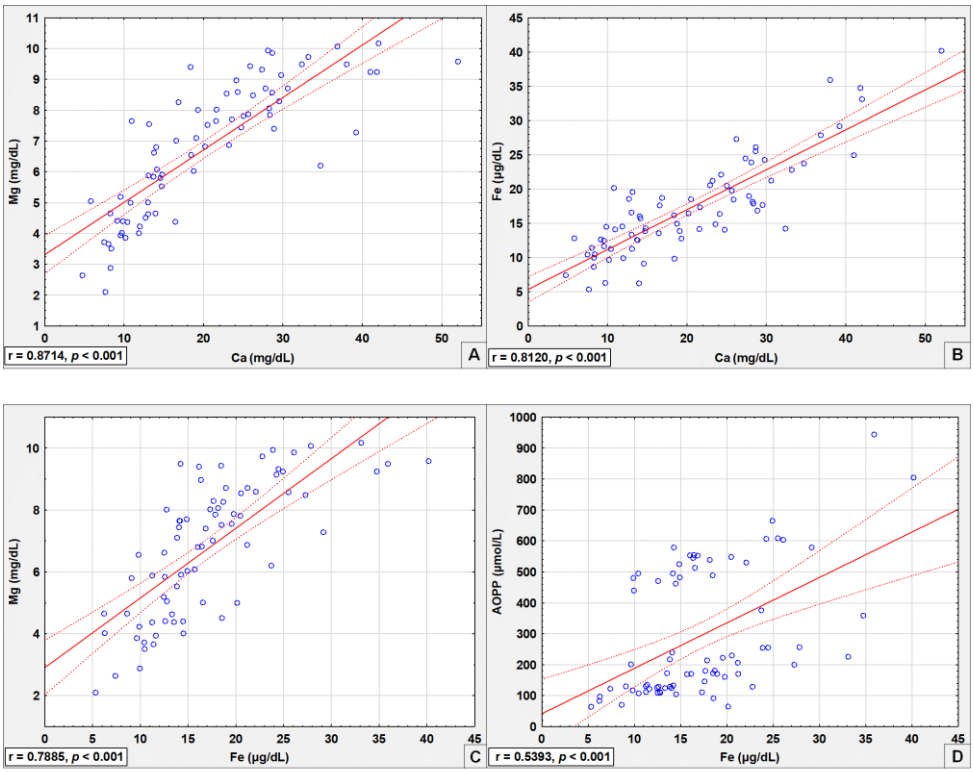

Figure 2. *Cont.*

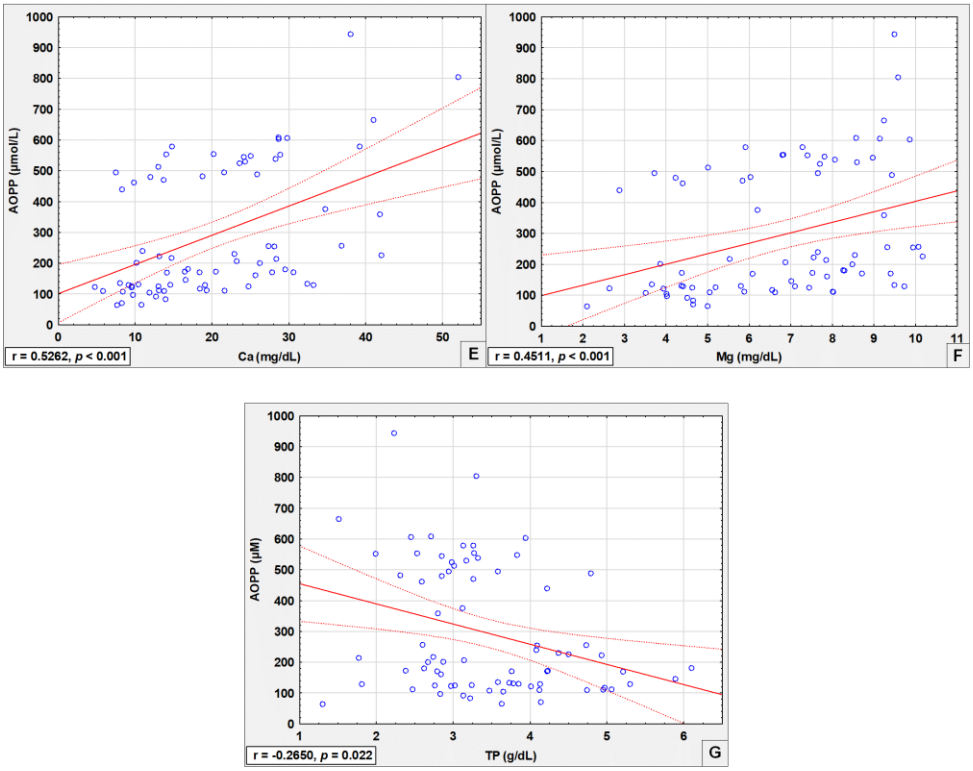

Figure 2. Correlations between concentrations of determined seminal plasma parameters (**A**–**G**). Mg—magnesium, Ca—calcium, Fe—iron, AOPP—advanced oxidation protein products, TP—total protein. The dashed line points indicate the 95% confidence interval. A two-tailed *p*-Value < 0.05 was considered significant.

3.3. ROC Curves Analysis

The receiver operating characteristic (ROC) curves analysis was performed only for parameters the levels of which showed significant differences between examined groups (Table 3). Figure 3 presents the results of the ROC curves analysis for parameters for which the area under the curve (AUC) was higher than 0.7. Based on the AUC, the clinical value of laboratory tests can be defined as: 0–0.5–zero, 0.5–0.7–limited, 0.7–0.9–moderate, and >0.9–high [32].

Table 3. Summary of receiver operating characteristic (ROC) curves analysis for seminal plasma parameters.

Parameter	Compared Groups	AUC	AUC with 95% Confidence Interval	Cut Off Point	Sensitivity	Specificity	*p*
AOPP	T vs. N	0.513	0.368–0.657	482.13	0.667	0.485	0.864
	AT vs. N	0.837	0.724–0.949	375.75	1.000	0.697	0.000
	OAT vs. N	0.855	0.752–0.958	254.47	0.964	0.758	0.000
	AT vs. T	0.838	0.717–0.959	375.75	1.000	0.767	0.000
	OAT vs. T	0.861	0.755–0.967	268.30	1.000	0.800	0.000
	OAT vs. AT	0.552	0.380–0.723	125.75	0.393	0.778	0.555

Table 3. *Cont.*

Parameter	Compared Groups	AUC	AUC with 95% Confidence Interval	Cut Off Point	Sensitivity	Specificity	*p*
TP	T vs. N	0.364	0.213–0.515	2.76	0.800	0.286	0.077
	AT vs. N	0.596	0.429–0.762	4.12	0.550	0.679	0.262
	OAT vs. N	0.531	0.373–0.690	2.62	0.958	0.214	0.699
	AT vs. T	0.728	0.571–0.884	3.63	0.700	0.800	0.004
	OAT vs. T	0.674	0.526–0.822	3.47	0.625	0.767	0.021
	OAT vs. AT	0.585	0.410–0.761	4.09	0.708	0.550	0.340
Fe	T vs. N	0.370	0.150–0.591	12.57	0.889	0.250	0.249
	AT vs. N	0.656	0.417–0.894	23.72	1.000	0.500	0.200
	OAT vs. N	0.725	0.526–0.923	23.88	0.957	0.500	0.027
	AT vs. T	0.541	0.361–0.721	23.72	1.000	0.185	0.657
	OAT vs. T	0.692	0.539–0.846	14.21	0.696	0.741	0.014
	OAT vs. AT	0.672	0.502–0.843	12.47	0.435	0.933	0.047

AOPP—advanced oxidation protein products, TP—total protein, Fe—iron, N—normozoospermia, T—teratozoospermia, AT—asthenoteratozoospermia, OAT—oligoasthenoteratozoospermia. The area under the ROC curve (AUC) is given with a 95% confidence interval. Data with AUC equal to or higher than 0.725 are marked in grey. Based on the AUC, the clinical value of laboratory tests can be defined as: 0–0.5–zero, 0.5–0.7–limited, 0.7–0.9–moderate, and >0.9–high.

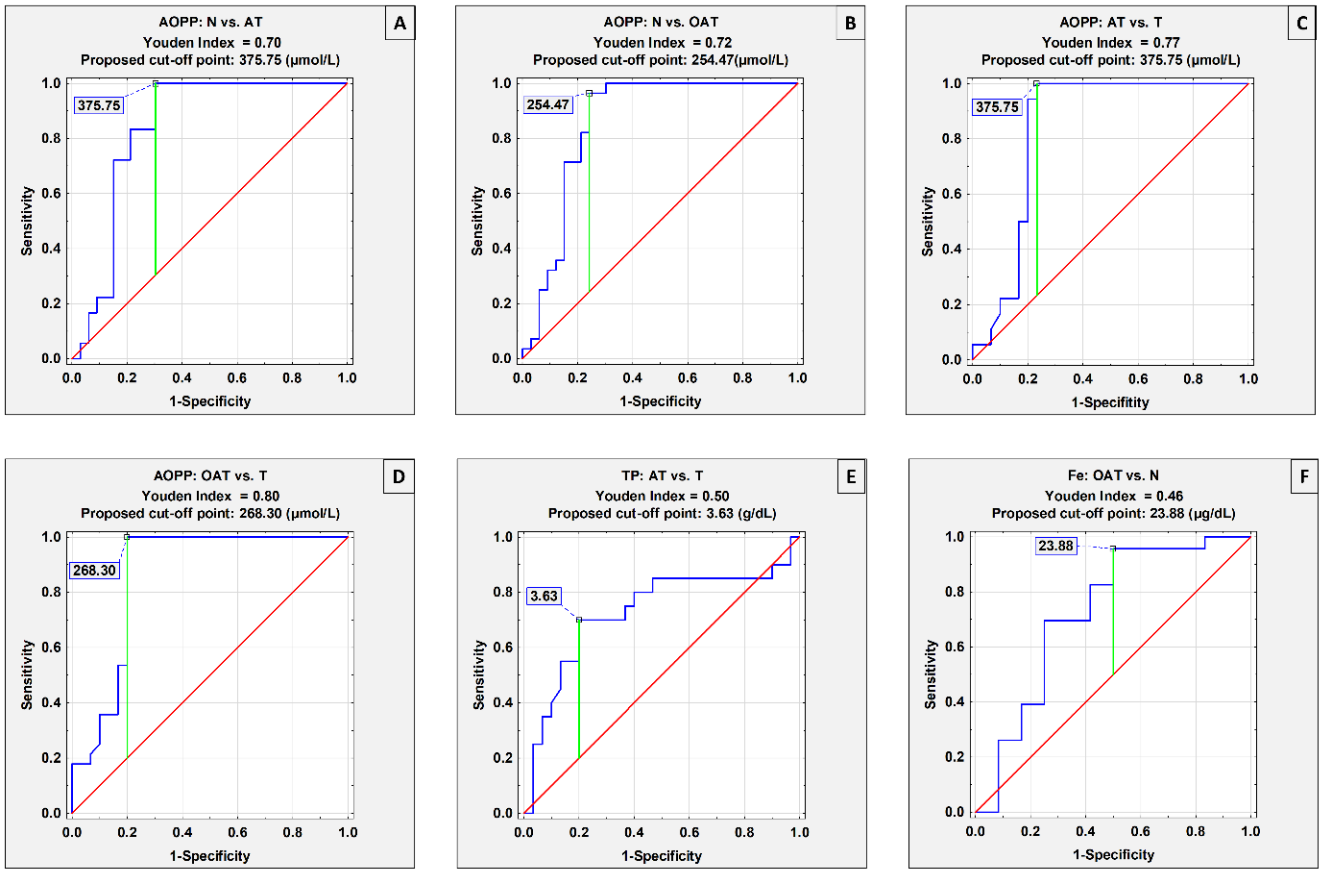

Figure 3. Receiver operating characteristic (ROC) curves for seminal plasma parameters with the area under the curve (AUC) equal to or higher than 0.725 (**A**–**F**). AOPP—advanced oxidation protein products, TP—total protein, Fe—iron. N—normozoospermia, T—teratozoospermia, AT—asthenoteratozoospermia, OAT—oligoasthenoteratozoospermia.

4. Discussion

Although seminal plasma AOPP have been investigated before [21,31,33], there is not much information about the associations between the levels of advanced oxidation protein products and concentrations of biochemical parameters in seminal plasma that may have an impact on the oxidative-antioxidant balance in this biological fluid. Physiologically, AOPP are formed throughout an individual's lifetime in small quantities and increase with age. Significantly higher serum concentrations of AOPP are observed in many pathological conditions. AOPP formation is induced by intensified glycooxidation processes, oxidative-antioxidant imbalance, and coexisting inflammation [34]. The results of our current research may suggest that sperm motility disorders are associated with decreased AOPP levels, which probably interfere with the oxidative-antioxidant balance of seminal plasma. We observed an analogous relationship for the value of the calculated AOPP/TP index (μmol of AOPP per gram of total protein), which was also significantly lower in the seminal plasma of patients with disturbed sperm motility (data not shown). Our previous study revealed that seminal plasma AOPP concentrations were significantly higher in the infertile teratozoospermic and azoospermic patients in comparison to the healthy fertile men [21]. In our other research, seminal plasma AOPP levels were significantly higher in infertile normozoospermic, oligozoospermic, and asthenozoospermic groups than in fertile normozoospermic men [31]. Interestingly, seminal plasma AOPP concentrations investigated by Demir and Ozdem [33] were significantly higher in oligoasthenoteratozoospermic, teratozoospermic, and azoospermic men in comparison to infertile normozoospermic patients. Based on the above information, we presume that the possible association between the formation of advanced oxidation protein products and the disorders of routinely examined sperm parameters is multidirectional and may involve additional factors which were not examined in our study. However, this hypothesis requires verification in future research involving a wider panel of biochemical parameters.

The seminal plasma total protein concentrations found suggest that sperm morphology abnormalities are the only sperm disorder that may be associated with decreased levels of this parameter. In contrast, Collodel et al. [35] reported that decreased seminal plasma total protein concentrations were associated with sperm motility $\leq$5th centile [35]. Mendeluk et al. [36] investigated whether seminal plasma total protein concentration is involved in the hyperviscosity of human seminal plasma. The authors did not observe significant differences between the analyzed groups (samples were analyzed according to the WHO criteria from 1998 and regarded as hyperviscous when the length of the thread formed on withdrawal of a glass rod exceeded 2 cm) in seminal plasma total protein concentrations [36]. Taking all this information into account, we may conclude that the measurements of seminal plasma total protein concentration are not sufficient for the identification of the cause of male infertility connected with sperm parameter disorders.

We observed the associations between decreased iron concentrations in seminal plasma with simultaneously existing sperm abnormalities, such as lower total sperm count, decreased motility, and morphology disorders. Our findings are quite similar to those obtained by Skandhan et al. [37], who reported a significant decrease in seminal plasma iron levels in asthenozoospermic and azoospermic patients in comparison to normozoospermic fertile men [37], and suggested that sperm motility disorders may be connected with the decrease in seminal plasma iron concentration. Additionally, a study by Jia et al. [38] reported a positive correlation between Fe levels and sperm concentration and total sperm count, and Shukla et al. [39] showed significantly decreased seminal plasma Fe levels in all infertile groups of patients in comparison to healthy men [39]. On the other hand, Pant and Srivastava [40] did not find significant differences in seminal plasma Fe concentrations between the studied azoospermic, oligoasthenozoospermic, oligozoospermic and asthenozoospermic groups [40]. Conversely, seminal plasma iron levels investigated by Marzec-Wróblewska et al. [41] were significantly higher in the teratozoospermic patients in comparison to patients with normal sperm parameters. Inconsistency in the results obtained by the abovementioned authors may be due to differences in the selection of the

types of male fertility disorders compared, as well as the methods of iron concentration assessment—many studies concerning iron concentration are based on mass spectrometry (MS) with modifications, whereas our research used spectrophotometry. Magali et al. [42] investigated the role of seminal plasma iron metabolism in oxidative stress, measuring, i.a., seminal plasma iron concentration, but the authors did not refer to the results concerning total seminal plasma iron concentration, suggesting the lack of a relationship between this parameter and other oxidative stress parameters measured [42]. However, the potential pro- and/or antioxidant role of iron in the male reproductive tract is still unknown.

In our study, the mean concentrations of uric acid were higher in the normozoospermic group than in the teratozoospermic group and asthenoteratozoospermic groups, but similar to the oligoasthenoteratozoospermic group; however, the differences were insignificant (Table 1). Lazzarino et al. [43] found no significant differences between groups of infertile patients, also in comparison to the fertile control group. Kand'ár et al. [44] investigated seminal plasma uric acid concentrations in smoking and non-smoking infertile men and also found no significant differences in seminal plasma UA levels between the studied groups; however, there is no data concerning the results of routine semen examination of those patients [44]. On the other hand, Allahkarami et al. [45] showed the presence of a negative correlation between seminal plasma uric acid concentration and sperm morphology [45]. Further experiments concerning the role of seminal plasma uric acid may demonstrate the particular role of uric acid in the context of male fertility.

We did not find significant differences in magnesium and calcium concentrations between examined groups of infertile patients; however, the levels of both elements were visibly higher in normozoospermic men than in patients with sperm disorders. Colagar et al. [46] found no significant differences in seminal plasma magnesium and calcium levels between infertile and fertile smoking and non-smoking groups. While Wong et al. [47] found no significant differences between seminal plasma magnesium and calcium concentrations between fertile and infertile men (semen analysis was performed according to WHO guidelines from 1992), the study revealed strong positive correlations between the seminal plasma concentrations of these elements [47], as does our present research. Sørensen et al. [48] investigated whether seminal plasma magnesium and calcium levels, among others, have an impact on time to pregnancy in healthy couples. No significant differences in the levels of these elements were shown for either group: men from couples with short time to pregnancy (one month), and men from couples with long time to pregnancy (ten months). The findings of Abdul-Rasheed [27] revealed significant decreases in seminal plasma magnesium concentrations in all studied infertile groups in comparison to normozoospermic fertile men. Based on the presented information, we may conclude that the role of calcium in the male reproductive tract in the context of maintenance of oxidative-antioxidant balance is complex. Achcińska and Kratz [4] underlined that calcium, especially its sufficient intracellular levels, is crucial in proper capacitation processes, as well as gamete fusion [4]. This raises the question of whether there are associations between seminal plasma and intracellular calcium levels in the groups of infertile men in comparison to healthy controls. Taking this information into account, together with the moderate positive correlation between seminal plasma calcium concentrations and AOPP levels, further investigation is needed in this field, especially with regards the comparison of results obtained for infertile and fertile men.

Our study revealed a very strong positive correlation between seminal plasma calcium and magnesium concentrations (Table 2, Figure 2A), which is consistent with the results of Wong et al. [47] but may be surprising, given the fact that magnesium is a calcium antagonist [49]. It is worth underlining that calcium plays a crucial role in the initiation of acrosomal reaction; moreover, during the capacitation process, sperm cells are modified, and the level of calcium ions physiologically increases [50]. Based on this information, we may presume that abnormal seminal plasma magnesium levels in infertile men may interrupt the proper biological function of calcium. A very strong positive correlation between seminal plasma calcium and iron concentrations was also found (Table 2, Figure 2B). Mito-

chondrial iron metabolism has been proven crucial for energy production and metabolism during spermatogenesis [51], but the relationship between calcium and iron levels in spermatogenesis should be investigated. Results of our research concerning the relationship between seminal plasma magnesium and iron concentrations contrast with the study results obtained by Srivastava et al. [40], who found a lack of significant correlations between these parameters. Our results, which reveal a strong positive correlation between Mg and Fe levels (Table 2, Figure 2C), seem surprising, given that lipid peroxidation is increased in the case of elevated iron levels and decreased magnesium concentrations [23,29]. Perhaps there is another mechanism that takes part in the regulation of magnesium and iron expression in the male reproductive tract. Moderate positive correlations between seminal plasma AOPP and iron, calcium, and magnesium were found (Table 2, Figure 2D–F), which may confirm the important role of these elements in protein oxidation. It may be hypothesized that weak negative correlations between seminal plasma AOPP and total protein concentrations are associated with the fact that AOPP is derived mainly from oxidation-modified albumin aggregates or fragments [34]. As albumin is the most abundant seminal plasma protein and a part of total protein amount, AOPP formation is reversely associated with the decrease in albumin concentration and, in consequence, with the decrease in total protein levels.

It is worth underlining that, as far as we know, this is the first study in which ROC curves analysis was performed for seminal plasma AOPP, TP and Fe concentrations in the context of decreased male fertility. ROC curves analysis for AOPP revealed this parameter to have moderate clinical value in the differentiation of normozoospermic infertile patients from the asthenoteratozoospermic (proposed cut-off point: 375.75 μmol/L; AUC = 0.837) with a sensitivity of 100% and specificity of 69.7%, and the oligoasthenoteratozoospermic (proposed cut-off point: 254.47 μmol/L; AUC = 0.855) with a sensitivity of 96.4% and specificity of 75.8% (Table 3, Figure 3A,B). Moreover, AOPP concentrations have moderate clinical value in the differentiation of teratozoospermic patients from the asthenoteratozoospermic (proposed cut-off point: 375.75 μmol/L, AUC = 0.838) with a sensitivity of 100% and specificity of 76.7%, and the oligoasthenoteratozoospermic (proposed cut-off point: 268.30 μmol/L, AUC = 0.861) with a sensitivity of 100% and specificity of 80% (Table 3, Figure 3C,D), which led us to conclude that levels of seminal plasma AOPP may be a usable marker of sperm motility disorders (reflected as asthenozoospermia in routine semen analysis). In this study, seminal plasma total protein concentration had a moderate clinical value and enabled differentiation of the AT group from the T group (proposed cut-off point 3.63 g/dL, AUC = 0.728), with a sensitivity of 70% and specificity of 80% (Table 3, Figure 3E), which may suggest that TP levels, similarly to AOPP, also differentiated asthenozoospermia from the other study groups. Seminal plasma iron concentration had moderate clinical value and enabled the differentiation of normozoospermic infertile patients from the oligoasthenoteratozoospermic (proposed cut-off point: 23.88 μg/dL, AUC = 0.725) with a sensitivity of 95.7% and specificity of 50% (Table 3, Figure 3F). Hence, we may presume that the decrease in sperm count is associated with a decreased level of seminal plasma iron.

5. Conclusions

Oxidative-antioxidant homeostasis is crucial for many physiological processes, including male fertility. Seminal plasma AOPP seems to be a promising parameter differentiating patients with sperm motility problems from men with other sperm disorders; however, the possible association between the formation of advanced oxidation protein products and sperm parameter disorders is multidirectional and may involve additional factors. Our study revealed a significant increase in seminal plasma total protein concentration in the OAT and AT groups in comparison to the T group, suggesting that asthenozoospermia may be connected with seminal plasma total protein levels. The results of our study led us to the conclusion that insufficient iron levels may reflect a decrease in sperm count. The lack of a representative control group of normozoospermic fertile men is the limitation of our study, and makes it impossible to determine if these parameters may be useful in the diagnostics

of idiopathic male infertility. Strong positive correlations between seminal plasma concentrations of iron, magnesium, and calcium may confirm that these elements play a very important role in the proper process of spermatozoa maturation, as well as capacitation and acrosomal reaction. The determination of factors associated with oxidative-antioxidant balance may provide valuable information towards the prediction of the effectiveness of AI. As we mentioned in our previous study [21], the antioxidant supplementation of male patients, in appropriate individual doses, may also be worthy of future investigation in the context of AI and IVF. Figure 4 summarizes the main findings from the present research.

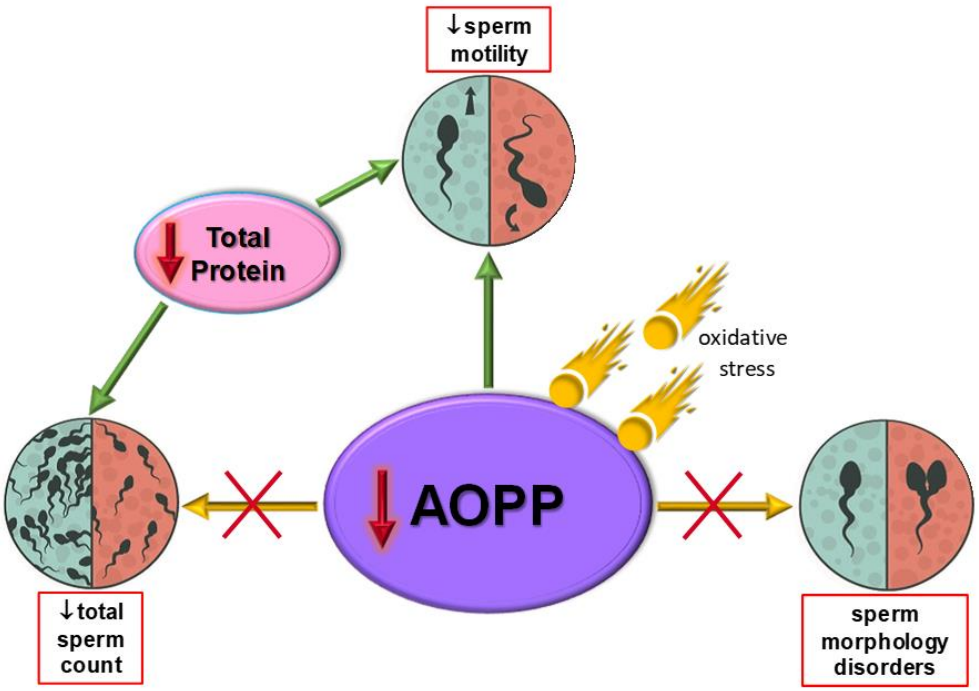

Figure 4. Relationships between the concentrations of advanced oxidation protein products (AOPP) and the levels of markers of oxidative-antioxidant balance in relation to semen parameters.

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Institutional Review Board Statement: The study procedures followed in the study were conducted in agreement with the Helsinki II declaration, and the protocol was approved by the Bioethics Human Research Committee of Wroclaw Medical University (permission no. KB 549/2019 and no. KB 707/2019).

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

Data Availability Statement: All data needed to evaluate the conclusions in the article are present in the article. Additional data related to this study are available upon reasonable request from the corresponding author or first author.

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

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15.5. Załącznik 5 – Całkowity dorobek naukowy

Ewa Magdalena Janiszewska Wrocław, 14.02.2023

Wykaz publikacji

1. Publikacje w czasopismach naukowych

1.1. Publikacje w czasopiśmie z IF

Lp	Opis bibliograficzny	IF	Punkty
1	**Janiszewska Ewa**, Kratz Ewa Maria: Could the glycosylation analysis of seminal plasma clusterin become a novel male infertility biomarker?, **Molecular Reproduction and Development**, 2020, vol. 87, nr 5, s. 515-524, DOI:10.1002/mrd.23340	2,609	100
2	**Janiszewska Ewa**, Kokot Izabela, Gilowska Iwona, Faundez Ricardo, Kratz Ewa Maria: The possible association of clusterin fucosylation changes with male fertility disorders, **Scientific Reports**, 2021, vol. 11, art.15674 [16 s.], DOI:10.1038/s41598-021-95288-w	4,997	140
3	**Janiszewska Ewa**, Kokot Izabela, Kmieciak Agnieszka, Gilowska Iwona, Faundez Ricardo, Kratz Ewa Maria: Are there associations between seminal plasma advanced oxidation protein products and selected redox-associated biochemical parameters in infertile male patients? A preliminary report, **Cells**, 2022, vol. 11, nr 22, art.3667 [15 s.], DOI:10.3390/cells11223667	7,666*	140
4	**Janiszewska Ewa**, Kokot Izabela, Kmieciak Agnieszka, Stelmasiak Zuzanna, Gilowska Iwona, Faundez Ricardo, Kratz Ewa Maria: The association between clusterin sialylation degree and levels of oxidative–antioxidant balance markers in seminal plasmas and blood sera of male partners with abnormal sperm parameters, **International Journal of Molecular Sciences**, 2022, vol. 23, nr 18, art.10598 [28 s.], DOI:10.3390/ijms231810598	6,208*	140
5	**Janiszewska Ewa**, Kmieciak Agnieszka, Kacperczyk Monika, Witkowska Aleksandra, Kratz Ewa Maria: The influence of clusterin glycosylation variability on selected pathophysiological processes in the human body, **Oxidative Medicine and Cellular Longevity**, 2022, vol. 2022, art.7657876 [25 s.], DOI:10.1155/2022/7657876	7,310*	100
	Podsumowanie	**28,790**	**620,00**

**IF 2021*

1.2 Publikacje w czasopiśmie bez IF

Lp	Opis bibliograficzny	Punkty
1	**Janiszewska Ewa**, Pluta Dominika, Siuta Jędrzej, Dobosz Tadeusz: Muzealnictwo: nowoczesne technologie w służbie historii. Część druga: Mokre preparaty muzealne jako źródło nowych informacji o przeszłości, **Opuscula Musealia**, 2018, vol. 25, s. 101-107, DOI:10.4467/20843852.OM.17.009.9606	13
2	Pluta Dominika, **Janiszewska Ewa**, Bonar Małgorzata, Alama Aneta, Dobosz Tadeusz: Muzealnictwo: nowoczesne technologie w służbie historii. Część pierwsza: Kopalne DNA - historia odkryć, perspektywy i problemy, **Opuscula Musealia**, 2018, vol. 25, s. 93-99, DOI:10.4467/20843852.OM.17.008.9605	13
3	**Janiszewska Ewa**, Pluta Dominika, Dobosz Tadeusz: Ocena stanu wiedzy młodych ludzi na temat HIV/AIDS, **Alergia Astma Immunologia**, 2019, vol. 24, nr 1, s. 24-29	20
	Podsumowanie	**46,00**

2. Rozdziały

Lp	Opis bibliograficzny	Punkty
1	Tokarski Miron, **Janiszewska Ewa**: Ocena dokładności diagnostycznej sekwencjonowania DNA z wykorzystaniem technologii nanoporów, W: Puzzel 2017 - VI Wrocławska Konferencja Studentów Nauk Technicznych i Ścisłych. Wrocław [1-2 kwietnia] 2017 : praca zbiorowa, (red.) Oskar Uchański, Wrocław 2017, Oskar Uchański, [83-85], ISBN 978-83-937278-6-5, [[Dostęp 24.01.2019]. Dostępny w: http://puzzel.plusuj.pl/wp-content/uploads/2017/09/Zapis-pokonf-2017.pdf]	0
	Podsumowanie	**0**

3. Abstrakty konferencyjne

Lp	Opis bibliograficzny
1	Tokarski Miron, Pencakowski Bartosz, Pluta Dominika, **Janiszewska Ewa**: Wykorzystanie technik biologii molekularnej w botanice sądowej, W: III Studencka Konferencja Kryminalistyczna "50 twarzy zbrodni". [Wrocław, 2-4 grudnia 2016]. Książka abstraktów 2016, [28]
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10	Pluta Dominika, **Janiszewska Ewa**, Tokarski Miron, Czechowicz Paulina, Cukierska Karolina: Medycyna personalizowana jako sposób na zmniejszenie działań niepożądanych w terapii, W: Konferencja Naukowa "Wpływ związków toksycznych na zdrowie ludzi i zwierząt". Wrocław, 30.03.2017 r. Książka abstraktów 2017, 9 poz.1
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20	**Janiszewska Ewa**, Kokot Izabela, Gilowska Iwona, Kratz Ewa Maria: Zmiany sjalilacji klasterynyu mężczyzn z obniżonym potencjałem rozrodczym - badanie pilotażowe, W: 11 Konferencja "Postępy w badaniach biomedycznych". Warszawa, 27.02.2021. Książka streszczeń, Warszawa 2021, 23 poz.7
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Sumaryczny Impact Factor: 28,790

Punktacja Ministerialna: 666,00 pkt.

15.6. **Załącznik 6 –** Oświadczenia współautorów publikacji wchodzących w skład cyklu

Wrocław, 08.02.2023 r.

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Oświadczam, że mój wkład w powstanie artykułów wchodzących do cyklu publikacji będących podstawą mojej rozprawy doktorskiej:

1. **Ewa Janiszewska**, Ewa Maria Kratz: Could the glycosylation analysis of seminal plasma clusterin become a novel male infertility biomarker? **Molecular Reproduction and Development, 2020**, vol. 87, nr 5, s. 515-524, DOI:10.1002/mrd.23340

   polegał na zebraniu i analizie piśmiennictwa, interpretacji informacji w nim zawartych, współtworzeniu tekstu manuskryptu oraz jego ostatecznej formy.

2. **Ewa Janiszewska**, Izabela Kokot, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The possible association of clusterin fucosylation changes with male fertility disorders. **Scientific Reports, 2021**, vol. 11, art.15674 [16 s.], DOI:10.1038/s41598-021-95288-w

   polegał na współudziale w doborze metod badawczych i ustaleniu warunków ich przeprowadzenia, wykonaniu części doświadczalnej, analizie oraz interpretacji uzyskanych wyników badań, współtworzeniu manuskryptu oraz jego ostatecznej formy, a także współudziale w pozyskaniu źródeł finansowania.

3. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Zuzanna Stelmasiak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The association between clusterin sialylation degree and levels of oxidative–antioxidant balance markers in seminal plasmas and blood sera of male partners with abnormal sperm parameters. **International Journal of Molecular Sciences, 2022**, vol. 23, nr 18, art.10598 [28 s.], DOI:10.3390/ijms231810598.

   polegał na współudziale w doborze metod badawczych i ustaleniu warunków ich przeprowadzenia, wykonaniu części doświadczalnej, analizie oraz interpretacji uzyskanych wyników badań, współtworzeniu manuskryptu oraz jego ostatecznej formy tekstowej oraz graficznej.

4. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: Are there associations between seminal plasma advanced oxidation protein products and selected redox-associated biochemical parameters in infertile male patients? A preliminary report. **Cells, 2022**, vol. 11, nr 22, art.3667 [15 s.], DOI:10.3390/cells11223667

   polegał na współudziale w doborze metod badawczych i ustaleniu warunków ich przeprowadzenia, wykonaniu części doświadczalnej, analizie oraz interpretacji uzyskanych wyników badań, współtworzeniu manuskryptu oraz jego ostatecznej formy tekstowej oraz graficznej.

Jednocześnie oświadczam, że wymienione powyżej artykuły, za zgodą wszystkich Współautorów, wchodzą w skład cyklu publikacyjnego stanowiącego podstawę mojej rozprawy doktorskiej.

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   polegał na ustaleniu koncepcji i założeń manuskryptu, zebraniu i analizie piśmiennictwa, interpretacji danych w nich zawartych, współtworzeniu, ocenie merytorycznej manuskryptu i korekcie manuskryptu oraz korespondencji z redakcją (autor korespondencyjny).

2. **Ewa Janiszewska**, Izabela Kokot, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The possible association of clusterin fucosylation changes with male fertility disorders. **Scientific Reports, 2021**, vol. 11, art.15674 [16 s.], DOI:10.1038/s41598-021-95288-w

   polegał na ustaleniu koncepcji i założeń badań, pozyskaniu materiału biologicznego do badań, planowaniu i nadzorze nad wykonywanymi badaniami, współudziale w krytycznej analizie i interpretacji uzyskanych wyników badań, współtworzeniu tekstu manuskryptu oraz jego ostatecznej formy, pozyskaniu źródeł finansowania oraz korespondencji z redakcją (autor korespondencyjny).

3. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Zuzanna Stelmasiak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The association between clusterin sialylation degree and levels of oxidative–antioxidant balance markers in seminal plasmas and blood sera of male partners with abnormal sperm parameters. **International Journal of Molecular Sciences, 2022**, vol. 23, nr 18, art.10598 [28 s.], DOI:10.3390/ijms231810598

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ostatecznej formy, pozyskaniu źródeł finansowania oraz korespondencji z redakcją (autor korespondencyjny).

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Jednocześnie wyrażam zgodę, aby wymienione powyżej artykuły zostały włączone do cyklu publikacyjnego będącego podstawą rozprawy doktorskiej Pani mgr Ewy Janiszewskiej.

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Oświadczam, że mój wkład w powstanie publikacji:

1. **Ewa Janiszewska**, Izabela Kokot, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The possible association of clusterin fucosylation changes with male fertility disorders. **Scientific Reports, 2021**, vol. 11, art.15674 [16 s.], DOI:10.1038/s41598-021-95288-w.

   polegał na współudziale w pozyskaniu i preparatyce materiału biologicznego do badań oraz współwykonaniu części oznaczeń.

2. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Zuzanna Stelmasiak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The association between clusterin sialylation degree and levels of oxidative–antioxidant balance markers in seminal plasmas and blood sera of male partners with abnormal sperm parameters. **International Journal of Molecular Sciences, 2022**, vol. 23, nr 18, art.10598 [28 s.], DOI:10.3390/ijms231810598.

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3. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: Are there associations between seminal plasma advanced oxidation protein products and selected redox-associated biochemical parameters in infertile male patients? A preliminary report. **Cells, 2022**, vol. 11, nr 22, art.3667 [15 s.], DOI:10.3390/cells11223667.

   polegał na współudziale w pozyskaniu materiału biologicznego do badań, walidacji metodyki części wykonywanych badań oraz współwykonaniu części oznaczeń, udziale w ostatecznej korekcie manuskryptu i odpowiedziach dla recenzentów.

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   polegał na współwykonaniu części badań, współudziale w korekcie edytorskiej manuskryptu.

2. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: Are there associations between seminal plasma advanced oxidation protein products and selected redox-associated biochemical parameters in infertile male patients? A preliminary report. **Cells, 2022**, vol. 11, nr 22, art.3667 [15 s.], DOI:10.3390/cells11223667.

   polegał na współwykonaniu części badań.

Jednocześnie wyrażam zgodę, aby wymienione powyżej artykuły zostały włączone do cyklu publikacyjnego będącego podstawą rozprawy doktorskiej Pani mgr Ewy Janiszewskiej.

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1. **Ewa Janiszewska**, Izabela Kokot, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The possible association of clusterin fucosylation changes with male fertility disorders. **Scientific Reports, 2021**, vol. 11, art.15674 [16 s.], DOI:10.1038/s41598-021-95288-w

   polegał na przeprowadzeniu analizy seminologicznej, wyselekcjonowaniu pacjentów do grup badanych, pozyskaniu plazm nasienia i surowic krwi oraz przygotowaniu bazy danych pacjentów.

2. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Zuzanna Stelmasiak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The association between clusterin sialylation degree and levels of oxidative–antioxidant balance markers in seminal plasmas and blood sera of male partners with abnormal sperm parameters. **International Journal of Molecular Sciences, 2022**, vol. 23, nr 18, art.10598 [28 s.], DOI:10.3390/ijms231810598

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danych pacjentów oraz współudziale w pozyskaniu źródeł finansowania na opublikowanie artykułu.

Jednocześnie wyrażam zgodę, aby wymienione powyżej artykuły zostały włączone do cyklu publikacyjnego będącego podstawą rozprawy doktorskiej Pani mgr Ewy Janiszewskiej.

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że mój wkład w powstanie publikacji:

1. **Ewa Janiszewska**, Izabela Kokot, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The possible association of clusterin fucosylation changes with male fertility disorders. **Scientific Reports, 2021**, vol. 11, art.15674 [16 s.], DOI:10.1038/s41598-021-95288-w

   polegał na przeprowadzeniu analizy seminologicznej, wyselekcjonowaniu pacjentów do grup badanych, pozyskaniu plazm nasienia oraz przygotowaniu bazy danych pacjentów.

2. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Zuzanna Stelmasiak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: The association between clusterin sialylation degree and levels of oxidative–antioxidant balance markers in seminal plasmas and blood sera of male partners with abnormal sperm parameters. **International Journal of Molecular Sciences, 2022**, vol. 23, nr 18, art.10598 [28 s.], DOI:10.3390/ijms231810598

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3. **Ewa Janiszewska**, Izabela Kokot, Agnieszka Kmieciak, Iwona Gilowska, Ricardo Faundez, Ewa Maria Kratz: Are there associations between seminal plasma advanced oxidation protein products and selected redox-associated biochemical parameters in infertile male patients? A preliminary report. **Cells, 2022**, vol. 11, nr 22, art.3667 [15 s.], DOI:10.3390/cells11223667

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Jednocześnie wyrażam zgodę, aby wymienione powyżej artykuły zostały włączone do cyklu publikacyjnego będącego podstawą rozprawy doktorskiej Pani mgr Ewy Janiszewskiej.

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podpis współautora

Wrocław, 08.02.2023

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   polegał współudziale w wykonaniu części badań.

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