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ROZPRAWA DOKTORSKA

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Analiza profilu i stopnia glikozylacji surowiczej immunoglobuliny G u kobiet z zaawansowaną endometriozą

The analysis of the profile and degree of serum immunoglobulin G glycosylation in women with advanced endometriosis

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Podziękowania

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Promotor mojej pracy doktorskiej,

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SPIS TREŚCI

1. PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY DOKTORSKIEJ

Rozprawa doktorska, stanowiąca podstawę do ubiegania się o stopień doktora nauk medycznych, powstała w oparciu o cykl trzech artykułów oryginalnych opublikowanych w czasopismach naukowych o zasięgu międzynarodowym, indeksowanych w bazie PubMed i uwzględnionych na liście Journal Citation Reports, o wysokim współczynniku wpływu IF.

Na rozprawę doktorską składają się następujące artykuły:

1. Variability of serum IgG sialylation and galactosylation degree in women with advanced endometriosis. **K. Sołkiewicz**, H. Krotkiewski, M. Jędryka, E.M. Kratz. Sci Rep. 2021 Mar 10;11(1):5586. doi: 10.1038/s41598-021-85200-x.

IF_{2021}: 4,996; MEiN: 140 pkt.

2. The alterations of serum IgG fucosylation as a potential additional new diagnostic marker in advanced endometriosis. **K. Sołkiewicz**, H. Krotkiewski, M. Jędryka, A. Czekański, E.M. Kratz. J Inflamm Res. 2022 Jan 13;15:251-266. doi: 10.2147/JIR.S341906. eCollection 2022.

IF_{2021}: 4,631; MEiN: 140 pkt.

3. O-glycosylation changes in serum immunoglobulin G are associated with inflammation development in advanced endometriosis. **K. Sołkiewicz**, M. Kacperczyk, H. Krotkiewski, M. Jędryka, E.M. Kratz. Int J Mol Sci. 2022 Jul 22;23(15):8087. doi: 10.3390/ijms23158087.

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Łączna wartość współczynnika wpływu (IF) dla powyższego cyklu artykułów wynosi: **15,835** oraz **420 punktów** według wykazu czasopism naukowych Ministerstwa Edukacji i Nauki (Komunikat Ministra Edukacji i Nauki z dnia 21 grudnia 2021 r. w sprawie wykazu czasopism naukowych i recenzowanych materiałów z konferencji międzynarodowych).

Wszystkie artykuły są dostępne w wersji ‘Open Access’.

2. OMÓWIENIE

2.1. Wstęp

Endometrioza to przewlekła choroba ginekologiczna, charakteryzująca się występowaniem komórek błony śluzowej macicy (endometrium) poza jamą macicy (Johnson & Hummelshoj, 2013). Choroba ta dotyka ok. 10% kobiet na całym świecie, a oprócz dolegliwości bólowych o różnym umiejscowieniu i stopniu nasilenia oraz dyskomfortu, endometrioza ma również istotny wpływ na płodność kobiety, u której występuje to schorzenie. Szacuje się, że od 30% do 50% kobiet w wieku rozrodczym, cierpiących na endometriozę, może mieć problemy z zajściem w ciążę, lecz jest to związane ze stopniem zaawansowania zmian chorobowych oraz ich lokalizacją (Colinet i wsp., 2018). Pomimo, że endometrioza nie jest chorobą nowotworową, wykazuje cechy charakterystyczne dla procesów nowotworzenia, takie jak np. naciekanie otaczających narządów (Berker & Seval, 2015). Zmiany endometrialne, ze względu na ich umiejscowienie, mogą mieć postać torbieli jajnika, implantów otrzewnowych i/lub powierzchownych lub endometriozy głęboko naciekającej, która wywołuje przewlekłą reakcję zapalną (Adamson i wsp., 2010). Zgodnie z klasyfikacją American Fertility Society (rAFS) (Andrews i wsp., 1985), obecnie American Society for Reproductive Medicine (ASRM) (Canis i wsp., 1997), w oparciu o zaawansowanie choroby i anatomiczne rozszerzenie zmian, endometriozę dzieli się na cztery stadia: łagodne, umiarkowane, ciężkie i rozległe (odpowiednio: I, II, III i IV stopień). Etiologia endometriozy nie jest w pełni poznana, a wiele czynników, takich jak hormonalne, zapalne czy immunologiczne, może być zaangażowanych w rozwój i progresję choroby (Wang i wsp., 2009; Farland i wsp., 2015). Uważa się, że patomechanizmy rozwoju endometriozy są związane z wrodzoną odpowiedzią immunologiczną, lecz nadal nie do końca zostały one poznane (Vlahos i wsp., 2010; Czyzyk i wsp., 2017; Ruderman & Pavone, 2017, Patel i wsp., 2018). Zmiany zachodzące w układzie immunologicznym, dotyczące rekrutacji komórek odpornościowych, adhezji komórek i regulacji procesów zapalnych, mogą ułatwiać implantację i „przeżycie" komórek endometrialnych (Symons i wsp., 2018). Wykazano, że kobiety z endometriozą mają zmienione wartości parametrów odpornościowych, zwłaszcza w zaawansowanych stadiach rozwoju choroby, z czym może wiązać się zmniejszony nadzór immunologiczny, zmniejszona cytotoksyczność zależna od limfocytów T dla autologicznych komórek endometrium oraz upośledzenie rozpoznawania ektopowych komórek endometrium

w wyniku nieprawidłowego działania komórek NK (ang. natural killers) (Lebovic i wsp., 2001; Patel i wsp., 2018). Do niedawna „złotym standardem diagnostycznym" w rozpoznaniu endometriozy było badanie laparoskopowe z potwierdzeniem histopatologicznym - badanie inwazyjne, wymagające podania pacjentce znieczulenia ogólnego i hospitalizacji po zabiegu, stwarzające ryzyko wystąpienia powikłań (Horne i wsp., 2014). W lutym 2022 roku ukazały się zaktualizowane wytyczne i zalecenia, opracowane przez Europejskie Towarzystwo Rozrodu Człowieka i Embriologii (ESHRE, ang. European Society of Human Reproduction and Embryology), dotyczące diagnozowania i leczenia endometriozy, zgodnie z którymi rekomendowaną metodą diagnostyki tej choroby jest ultrasonografia transwaginalna i obrazowanie z wykorzystaniem rezonansu magnetycznego. Brak swoistych objawów somatycznych, bagatelizowanie przez pacjentki niektórych dolegliwości związanych z rozwojem choroby powodują, że diagnostyka endometriozy nadal stanowi duże wyzwanie dla lekarzy i niejednokrotnie może trwać latami. Dodatkowym utrudnieniem w diagnozowaniu endometriozy jest brak markerów biochemicznych o wysokiej czułości i swoistości, które mogłyby posłużyć do nieinwazyjnych badań przesiewowych, pozwalających na zdiagnozowanie endometriozy na wczesnym etapie rozwoju choroby. Dlatego też poszukiwanie nowych czułych i swoistych biomarkerów, których oznaczanie wspomogłoby współczesną diagnostykę endometriozy, jest nadal aktualne i pożądane.

Glikozylacja to proces enzymatyczny, katalizowany przez glikozylotransferazy i glikozydazy, jest jedną z najczęściej występujących potranslacyjnych modyfikacji białek wydzielniczych, tj. modyfikacji polegającej na przyłączeniu łańcuchów cukrowych o określonej budowie do białek i lipidów za pomocą wiązania N- i/lub O-glikozydowego (Marth & Grewal, 2008). W zależności od miejsca i rodzaju przyłączenia pierwszej reszty cukrowej do łańcucha polipeptydowego Asparaginy (Asn) lub seryny/treoniny (Ser/Thr) wyróżniamy N- lub O-glikoproteiny. N-glikozylacja polega na przyłączeniu cząsteczki cukru N-acetyloglukozoaminy (GlcNAc) za pomocą wiązania glikozydowego do atomu azotu łańcucha bocznego Asn, obecnej w sekwencji Asn-X-Ser/Thr, gdzie X jest dowolnym aminokwasem za wyjątkiem proliny. Grupy oligosacharydów przyłączone do białek wiązaniem N-glikozydowym mają wspólny rdzeń pentasacharydowy składający się z trzech mannoz i dwóch reszt N-acetyloglukozoaminy (Man3GlcNac2), różnią się natomiast składem monosacharydowym oraz rodzajem wiązań jakim monosacharydy są przyłączone do części rdzeniowej (Bieberich, 2014). O-glikozylacja polega na przyłączeniu cząsteczki

cukru do atomu tlenu reszt Ser lub Thr łańcucha polipeptydowego. Ten typ glikozylacji może dotyczyć dowolnego białka i jest inicjowany przez ok. 20 transferaz polipeptydowych N-acetylogalaktozoaminy (GalNAc), które są odpowiedzialne za przyłączenie do białka reszt GalNAc (GalNAcα1-O-Ser/Thr; antygen Tn) (Stanley, 2011). Powstawanie antygenu Tn zapoczątkowuje szlak O-glikozylacji, a utworzony O-glikan może być dalej wydłużany na trzy różne sposoby: poprzez przyłączenie kwasu sjalowego wiązaniem α2,6 (antygen sjalo-Tn; sTn), galaktozy (antygen T - rdzeń 1; Galβ1,3GalNAcα-Ser/Thr) lub N-acetyloglukozoaminy (rdzeń 3; GlcNAcβ1-3GalNAcα-Ser/Thr) (Iwai i wsp., 2005).

Glikozylacja odgrywa ważną rolę w procesach biologicznych, takich jak rozpoznawanie i adhezja komórek, komunikacja i interakcje na poziomie komórka-komórka (Cummings, 2019), a także odgrywa kluczową rolę w funkcjonowaniu przeciwciał (Kozłowska i wsp., 2018). Immunoglobulina G (IgG) to glikoproteina o masie cząsteczkowej około 150 kDa, jest najpowszechniejszą immunoglobuliną we krwi (stanowi około 75% puli immunoglobulin w surowicy), zaangażowaną w patogenezę i progresję wielu chorób. Immunoglobulina G odgrywa kluczową rolę w obronie gospodarza przed infekcjami bakteryjnymi i wirusowymi, ale także uczestniczy w procesach patologicznych (m.in. w stanach zapalnych) będących przyczyną lub następstwem wielu chorób. Immunoglobulina ta może aktywować różne mechanizmy efektorowe, takie jak cytotoksyczność zależną od dopełniacza (CDC, ang. complement dependent cytotoxicity), cytotoksyczność komórkową zależną od przeciwciał (ADCC, ang. antibody dependent cellular cytotoxicity) oraz fagocytozę (Schroeder & Cavacini, 2010; Quast & Lünemann, 2014). Glikany IgG są niezbędne do prawidłowej pracy układu odpornościowego. Najlepiej poznanym i opisanym rodzajem glikozylacji IgG jest N-glikozylacja, obserwowana we wszystkich podklasach ludzkich immunoglobulin G, w której reszty węglowodanowe są przyłączone do asparaginy 297 (Asn 297) w drugiej domenie stałej CH2 łańcucha ciężkiego IgG (ang. second constant domain of the heavy chain) (Böhm i wsp., 2012; Seeling i wsp., 2021). N-glikany mogą wpływać na stabilność przeciwciał (Mimura i wsp., 2000) wiążąc się z receptorem Fcγ (FcγR) i dopełniaczem (Lee, 2017), w konsekwencji modulując funkcje efektorowe, takie jak ADCC i CDC (Niwa i wsp., 2005; Chung i wsp., 2014; Lin i wsp., 2015; Wang i wsp., 2017). Typowe N-glikany obecne na IgG są dwuantenowymi kompleksami, składającymi się ze stałego heptamerycznego rdzenia zawierającego trzy reszty mannozy i cztery reszty

N-acetyloglukozoaminy (GlcNAc), do którego dodatkowo może być przyłączona zarówno fukoza (tzw. fukoza rdzeniowa), jak również dodatkowa, rozdzielająca, GlcNAc. Około 10% endogennych glikoform IgG znajdujących się w ludzkiej surowicy posiada rozdzielającą GlcNAc, której obecność zwiększa powinowactwo wiązania IgG do FcγRIIIa, powodując 10-30-krotnie wyższą aktywność ADCC (Ruhaak i wsp., 2010). Dodatkowo, anteny cukrowe (α2,6 i α2,3) mogą mieć zmienny profil glikozylacji dotyczący ekspresji końcowej galaktozy i/lub kwasów sjalowych (Arnold, 2007; Böhm i wsp., 2012; Kiyoshi i wsp., 2017). Drugie miejsce N-glikozylacji znajduje się na łańcuchach ciężkim i lekkim regionów zmiennych IgG (odpowiednio: ang. heavy chain-variable region – VH oraz ang. light chain-variable region - VL). N-glikozylację w regionie Fab (ang. antigen-binding fragment) zaobserwowano w 15-25% surowiczej puli IgG. Obecność glikanów w regionie Fab IgG może przyczyniać się do wyższej stabilności tych przeciwciał i zwiększać ich powinowactwo do antygenu ([1]van de Bovenkamp i wsp., 2018; [2]van de Bovenkamp i wsp., 2018). Profil i stopień glikozylacji IgG może zmieniać się w różnych stanach patologicznych, a skład glikanów może zmieniać funkcje efektorowe tej immunoglobuliny poprzez modulowanie jej powinowactwa do ligandów, takich jak receptory Fcγ (Schroeder & Cavacini, 2010). W wielu doniesieniach naukowych opisano zmienność glikozylacji IgG, zwłaszcza stopnia ekspresji jej glikanów, związaną z wiekiem, płcią oraz ciążą. Udokumentowano udział zmian glikozylacji regionu Fc IgG w patogenezie chorób autoimmunologicznych (reumatoidalnego zapalenia stawów, choroby Leśniowskiego-Crohna, tocznia rumieniowatego) i chorobach nowotworowych (Saldova i wsp., 2007; Huhn i wsp., 2009; van de Geijn i wsp., 2009; Ruhaak i wsp., 2010; Bones i wsp., 2010; Pucic i wsp., 2011; Ercan i wsp., 2012). Wykazano, że u pacjentów z reumatoidalnym zapaleniem stawów stopień galaktozylacji i sjalilacji konserwatywnych N-glikanów (Asn 297) w domenach CH2 IgG jest obniżony, a obniżona ekspresja końcowej galaktozy wpływa na aktywację komórek efektorowych i inicjuje odpowiedź zapalną. Dodatkowo wykazano, że stopień agalaktozylacji IgG jest wprost proporcjonalny do ciężkości przebiegu choroby (Pasek i wsp., 2006; Kozłowska i wsp., 2018).

Drugim typem glikozylacji IgG, zdecydowanie mniej poznanym niż N-glikozylacja, jest O-glikozylacja. Wykazano obecność O-glikanów w regionie zawiasowym ludzkich immunoglobulin IgA1, IgD oraz IgG, lecz brak jest informacji na temat ekspresji O-glikanów w surowiczej IgG w przebiegu zaawansowanej endometriozy. Region zawiasowy to fragment przeciwciała, znajdujący się pomiędzy

łańcuchami ciężkimi fragmentów Fab i Fc, w którym znajdują się mostki dwusiarczkowe. Różni się on budową i długością między klasami immunoglobulin, a jego unikalna struktura i położenie zapewniają segmentową elastyczność, która jest niezbędna do prawidłowego funkcjonowania przeciwciał (Takahashi, 1982; Mattu i wsp., 1998; Wada i wsp., 2010). Spośród czterech podklas immunoglobuliny G (IgG1-IgG4), IgG3 ma wyjątkowo wydłużony region zawiasowy, lecz jego rola w pełnieniu przez immunoglobulinę jej funkcji biologicznych nie jest w pełni poznana. Wykazano, że ludzka IgG3, skuteczniej niż jakakolwiek inna podklasa IgG, aktywuje reakcje, w których pośredniczy dopełniacz i FcγR (Jefferis, 2007; Bruhns i wsp., 2009, Plomp i wsp., 2015). IgG3 oprócz N-glikanów obecnych w regionach Fab i Fc, posiada również O-glikany w regionie zawiasowym. Uważa się, że w surowicy krwi około 10% przeciwciał poliklonalnych IgG3 i około 13% przeciwciał monoklonalnych IgG3 zawiera O-glikany. Każdy łańcuch ciężki IgG3 zawiera trzy sekwencje powtórzeń tworzących region zawiasowy, który zawiera sześć potencjalnych miejsc O-glikozylacji: trzy reszty treoniny i trzy reszty seryny, lecz tylko dwa lub trzy z tych miejsc są zajęte przez O-glikany, które zazwyczaj są przyłączone do treoniny (Plomp i wsp., 2015). Ze względu na różnorodność funkcji O-glikanów w organizmie człowieka, wszelkie zmiany profilu i stopnia O-glikozylacji glikoprotein są istotne, ponieważ wiążą się z rozwojem wielu chorób, w tym progresji nowotworów (Pinho & Reis, 2015; Stowell i wsp., 2015), cukrzycy (Żurawska-Płaksej i wsp., 2016) i choroby Alzheimera (Robertson i wsp., 2004; Akasaka-Manya & Manya, 2020). Wykazano, że nadekspresja na glikoproteinach antygenu Tn bezpośrednio indukuje cechy onkogenne, w tym zwiększoną proliferację komórek, zmniejszoną apoptozę, zwiększoną adhezję i zdolność komórek do migracji. Nieprawidłową ekspresję skróconych form O-glikanów -antygenów Tn - zaobserwowano m.in. na nabłonkowych komórkach nowotworowych i we wczesnych zmianach przednowotworowych nabłonka, które poprzedzają rozwój gruczolakoraka (Radhakrishnan i wsp., 2014; Hofmann i wsp., 2015; Dong i wsp., 2018; Sletmoen i wsp., 2018). Zmiany glikozylacji immunoglobuliny G, zwłaszcza te dotyczące stopnia ekspresji glikanów, są związane z powstawaniem i rozwojem wielu procesów patologicznych dotyczących m.in. adhezji komórek, angiogenezy, zapłodnienia, rozwoju i przerzutów nowotworów złośliwych, a także chorób autoimmunologicznych oraz endometriozy (Saldova i wsp., 2007; van de Geijn i wsp., 2009; Bones i wsp., 2010; Pucić i wsp., 2011; Ercan i wsp., 2012; Zhang i wsp., 2015; Chung i wsp., 2020; Sołkiewicz i wsp., 2021; Zheng i wsp., 2021; Sołkiewicz i wsp., 2022).

2.2. Cele i założenia badań

2.2.1. Cele badań

Wyodrębniono następujące główne cele badawcze:

- Izolacja immunoglobuliny G z każdej z surowic wchodzących w skład danej grupy badanej: pacjentek z zaawansowaną endometriozą, pacjentek bez endometriozy, lecz cierpiących na inne schorzenia ginekologiczne (grupa odniesienia) oraz grupy kontrolnej kobiet zdrowych.
- Analiza profilu i stopnia N- i O-glikozylacji, przeprowadzona zarówno dla preparatów wyizolowanej IgG, jak i dla IgG w surowicy (bez uprzedniej izolacji), w każdej z trzech grup badanych, a następnie porównanie otrzymanych wyników oznaczeń między grupą pacjentek z zaawansowaną endometriozą a tymi otrzymanymi dla kobiet z pozostałych dwóch analizowanych grup.
- Określenie charakterystycznego dla zaawansowanej endometriozy profilu N- i O-glikozylacji surowiczej IgG oraz wytypowanie parametrów glikozylacji o wysokiej wartości klinicznej, które mogłyby stanowić dodatkowy marker diagnostyczny tej choroby.
- Porównanie czułości oznaczeń profilu i stopnia glikozylacji IgG wykonanych dla wyizolowanych z surowic preparatów IgG, z tymi wykonanymi dla IgG w surowicy.

2.2.2. Założenia badań

Do głównych założeń zrealizowanych badań należało:

- Określenie charakterystycznego dla kobiet z zaawansowaną endometriozą profilu N- i O-glikozylacji surowiczej immunoglobuliny G oraz, w oparciu o uzyskane wyniki, wskazanie ewentualnych cech charakterystycznych w profilu glikanowym IgG dla tego schorzenia.
- Sprawdzenie, czy istnieją różnice w ekspresji N- i O-glikanów surowiczej IgG między grupą pacjentek z zaawansowaną endometriozą a pozostałymi analizowanymi grupami kobiet, które pozwoliłyby na różnicowanie tych grup.

- Obliczenie współczynnika galaktozylacji dla N-glikanów surowiczej IgG (wyizolowana oraz surowicza IgG) we wszystkich analizowanych grupach kobiet oraz sprawdzenie czy parametr ten pozwoli na odróżnienie pacjentek z zaawansowaną endometriozą od pozostałych grup badanych.
- Podjęcie próby poszerzenia panelu parametrów diagnostycznych służących do rozpoznania zaawansowanej endometriozy o dodatkowe, nowe biomarkery pomocne w nieinwazyjnej diagnostyce tego schorzenia.
- Sprawdzenie czy czułość analizy profilu i stopnia glikozylacji IgG w surowicy jest wystarczająca na tyle, aby można było wykorzystać łatwo dostępny materiał biologiczny jakim jest surowica do rutynowych oznaczeń gliko-markerów w procesie diagnostycznym zaawansowanej endometriozy, z pominięciem pracochłonnego i czasochłonnego procesu izolacji IgG.

2.3. Materiały i metody zastosowane w badaniach

2.3.1. Materiały

Materiał do badań stanowiły surowice krwi pochodzące od kobiet z rozpoznaniem III i IV stadium endometriozy (E; n=40, mediana wieku: 34 lata, z przedziałem między górnym a dolnym kwartylem [30,5–40,5]) oraz od kobiet bez endometriozy, lecz cierpiących na inne, łagodne schorzenia ginekologiczne (NE; n=36, mediana wieku 39 lat, z przedziałem między górnym a dolnym kwartylem [33,5–42,0]). Surowice uzyskano od pacjentek leczących się w Klinice Ginekologii Onkologicznej Wrocławskiego Centrum Onkologii. Pacjentki z grup E i NE poddano zabiegom chirurgicznym, głównie laparoskopowym, a po weryfikacji histologicznej i ustaleniu rozpoznania zakwalifikowano do właściwej grupy i pobrano krew na aktywator krzepnięcia w celu uzyskania surowicy stanowiącej materiał do dalszych badań. Kobiety z endometriozą zaklasyfikowano według rozległości i zaawansowania choroby, zgodnie ze zaktualizowaną klasyfikacją Amerykańskiego Towarzystwa Płodności (rAFS, ang. revised American Fertility Society). W grupie pacjentek bez endometriozy potwierdzono histologicznie występowanie torbieli jajnika, dysplazję - CIN 3 (śródnabłonkowa neoplazja szyjki macicy 3. stopnia) lub obecność mięśniaków gładkich. Grupę kontrolną stanowiły kobiety zdrowe (C; n=19, mediana wieku 39 lat, z przedziałem między górnym a dolnym kwartylem [35,0–48,0 lat]), regularnie miesiączkujące, bez objawów lub historii związanej z endometriozą, niebędące w ciąży, bez chorób ginekologicznych. Termin uzyskania surowicy do badań nie był skorelowany z dniem cyklu menstruacyjnego, w którym znajdowała się uczestniczka projektu w dniu pobrania krwi. Przed rozpoczęciem badań wszystkie kobiety wyraziły świadomą pisemną zgodę na udział w badaniach, a projekt badań został pozytywnie zaopiniowany przez Komisję Bioetyczną przy Uniwersytecie Medycznym we Wrocławiu (Nr KB-293/2016; Nr KB-297/2022).

2.3.2. Metody

Pierwszy etap badań obejmował izolację IgG z każdej z badanych surowic, którą przeprowadzono z wykorzystaniem kolumny powinowactwa ze złożem Protein A/G-Sepharose (BioVision Inc., Milpitas, USA). W wyizolowanych i oczyszczonych preparatach IgG stężenie białka (IgG) oznaczono metodą bicynchoninową (BCA).

W surowicach stężenie IgG oznaczono metodą turbidymetryczną z wykorzystaniem analizatora biochemicznego Konelab 20i (ThermoScientific, Vantaa, Finland).

Kolejny etap realizacji projektu obejmował analizę profilu i stopnia N-glikozylacji IgG, zarówno w wyizolowanych preparatach IgG jak i w IgG w surowicy, za pomocą zmodyfikowanego testu fazy stałej lektyno-ELISA z zastosowaniem specyficznych biotynylowanych lektyn (Vector Laboratories Inc., Burlingame, CA, USA). Analiza stopnia sjalilacji oraz galaktozylacji/agalaktozylacji IgG została oparta na względnej reaktywności glikanów IgG z biotynylowanymi lektynami specyficznymi wobec końcowego kwasu sjalowego (*Maackia amurensis* agglutinin - MAA, reaguje z kwasem sjalowym przyłączonym wiązaniem α2,3 i *Sambucus nigra* agglutinin - SNA, specyficzna wobec kwasu sjalowego przyłączonego wiązaniem α2,6) oraz końcowych reszt galaktozy (Gal) i N-acetyloglukozoaminy (GlcNAc), (odpowiednio: *Ricinus communis* agglutinin I - RCA-I oraz *Griffonia simplicifolia* lectin II - GSL-II). Do analizy profilu i stopnia fukozylacji IgG zastosowano biotynylowane lektyny specyficzne zarówno wobec fukozy antenowej jak i rdzeniowej: *Lotus tetragonolobus* agglutinin - LTA, reaguje specyficznie z fukozą przyłączoną wiązaniem α1,3 do GlcNAc, *Ulex europaeus* agglutinin - UEA, specyficzna wobec fukozy antenowej przyłączonej wiązaniem α1,2 do galaktozy oraz fukozy połączonej wiązaniem α1,3 z GlcNAc, *Aleuria aurantia* lectin - AAL i *Lens culinaris* agglutinin - LCA, reagujące z fukozą przyłączoną wiązaniem α1,6 do rdzenia cukrowego. Analizę O-glikozylacji IgG przeprowadzono z wykorzystaniem biotynylowanych lektyn (Vector Laboratories Inc., Burlingame, CA, USA) specyficznych wobec O-glikanów: *Maclura pomifera* lectin - MPL i *Vicia villosa* lectin – VVL, odpowiednio rozpoznających kompletne struktury O-glikanów (antygen T) oraz ich formy skrócone do pojedynczej GalNAc (antygen Tn), a także Jacaliny rozpoznającej antygen T. Dodatkowym aspektem badań było sprawdzenie, czy poza obecnością typowych dla surowiczej IgG N-glikanów dwuantenowych w przebiegu endometriozy, w surowiczej IgG obecne są również glikany wielorozgałęzione. Oznaczenia przeprowadzono z zastosowaniem biotynylowanej leukoaglutyniny z *Phaseolus vulgaris* (PHA-L), specyficznej wobec GlcNAc przyłączonej wiązaniem β1,6, obecnej na trzeciej antenie N-glikanów. Do analizy statystycznej uzyskanych wyników zastosowano program Statistica 13.3 PL (StatSoft), a za istotne uznano wyniki, dla których współczynnik istotności $p<0,05$.

Szczegóły dotyczące aspektów metodologicznych, w tym rodzajów testów statystycznych zastosowanych do analizy uzyskanych wyników oznaczeń, opisano w publikacjach stanowiących podstawę niniejszej rozprawy doktorskiej.

2.4. Podsumowanie otrzymanych wyników badań

Poniżej krótko omówiono najważniejsze wyniki zrealizowanych badań oraz przedstawiono płynące z nich wnioski, zawarte w artykułach stanowiących podstawę tej dysertacji.

2.4.1. Artykuł nr 1, pt.: Variability of serum IgG sialylation and galactosylation degree in women with advanced endometriosis

Wykazano, że ekspresja kwasu sjalowego przyłączonego wiązaniem α2,3 była istotnie wyższa u kobiet zdrowych, zarówno w przypadku surowiczej IgG, jak i jej wyizolowanej formy (odpowiednio: 0,023±0,013 AU i 0,051±0,0032 AU), w porównaniu z pacjentkami z zaawansowaną endometriozą (E, odpowiednio: 0,011±0,012 AU i 0,0009±0,031 AU) oraz kobietami bez endometriozy, lecz cierpiącymi z powodu innych chorób ginekologicznych (NE, odpowiednio: 0,016±0,021 AU i 0,004±0,009 AU). W grupie kobiet zdrowych wykazano także istotnie wyższą ekspresję końcowego kwasu sjalowego przyłączonego wiązaniem α2,6 w wyizolowanych preparatach IgG (0,546±0,165 AU) w porównaniu do grupy E i NE (odpowiednio: 0,263±0,144 AU i 0,250±0,115 AU). W przypadku surowiczej IgG, istotnie niższą ekspresję kwasu sjalowego przyłączonego wiązaniem α2,6, wykazano w grupie odniesienia NE (0,227±0,098 AU) w porównaniu do grupy kontrolnej kobiet zdrowych (0,301±0,071 AU) i grupy z zaawansowaną endometriozą (0,288±0,105 AU). Obliczono również współczynnik sjalilacji MAA/SNA (współczynnik względnej reaktywności glikanów IgG z MAA do względnej reaktywności glikanów IgG z SNA), w przypadku surowiczej IgG istotne różnice zaobserwowano jedynie między grupą kobiet z zaawansowaną endometriozą, gdzie wartość współczynnika sjalilacji wynosiła: 0,042±0,048 AU i była istotnie niższa od wartości uzyskanych w grupie kobiet zdrowych (0,081±0,048 AU). W przypadku izolowanych preparatów IgG wartość współczynnika MAA/SNA była istotnie niższa zarówno w grupie E jak i NE w porównaniu do grupy kontrolnej. Ponadto zaobserwowano obniżoną reaktywność glikanów IgG z lektyną wykrywającą galaktozę (RCA-I) w grupie kobiet z zaawansowaną endometriozą oraz u kobiet z grupy odniesienia bez endometriozy, w porównaniu do grupy kontrolnej kobiet zdrowych, a wykazane

różnice były istotne zarówno dla surowiczej IgG jak i dla wyizolowanych preparatów IgG. Istotne różnice we względnej reaktywności glikanów IgG z GSL-II wykazano jedynie dla IgG w surowicy, a otrzymane wartości dla grupy kontrolnej były istotnie niższe (0,023±0,018 AU) w porównaniu do grupy z zaawansowaną endometriozą i grupy odniesienia, odpowiednio: 0,037±0,041 AU i 0,036±0,028 AU. Wartość współczynnika agalaktozylacji (GSL-II/RCA-I), obliczonego jako indeks wartości względnej reaktywności N-glikanów IgG z lektyną wykrywającą GlcNAc do względnej reaktywności N-glikanów IgG z lektyną specyficzną wobec galaktozy, była istotnie wyższa w grupie pacjentek z zaawansowaną endometriozą oraz w grupie pacjentek z innymi niż endometrioza chorobami ginekologicznymi w porównaniu do kobiet zdrowych, co jest związane z obserwowaną obniżoną ekspresją galaktozy na glikanach w IgG w obydwu grupach pacjentek. Ocenę wartości klinicznej oznaczeń względnych reaktywności glikanów IgG z zastosowanymi lektynami oraz obliczonych współczynników sjalilacji - MAA/SNA i agalaktozylacji - GSL-II/RCA-I, w różnicowaniu kobiet z zaawansowaną endometriozą oraz kobiet z grupy kontrolnej, przeprowadzono przy pomocy analizy krzywych ROC, która pozwoliła na określenie dla badanych parametrów poziomu czułości i swoistości diagnostycznej oraz na wytypowanie tych spośród nich, które różnicowały obydwie grupy. Na podstawie wartości pola powierzchni pod krzywą (AUC≥0,762) wytypowano 3 parametry różnicujące, którymi były wartości względnej reaktywności glikanów IgG z MAA oraz wartości współczynników MAA/SNA i GSL-II/RCA, zarówno dla IgG w surowicy, jak i dla IgG w izolowanych preparatach, które pozwoliły na różnicowanie kobiet z zaawansowaną endometriozą od grupy kobiet zdrowych. Przeprowadzona analiza skupień dla wybranych w analizie krzywych ROC parametrów różnicujących obydwie grupy, dodatkowo potwierdziła, że dla IgG w surowicy panel tych 3 parametrów różnicuje grupę E od grupy kobiet zdrowych.

2.4.2. Artykuł nr 2, pt.: The alterations of serum IgG fucosylation as a potential additional new diagnostic marker in advanced endometriosis

Wykazano, że w grupie kobiet zdrowych reaktywność N-glikanów IgG (zarówno w surowicy jak i w wyizolowanych preparatach) z lektyną specyficzną wobec fukozy rdzeniowej (AAL) jest istotnie wyższa, odpowiednio: 0,056±0,015 AU i 0,140±0,043 AU, niż w grupie pacjentek z zaawansowaną endometriozą (E, odpowiednio: 0,029±0,010 AU i 0,055±0,033 AU) oraz w grupie pacjentek bez endometriozy, lecz

cierpiących z powodu innych, łagodnych schorzeń ginekologicznych (NE, odpowiednio: 0,028±0,014 AU i 0,093±0,022 AU). Względne reaktywności glikanów IgG z LTA, lektyną swoistą wobec antenowej fukozy przyłączonej wiązaniem α1,3 do GlcNAc, były istotnie niższe w grupie kobiet zdrowych w porównaniu do dwóch pozostałych grup badanych (E i NE), a zależność tę zaobserwowano zarówno dla IgG w surowicy, jak i dla jej wyizolowanej formy. Jedynie dla IgG w wyizolowanych preparatach wykazano istotnie wyższe względne reaktywności jej glikanów z UEA, lektyną swoistą wobec antenowej fukozy przyłączonej wiązaniem α1,2, w grupie zdrowych kobiet w porównaniu do grup E i NE. Analiza krzywych ROC przeprowadzona dla względnych reaktywności glikanów IgG z fukozo-specyficznymi lektynami w każdej z trzech grup badanych, pozwoliła na określenie wartości klinicznej dla analizowanych parametrów oraz poziomu ich czułości i swoistości diagnostycznej, a także na wytypowanie parametrów różnicujących grupy badane. Dla IgG w surowicy wartości względnych reaktywności z trzema z czterech zastosowanych lektyn: AAL, LTA i LCA charakteryzowały się wysoką wartością kliniczną (AUC≥0,775), a czułość i swoistość diagnostyczna wynosiły odpowiednio: dla AAL 0,950 i 0,737, dla LTA 0,925 i 0,842 oraz dla LCA 0,825 i 0,579. Dla IgG w wyizolowanych preparatach wartości względnych reaktywności ze wszystkimi zastosowanymi fukozo-specyficznymi lektynami były istotne klinicznie (AUC≥0,923), a wartości czułości i swoistości diagnostycznej mieściły się odpowiednio w granicach: 0,875 i 1,000 dla AAL, 0,825 i 0,947 dla LTA oraz 0,950 i 0,947 dla LCA. Przeprowadzona analiza klastrowa, do której wybrano parametry istotnie różnicujące pacjentki z zaawansowaną endometriozą od kobiet zdrowych, których wartość kliniczna była równocześnie co najmniej umiarkowana (AUC≥0,775), pozwoliła na wytypowanie panelu parametrów różnicujących obydwie grupy. Zarówno dla IgG w surowicy jak i w wyizolowanych preparatach IgG, w skład tego panelu weszła względna reaktywność glikanów IgG z lektynami rozpoznającymi fukozę rdzeniową (AAL i LCA) oraz fukozę antenową wchodzącą w skład struktur cukrowych typu Lewis^x (LTA).

2.4.3. Artykuł nr 3, pt.: O-glycosylation changes in serum immunoglobulin G are associated with inflammation development in advanced endometriosis

Przeprowadzone badania pozwoliły na wykazanie, że względna reaktywność O-glikanów w IgG w surowicy z lektyną MPL specyficzną wobec antygenu T, była istotnie wyższa w grupie kobiet z łagodnymi schorzeniami ginekologicznymi

(NE: 0,169±0,111 AU) niż w grupie pacjentek z zaawansowaną endometriozą (E: 0,118±0,045 AU) oraz w grupie kobiet zdrowych (0,103±0,042 AU). Dla IgG w surowicy względne reaktywności jej glikanów z lektyną VVL wykrywającą skrócone formy O-glikanów (antygen Tn) oraz wartości obliczonego współczynnika O-glikozylacji MPL/VVL, a także względne reaktywności z Jacaliną, reagującą zarówno z kompletnymi, jak i skróconymi formami O-glikanów, nie różniły się istotnie między badanymi grupami. Brak było także istotnych różnic między analizowanymi grupami kobiet we względnych reaktywnościach glikanów IgG w surowicy z lektyną PHA-L, specyficzną wobec wielorozgałęzionych form N-glikanów. Względne reaktywności O-glikanów IgG w wyizolowanych preparatach z lektynami specyficznymi wobec antygenu T i antygenu Tn, były istotnie wyższe u kobiet z zaawansowaną endometriozą i u kobiet z grupy NE w porównaniu z wartościami uzyskanymi dla grupy zdrowych uczestniczek. Wartości współczynnika O-glikozylacji MPL/VVL dla wyizolowanych preparatów IgG były istotnie niższe w grupie kobiet zdrowych (2,750±5,546 AU) w porównaniu do grup E (7,465±4,439 AU) i NE (5,885±3,383 AU). Względne reaktywności O-glikanów IgG w wyizolowanych preparatach z Jacaliną w grupie kobiet z zaawansowaną endometriozą (1,025±0,094 AU) i w grupie NE (1,103±0,138 AU) były istotnie wyższe niż te obserwowane w grupie kontrolnej kobiet zdrowych (0,106±0,140 AU). Dodatkowo, dla IgG w wyizolowanych preparatach, ekspresja O-glikanów reaktywnych z Jacaliną była istotnie niższa w grupie NE niż w grupie E (p=0.006401). Ekspresja wielorozgałęzionych N-glikanów IgG w wyizolowanych preparatach, była istotnie wyższa w grupach E i NE w porównaniu z grupą kontrolną kobiet zdrowych, dla której uzyskane wartości względnej reaktywności z PHA-L były bliskie zeru. Ocenę użyteczności klinicznej oznaczeń względnych reaktywności glikanów IgG ze wszystkimi lektynami przeprowadzono przy pomocy analizy krzywych ROC, która pozwoliła na określenie dla badanych parametrów poziomu czułości i swoistości diagnostycznej oraz na wytypowanie parametrów różnicujących grupy badane. W przypadku względnych reaktywności glikanów IgG w surowicy ze wszystkimi zastosowanymi lektynami ich wartość kliniczna była niska, natomiast dla IgG w wyizolowanych preparatach zastosowany do analizy glikozylacji panel lektyn specyficznych wobec O-glikanów (MPL, VVL, Jacalina), jak i wobec wielorozgałęzionych form N-glikanów (PHA-L) pozwolił na różnicowanie pacjentek z zaawansowaną endometriozą od grupy kobiet zdrowych (AUC≥0,811), a także

na różnicowanie pacjentek bez endometriozy, lecz z łagodnymi schorzeniami ginekologicznymi, od kobiet zdrowych (AUC≥0,801).

2.5. Wnioski

Poniżej przedstawiono najważniejsze wnioski płynące z przeprowadzonych badań.

1. Panel markerów glikozylacji IgG w surowicy (ekspresja końcowego kwasu sjalowego przyłączonego wiązaniem α2,3, wartości współczynnika sjalilacji MAA/SNA i współczynnika agalaktozylacji GSL-II/RCA-I) może być pomocny w różnicowaniu i diagnostyce zaawansowanego stadium endometriozy. Wykazano, że analiza sjalilacji i stopnia galaktozylacji/agalaktozylacji glikanów IgG w surowicy, niewymagająca czasochłonnej i skomplikowanej procedury jej izolacji, może być użyteczna jako marker różnicujący kobiety z wysokim ryzykiem rozwoju zaawansowanej endometriozy.
2. Analiza profilu i stopnia fukozylacji N-glikanów dla IgG w surowicy ma szansę stać się uzupełniającym nieinwazyjnym narzędziem diagnostycznym w zaawansowanej endometriozie. Panel parametrów, którymi są: ekspresja AAL- i LCA-reaktywnej fukozy rdzeniowej oraz LTA-reaktywnej fukozy antenowej wchodzącej w skład struktur cukrowych Lewis^x, może być brany pod uwagę jako użyteczne narzędzie w diagnostyce kobiet z zaawansowaną endometriozą, jednakże jego kliniczna użyteczność w codziennej praktyce wymaga oceny w kolejnych, szerzej zakrojonych badaniach.
3. Uzyskane wyniki wskazują jednoznacznie na obecność O-glikanów w surowiczej IgG, zarówno u pacjentek z zaawansowaną endometriozą, jak i w grupie kobiet bez endometriozy, lecz z innymi chorobami ginekologicznymi, co wykazano po raz pierwszy. Wartości współczynnika O-glikozylacji MPL/VVL oraz względne reaktywności O-glikanów IgG w wyizolowanych preparatach z MPL i VVL, które odzwierciedlają ekspresję odpowiednio antygenu T i antygenu Tn, były istotnie wyższe u kobiet z zaawansowaną endometriozą niż u zdrowych kobiet. Analogiczną zależność zaobserwowano między tymi grupami we względnych reaktywnościach O-glikanów IgG w wyizolowanych preparatach z Jacaliną.
4. U pacjentek bez endometriozy, lecz z innymi chorobami ginekologicznymi, względne reaktywności glikanów IgG w wyizolowanych preparatach z lektynami swoistymi wobec O-glikanów, a także wartości współczynnika MPL/VVL, były istotnie wyższe niż w grupie kontrolnej kobiet zdrowych.

5. U pacjentek cierpiących na schorzenia ginekologiczne inne niż endometrioza zaobserwowano istotnie wyższą względną reaktywność wyizolowanych preparatów IgG z Jacaliną, w porównaniu z grupą kobiet zdrowych oraz pacjentek z zaawansowaną endometriozą, co jest szczególnie ważne z diagnostycznego punktu widzenia, ponieważ poszukiwane były również parametry, które mogłyby odróżnić zaawansowaną endometriozę od innych chorób ginekologicznych. Takim parametrem wydaje się być stopień ekspresji O-glikanów reagujących z Jacaliną, najprawdopodobniej typu 'core 3', których obecność manifestowała się w wyizolowanych preparatach IgG.
6. Oprócz obecności typowych dla surowiczej IgG dwuantenowych N-glikanów, wykazano również występowanie ich wielorozgałęzionych form. Istotnie wyższa ekspresja wielorozgałęzionych N-glikanów surowiczej IgG u kobiet z zaawansowaną endometriozą oraz u pacjentek z innymi niż endometrioza chorobami ginekologicznymi, w porównaniu z grupą kobiet zdrowych, wskazuje, że obecność N-glikanów wielorozgałęzionych IgG jest związana ze stanem zapalnym towarzyszącym rozwojowi chorób dotyczących kobiecych narządów rozrodczych, niezależnie od jednostki chorobowej.
7. Przeprowadzone badania wykazały także, że grupa kobiet bez endometriozy, lecz cierpiących na łagodne choroby ginekologiczne, nie jest odpowiednią grupą porównawczą dla kobiet z zaawansowaną endometriozą, gdyż zaobserwowane w obydwu grupach zmiany ekspresji glikanów IgG, istotnie różne od tych wykazanych dla kobiet zdrowych, najprawdopodobniej były związane z rozwojem stanu zapalnego, który zazwyczaj towarzyszy chorobom ginekologicznym. Dlatego też w przeprowadzonych badaniach, w przeciwieństwie do doniesień wielu autorów, których przedmiotem badań była endometrioza, grupę kontrolną stanowiły zdrowe kobiety, bez jakichkolwiek zdiagnozowanych schorzeń ginekologicznych i stanu zapalnego w obrębie narządów rodnych.

Podsumowując, uzyskane wyniki badań pozwoliły na wytypowanie gliko-markerów pomocnych w diagnostyce zaawansowanej endometriozy, co może ukierunkować przyszłe badania w tej dziedzinie. Wierzę, że wyniki moich badań przyczynią się również do lepszego zrozumienia mechanizmów molekularnych towarzyszących tej chorobie i w przyszłości pozwolą na opracowanie swoistych i czułych algorytmów diagnostycznych zaawansowanej endometriozy. Markery glikozylacji, analizowane

dla IgG bezpośrednio w surowicy, mogą służyć jako narzędzie kliniczne do różnicowania kobiet z wysokim ryzykiem rozwoju zaawansowanej endometriozy, zakwalifikowanych do zabiegu laparoskopowego. Ponadto, uzyskane wyniki mogą stanowić podstawę do dalszych badań mających na celu poszukiwanie nieinwazyjnych markerów diagnostycznych nie tylko zaawansowanych stadiów rozwoju endometriozy, ale także, co szczególnie istotne z punktu widzenia pacjentek, wczesnego stadium rozwoju choroby.

3. WYKAZ SKRÓTÓW

AAL	*Aleuria aurantia* lectin - lektyna z dzieżki pomarańczowej
ADCC	cytotoksyczność komórkowa zależna od przeciwciał (ang. antibody dependent cellular cytotoxicity)
Asn 297	asparagina 297
ASRM	Amerykańskie Towarzystwo Medycyny Rozrodu (ang. American Society for Reproductive Medicine)
BCA	metoda bicynchoninowa (ang. bicinchoninic acid protein assay)
C	grupa kontrolna kobiet zdrowych
CDC	cytotoksyczność zależna od dopełniacza (ang. complement-dependent cytotoxicity)
CH	domena stała łańcucha ciężkiego przeciwciała (ang. constant domain of the heavy chain)
CH2	druga domena stała łańcucha ciężkiego przeciwciała (ang. second constant domain of the heavy chain)
CIN 3	śródnabłonkowa neoplazja szyjki macicy 3. stopnia (ang. cervical intraepithelial neoplasia)
CL	domena stała łańcucha lekkiego przeciwciała (ang. constant domain of the light chain)
E	grupa kobiet z zaawansowaną endometriozą
ESHRE	Europejskie Towarzystwo Rozrodu Człowieka i Embriologii (ang. European Society of Human Reproduction and Embryology)
Fab	fragment zmienny przeciwciała wiążący antygen (ang. antigen-binding fragment)
Fc	fragment stały przeciwciała, krystalizujący (ang. crystallizable fragment)
FcγR	receptor Fc-γ

Gal	galaktoza
GalNAc	N-acetylogalaktozoamina
GlcNAc	N-acetyloglukozoamina
GSL-II	*Griffonia simplicifolia* II lectin - lektyna z czarnej fasoli afrykańskiej
IgA	immunoglobulina klasy A
IgD	immunoglobulina klasy D
IgG	immunoglobulina klasy G
LCA	*Lens culinaris* agglutinin - lektyna z soczewicy jadalnej
LTA	*Lotus tetragonolobus* agglutinin - lektyna z głąbigroszku szkarłatnego
MAA	*Maackia amurensis* agglutinin - lektyna z makii amurskiej
MPL	*Maclura pomifera* lectin - lektyna z żółtnicy pomarańczowej
NE	grupa odniesienia kobiety bez endometriozy, z łagodnymi schorzeniami ginekologicznymi
NK	naturalni zabójcy (ang. natural killers)
PHA-L	*Phaseolus vulgaris* leucoagglutinin - lektyna z fasoli zwykłej
rAFS	klasyfikacja stopnia zaawansowania endometriozy na podstawie kryteriów Amerykańskiego Towarzystwa Płodności, ang. revised American Fertility Society classification
RCA-I	*Ricinus communis* agglutinin I - lektyna z rącznika pospolitego
Ser	seryna
SNA	*Sambucus nigra* agglutinin - lektyna z bzu czarnego
Thr	treonina
UEA	*Ulex europaeus* agglutinin - lektyna z kolcolistu zachodniego
VH	część zmienna łańcucha ciężkiego przeciwciała (ang. heavy-chain-variable region)

VL część zmienna łańcucha lekkiego przeciwciała (ang. light chain-variable region)

VVL *Vicia villosa* lectin - lektyna z wyki kosmatej

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5. STRESZCZENIE PRACY W JĘZYKU POLSKIM

Wprowadzenie: Endometrioza, to choroba, u podłoża której leży rozrost błony śluzowej macicy (endometrium) poza jamą macicy. Brak swoistych objawów powoduje, że endometrioza jest trudna do zdiagnozowania, a obecnie zalecane metody diagnostyki obrazowej (USG, MRI) mogą być niewystarczające do ustalenia właściwego rozpoznania, zwłaszcza w przypadku zmian zaotrzewnowych i głęboko naciekających. Obecnie brak jest czułych i swoistych markerów, które umożliwiłyby skuteczną diagnostykę endometriozy. Dlatego istnieje potrzeba poszukiwania nieinwazyjnych biomarkerów charakterystycznych dla tej choroby, które mogłyby być zastosowane w rutynowej diagnostyce laboratoryjnej.

Cel badań: Głównym celem przeprowadzonych badań było określenie profilu i stopnia glikozylacji surowiczej immunoglobuliny G (IgG) u kobiet z zaawansowaną endometriozą oraz sprawdzenie czy występują różnice w ekspresji glikanów IgG między grupą pacjentek z zaawansowaną endometriozą a grupą kobiet z innymi niż endometrioza, łagodnymi chorobami ginekologicznymi oraz kobietami zdrowymi, co pozwoliłoby na ich różnicowanie oraz na określenie ewentualnych cech charakterystycznych dla zaawansowanej endometriozy w profilu glikanowym IgG obecnej w surowicy. Dodatkowym aspektem przeprowadzonych badań było również porównanie czułości oznaczeń profilu i stopnia glikozylacji IgG między oznaczeniami wykonanymi dla IgG obecnej w surowicy i w wyizolowanych preparatach IgG, w kontekście wykorzystania łatwo dostępnego materiału biologicznego jakim jest surowica krwi do rutynowych oznaczeń gliko-markerów w procesie diagnostycznym endometriozy, z pominięciem pracochłonnego i czasochłonnego procesu izolacji IgG.

Materiał i metody: Surowice badane pochodziły od pacjentek z zaawansowaną endometriozą (E; n=40) oraz od pacjentek bez endometriozy, z łagodnymi schorzeniami ginekologicznymi (NE; n=36). Grupa kontrolna to kobiety zdrowe (C; n=19), bez jakichkolwiek zdiagnozowanych chorób ginekologicznych. Izolację IgG z surowic przeprowadzono z użyciem kolumny powinowactwa ze złożem Protein A/G-Sepharose. Oznaczenie stężenia IgG przeprowadzono z wykorzystaniem następujących metod: 1) dla wyizolowanych preparatów IgG metodą bicynchoninową, 2) dla surowiczej IgG metodą turbidymetryczną. Analizę profilu i stopnia glikozylacji IgG przeprowadzono

z wykorzystaniem testu lektyno-ELISA z zastosowaniem lektyn specyficznych wobec wybranych N- i O-glikanów. Do analizy statystycznej uzyskanych wyników zastosowano program Statistica 13.3 PL, a za istotne uznano wyniki, dla których współczynnik istotności $p<0,05$.

Wyniki: Ekspresja końcowego kwasu sjalowego na glikanach IgG w surowicy, jak i w wyizolowanych preparatach IgG, była istotnie wyższa w grupie kobiet zdrowych w porównaniu z pacjentkami z grup E i NE. Zaobserwowano także obniżoną ekspresję galaktozy na N-glikanach IgG (surowica i wyizolowane preparaty) w grupach E i NE w odniesieniu do grupy C, a wartość współczynnika agalaktozylacji (GSL-II/RCA-I), była istotnie wyższa w grupach E i NE w porównaniu do grupy C. Wartość współczynnika sjalilacji (MAA/SNA) była istotnie niższa w grupie E w porównaniu do C. Ekspresja fukozy rdzeniowej była istotnie wyższa w grupie C w porównaniu do grupy E i NE, zarówno dla IgG w surowicy jak i dla IgG w wyizolowanych preparatach. Natomiast w przypadku fukozy antenowej LTA- i UEA-reaktywnej zależności te były odwrotne. Względne reaktywności O-glikanów w wyizolowanych preparatach IgG ze specyficznymi lektynami oraz wartości współczynnika O-glikozylacji MPL/VVL były istotnie wyższe u pacjentek z grup E i NE w porównaniu z grupą kontrolną kobiet zdrowych. Ponadto dla wyizolowanej IgG w grupach E i NE wykazano również istotnie wyższą ekspresję wielorozgałęzionych N-glikanów. Wyniki analiz krzywych ROC i analizy skupień pozwoliły na wytypowanie panelu gliko-markerów (względna reaktywność glikanów IgG z MPL, VVL, Jacaliną oraz współczynnik MPL/VVL), które mogą stanowić pomocne narzędzie diagnostyczne w różnicowaniu kobiet z zaawansowaną endometriozą od kobiet zdrowych.

Wnioski: Wartości względnej reaktywności glikanów IgG w surowicy i w wyizolowanych preparatach IgG, ze sjalo-specyficzną lektyną MAA, wykrywającą kwas sjalowy przyłączony wiązaniem α2,3, oraz wartości współczynników sjalilacji i agalaktozylacji pozwoliły na różnicowanie kobiet z zaawansowaną endometriozą od kobiet zdrowych. Reaktywność glikanów IgG w surowicy z panelem fukozo-specyficznych lektyn AAL, LCA i LTA może być brana pod uwagę jako przydatne narzędzie diagnostyczne i kliniczne do różnicowania pacjentek z zaawansowaną endometriozą od grupy zdrowych kobiet. Ponadto wykazano, że analiza fukozylacji surowiczej IgG, bez uprzedniej czasochłonnej, pracochłonnej i kosztownej izolacji IgG, jest wystarczająca do różnicowania zaawansowanych stadiów endometriozy od grupy

kontrolnej zdrowych kobiet. Zmiany profilu i stopnia O-glikozylacji IgG pozwalają na różnicowanie kobiet z zaawansowaną endometriozą od kobiet zdrowych. Analiza ekspresji O-glikanów specyficznie wiążących się z Jacaliną, najprawdopodobniej typu 'core 3', obecnych w wyizolowanych preparatach IgG, może być pomocna w różnicowaniu kobiet z zaawansowaną endometriozą od pacjentek z innymi chorobami ginekologicznymi o podłożu zapalnym, co ma szczególną wartość diagnostyczną. Wykazana istotnie wyższa ekspresja wieloantenowych N-glikanów w surowiczej IgG u kobiet z grupy E i NE w porównaniu z kobietami zdrowymi wskazuje, że obecność wielorozgałęzionych N-glikanów w IgG towarzyszy stanom zapalnym występującym w wielu różnych chorobach ginekologicznych.

6. STRESZCZENIE PRACY W JĘZYKU ANGIELSKIM

Introduction: Endometriosis is a disease caused by the growth of the lining of the uterus (endometrium) outside the uterus. The lack of specific symptoms makes endometriosis difficult to diagnose, and currently recommended diagnostic imaging methods (USG, MRI) may be insufficient to establish the correct diagnosis, especially in the case of retroperitoneal and deep infiltrating lesions. Currently, there are no sensitive and specific markers that would enable an effective diagnosis of endometriosis. Therefore, there is a need to search for non-invasive biomarkers characteristic of this disease, which could be used in routine laboratory diagnostics.

Aim of the study: The main objective of the study was to determine the profile and degree of glycosylation of serum immunoglobulin G (IgG) in women with advanced endometriosis and to check whether there are differences in the expression of IgG glycans between the group of patients with advanced endometriosis and the group of women with other than endometriosis, mild gynecological diseases and healthy women, which would allow for their differentiation, and to determine possible features characteristic of advanced endometriosis in the IgG glycan profile present in the serum. An additional aspect of the conducted research was also the comparison of the sensitivity of the determination of the profile and degree of glycosylation of IgG between the determinations made in serum and in isolated IgG preparations, in the context of the use of readily available biological material, i.e. blood serum, for routine determinations of glycomarkers in the diagnostic process of endometriosis, omitting the laborious and time-consuming process of IgG isolation.

Materials and methods: The tested sera came from patients with advanced endometriosis (E; n=40) and from patients without endometriosis with mild gynecological diseases (NE; n=36). The control group consisted of healthy women (C; n=19), without any diagnosed gynecological diseases. IgG isolation from the sera was performed using an affinity column with Protein A/G-Sepharose. IgG concentration was determined respectively: 1) for isolated IgG preparations by bicinchoninic method, 2) for serum IgG by turbidimetric method. The analysis of the profile and degree of glycosylation of IgG was carried out using the lectin-ELISA test with the use of lectins specific for selected N- and O-glycans. The Statistica 13.3 PL program was used

for the statistical analysis of the obtained results, and the results for which the significance coefficient $p<0.05$ were considered significant.

Results: Expression of terminal sialic acid on serum IgG glycans and in isolated IgG preparations were significantly higher in the group of healthy women compared to patients from groups E and NE. There was also a reduced expression of galactose on IgG glycans in groups E and NE in relation to group C, and the value of the agalactosylation coefficient (GSL-II/RCA-I) was significantly higher in groups E and NE compared to group C. The value of the sialylation coefficient (MAA/SNA) was significantly lower in group E compared to C. Expression of core fucose was significantly higher in group C compared to groups E and NE for both serum IgG and IgG in isolated preparations. However, these relationships were reversed in the case of LTA- and UEA-reactive antennary fucose. The relative reactivities of O-glycans in isolated IgG preparations with specific lectins and the values of the MPL/VVL O-glycosylation coefficient were significantly higher in patients from groups E and NE compared to the control group of healthy women. In addition, a significantly higher expression of multibranched N-glycans was also demonstrated for the isolated IgG in groups E and NE. The results of the analysis of ROC curves and cluster analysis allowed to select of a panel of glycomarkers (MPL, VVL, MPL/VVL, Jacalina), which can be a helpful diagnostic tool in differentiating women with advanced endometriosis from healthy women.

Conclusions: The values of the relative reactivity of IgG glycans in the serum and in the isolated IgG preparations with the sialyl-specific MAA lectin as well as the values of the sialylation and agalactosylation coefficients allowed for the differentiation of women with advanced endometriosis from healthy women. The reactivity of serum IgG glycans with a panel of fucose-specific AAL, LCA and LTA lectins can be considered a useful diagnostic and clinical tool to differentiate patients with advanced endometriosis from healthy patients women. In addition, it has been shown that the analysis of serum IgG fucosylation, without prior time-consuming, laborious and costly isolation of IgG, is sufficient to differentiate advanced stages of endometriosis from a control group of healthy women. Changes in the profile and degree of IgG O-glycosylation allow differentiating women with advanced endometriosis from healthy women. Analysis of the expression of O-glycans specifically binding to Jacalin, most likely of the 'core 3' type, present in isolated IgG preparations, may help differentiate

women with advanced endometriosis from patients with other inflammatory gynecological diseases. The significantly higher expression of multi-antennary N-glycans in serum IgG in women from groups E and NE compared to healthy women indicates that the presence of multi-branched N-glycans in IgG accompanies inflammation in various gynecological diseases.

7. PUBLIKACJE

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Variability of serum IgG sialylation and galactosylation degree in women with advanced endometriosis

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Endometriosis is an inflammatory disease which diagnostics is difficult and often invasive, therefore non-invasive diagnostics methods and parameters are needed for endometriosis detection. The aim of our study was to analyse the glycosylation of native serum IgG and IgG isolated from sera of women classified as: with endometriosis, without endometriosis but with some benign ginecological disease, and control group of healthy women, in context of its utility for differentiation of advanced endometriosis from the group of healthy women. IgG sialylation and galactosylation/agalactosylation degree was determined using specific lectins: MAA and SNA detecting sialic acid α2,3- and α2,6-linked, respectively, RCA-I and GSL-II specific to terminal Gal and terminal GlcNAc, respectively. The results of ROC and cluster analysis showed that the serum IgG MAA-reactivity, sialylation and agalactosylation factor may be used as supplementary parameters for endometriosis diagnostics and could be taken into account as a useful clinical tool to elucidate women with high risk of endometriosis development. Additionally, we have shown that the analysis of native serum IgG glycosylation, without the prior time-consuming and expensive isolation of the protein, is sufficient to differentiation endometriosis from a group of healthy women.

Endometriosis, defined as the presence of endometrial glands and stroma like lesions outside of the uterus[1], has been diagnosed in 176 million women worldwide[2]. The disease has a significant impact on women fertility, as it occurs in more than one-third of infertile women and two-thirds of women with chronic pelvic pain[3]. Endometriosis, although not a malignant disease, invades surrounding organs[4] and the lesions can appear as ovarian cyst, peritoneal or superficial implants, or deep infiltrating disease, which induces a chronic, inflammatory reaction[2]. According to the revised American Fertility Society (rAFS) classification[5] and up-to-dated by American Society for Reproductive Medicine (ASRM)[6], based on severity and anatomical extension of changes, endometriosis is graded into four stages: minimal, mild, moderate and severe (I, II, III and IV stage, respectively). The etiology of this disease remains obscure despite several hypotheses how endometriosis develop. Therefore, many various factors, hormonal, inflammatory or immunological, may determine whether deposits in the pelvic cavity may implant and persist[7–9]. Endometriosis seems to be associated with antibody self-reactivity and chronic local inflammation, coexisting also with autoimmune disease, but this does not seem to be a common phenomenon[10]. The presence of autoreactive antibodies in serum of some endometriosis women may be a natural by-product of inflammation and local tissue destruction. The finding of antibodies to endometrial and ovarian nuclear antigens in women with endometriosis supports the hypothesis of endometriosis being a multiple antibody autoimmune condition[10]. The 'gold diagnostic standard' for endometriosis identification is laparoscopic inspection with histologic confirmation after biopsy, however, the correlation between clinical symptoms and disease stage is poor, and invasive testing carries required anaesthesia and surgical skills and produces potential risk of complications[11,12]. Many studies focus on searching for biochemical markers with high sensitivity and specificity for non-invasive diagnostics or screening of endometriosis[11–13] as well as aim better understanding molecular mechanisms responsible for disease development[14–16]. Proper identification of endometriosis is still problematic

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Groups	Relative reactivity with lectins (AU)											
	MAA (s)	MAA (i)	SNA (s)	SNA (i)	MAA/SNA ratio (s)	MAA/SNA ratio (i)	GSL-II (s)	GSL-II (i)	RCA-I (s)	RCA-I (i)	GSL-II / RCA-I ratio (s)	GSL-II / RCA-I ratio (i)
E n = 40	0.011 ± 0.012	0.009 ± 0.031	0.288 ± 0.105	0.263 ± 0.144	0.042 ± 0.048	0.030 ± 0.099	0.037 ± 0.041	0.034 ± 0.028	0.331 ± 0.343	0.341 ± 0.325	0.169 ± 0.200	0.145 ± 0.132
NE n = 36	0.016 ± 0.021	0.004 ± 0.009	0.227 ± 0.098	0.250 ± 0.115	0.063 ± 0.060	0.018 ± 0.034	0.036 ± 0.028	0.032 ± 0.014	0.232 ± 0.147	0.448 ± 0.443	0.197 ± 0.147	0.135 ± 0.120
			$^{1}p = 0.004$						$^{1}p = 0.047$			
Control n = 19	0.023 ± 0.013	0.051 ± 0.032	0.301 ± 0.071	0.546 ± 0.165	0.081 ± 0.048	0.107 ± 0.087	0.023 ± 0.018	0.025 ± 0.013	0.468 ± 0.259	0.925 ± 0.419	0.054 ± 0.058	0.035 ± 0.028
	$^{1}p = 0.0001$	$^{1}p = 0.000000$		$^{1}p = 0.000000$	$^{1}p = 0.001$	$^{1}p = 0.000000$	$^{1}p = 0.026$		$^{1}p = 0.013$	$^{1}p = 0.000000$	$^{1}p = 0.0001$	$^{1}p = 0.000001$
	$^{2}p = 0.004$	$^{2}p = 0.000000$	$^{2}p = 0.001$	$^{2}p = 0.000000$		$^{2}p = 0.000000$	$^{2}p = 0.022$		$^{2}p = 0.00007$	$^{2}p = 0.00007$	$^{2}p = 0.00002$	$^{2}p = 0.00001$

Table 1. Relative reactivity of s-IgG and i-IgG glycans with specific lectins. The results are expressed in absorbance units (AU) as mean values ± SD. Serum IgG glycan reactivities with lectins were estimated by direct lectin-ELISA and expressed in absorbance units (AU). MAA: *Maackia amurensis* agglutinin, recognizing sialic acid α2,3-linked; SNA: *Sambucus nigra* agglutinin, recognizing sialic acid α2,6-linked; GSL-II: *Griffonia simplicifolia* lectin II, recognizing terminal GlcNAc; RCA-I: *Ricinus communis* agglutinin I, recognizing terminal Gal. Significant differences versus groups: [1]with endometriosis (E), [2]non-endometriosis (NE). Control—group of healthy women. s-IgG—serum native IgG; i-IgG—serum IgG isolates. Statistically significant differences were accepted for a *p*-value of less than 0.05.

in gynaecologic practice, therefore extensive research for new biomarkers give hope for more accurate diagnostics, especially in the advanced stages of the disease.

Glycosylation, one of the most common post-translational modifications of secretory and membrane proteins, plays an important role in biological processes, such as cell recognition and adhesion, cell–cell communication and cell–cell interactions[17], and plays a key role in antibodies function[18]. Immunoglobulin G (IgG), 150-kDa glycoprotein, is the most abundant immunoglobulin in blood (represents about 75% of serum immunoglobulins), involved in the pathogenesis and progression of many diseases. IgG plays a key role in defending the host against microbial infections, but also in pathological events (*inter alia* inflammation) being a cause of various diseases. Immunoglobulin G can activate a variety of effector mechanisms, such as complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity and phagocytosis[19,20]. IgG glycans are essential for the proper activity of the immune system. The presence or absence of one sugar moiety in oligosaccharide structure of N-glycan may result in the stimulation or suppression of immune response. The contribution of IgG Fc glycosylation changes was documented for the pathogenesis of rheumatoid arthritis, Crohn's disease and lupus erythematosus. Found in these diseases the decreased IgG galactosylation and sialylation, activates effector cells and initiates an inflammatory response[18]. IgG has one biantennary N-linked glycan attached to asparagine 297[14,21], which consists of a constant heptameric core structure containing three mannose residues and four GlcNAc residues and may contain additional core fucose, as well as bisecting GlcNAc. Additionally, the branching arms (α-6 and α-3) may have variable glycosylation pattern consisting of terminal galactose and sialic acids[14,22,23]. The profile and degree of IgG glycosylation can vary in various pathological conditions and its glycans composition can alter the effector functions of immunoglobulin by modulating its affinity for ligands, such as Fcγ receptors (FcγRs)[19]. Many reports described variations of IgG glycosylation, especially the degree of glycosylation, related to age, sex, heritability and pregnancy, as well as to autoimmune diseases and cancers[24–30]. It is already documented, that in rheumatoid arthritis patients, serum IgG galactosylation of its conservative N-glycans (Asn-297) in CH_2 domains of the heavy chains is decreased, and that IgG agalactosylation is proportional to disease severity[31].

In the present study we were interested, if the expression of serum IgG sialylated and galactosylated/agalactosylated glycoforms is characteristic for advanced stages of endometriosis. Another important aspect was the comparison of native serum IgG glycosylation with glycosylation of IgG isolated from serum, and evaluation the potential diagnostic suitability of glycan analysis when glycoprotein is not isolated earlier from biological fluid. The degree of IgG sialylation and galactosylation/agalactosylation was analysed using a modified solid phase enzyme-linked immunosorbent assay, lectin-ELISA[31–34].

Results

The relative reactivities of serum IgG glycans with selected panel of biotinylated lectins, specific to sialic acid (MAA, SNA), terminal Gal (RCA-I) and terminal GlcNAc (GSL-II) are presented in Table 1 as mean values of absorbances and standard deviations (SD) for each analysed group. The distributions and median values of IgG relative reactivities with lectins tested, measured for the control, E and NE groups, are presented in Fig. 1. The additional Tables and Figures are presented in Supplementary materials (Tables 1S, 2S, 3S and Figs. 1S, 2S, 3S, 4S, 5S).

Relative reactivity of IgG with MAA and SNA. The expression of MAA-reactive α2,3-linked sialic acid in s-IgG was significantly lower in E and NE groups (0.011 ± 0.012 AU, median 0.007 AU, $p = 0.0001$ and 0.016 ± 0.021 AU, median 0.012 AU, $p = 0.004$, respectively) than for the control group (0.023 ± 0.013 AU, median 0.020 AU). Also the relative reactivities of i-IgG with MAA were higher for control group (0.051 ± 0.0032 AU, median 0.037 AU) than in E and NE groups (0.009 ± 0.031 AU, median 0.000 AU, $p = 0.000000$ and 0.004 ± 0.009 AU, median 0.000 AU, $p = 0.000000$, respectively).

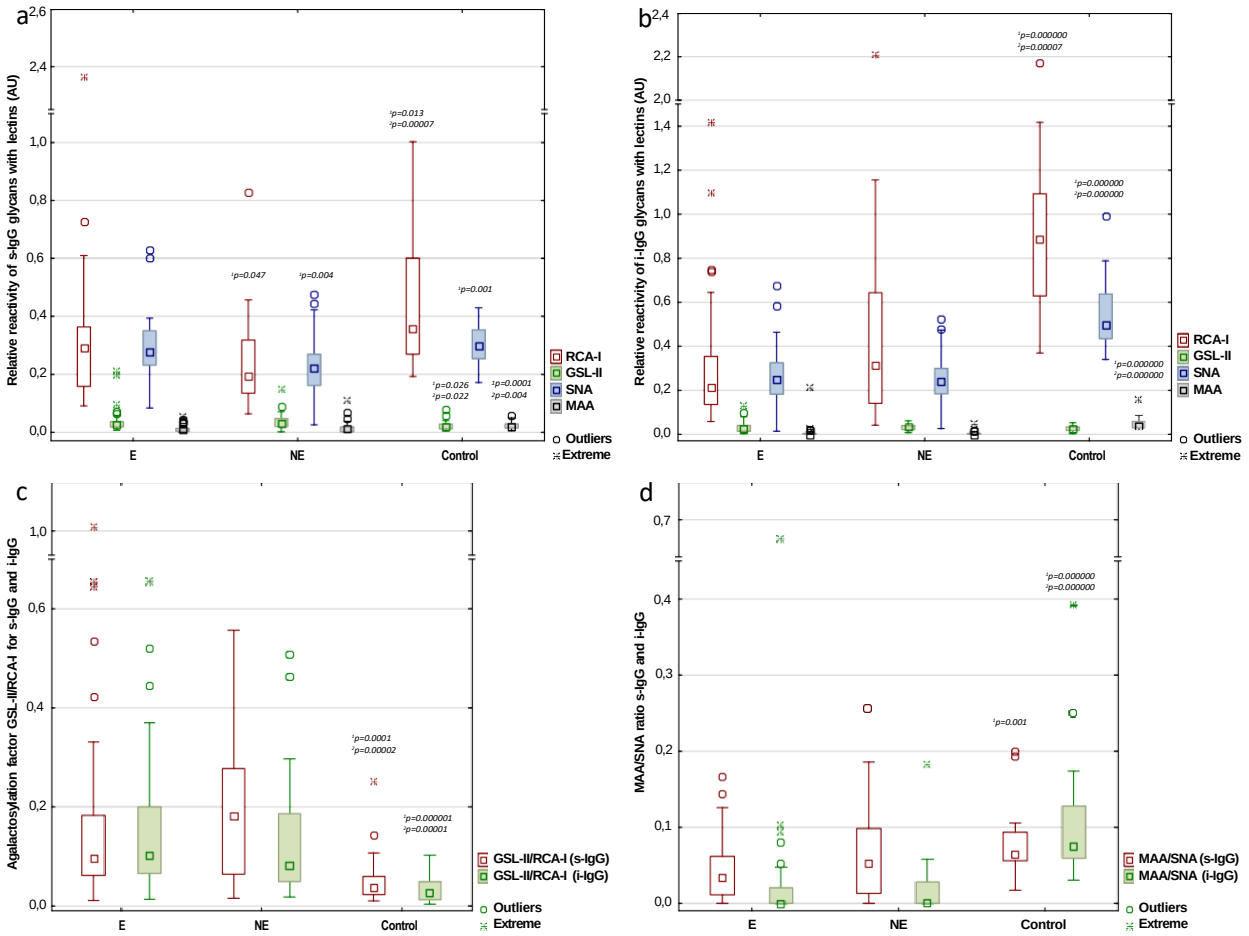

Figure 1. Relative reactivities of serum native IgG (s-IgG)—(**a**), and isolated serum IgG (i-IgG)—(**b**) glycans with specific lectins. GSL-II: *Griffonia simplicifolia* lectin II, recognizing terminal GlcNAc; RCA-I: *Ricinus communis* agglutinin I, recognizing terminal Gal; MAA: *Maackia amurensis* agglutinin, recognizing sialic acid α2,3-linked; SNA: *Sambucus nigra* agglutinin, recognizing sialic acid α2,6-linked. E—endometriosis, NE—no endometriosis and Control—group of healthy women. The relative reactivities with lectins were expressed in absorbance units (AU). (**c**)—the values of agalactosylation factor GSL-II/RCA-I for IgG glycans. (**d**)—the values of MAA/SNA ratio for IgG glycans. MAA: *Maackia amurensis* agglutinin, recognizing sialic acid α2,3-linked; SNA: *Sambucus nigra* agglutinin, recognizing sialic acid α2,6-linked. Significant differences versus groups: [1]E, [2]NE. Median is indicated as square. A two-tailed *p*-value of less than 0.05 was considered as significant.

The relative reactivities of s-IgG with SNA-reactive sialic acid α2,6-linked were significantly lower in NE group (0.227 ± 0.098 AU, median 0.222 AU) than those measured for the control group (0.301 ± 0.071 AU, median 0.298 AU, $p = 0.001$) and endometriosis (0.288 ± 0.105 AU, median 0.278 AU, $p = 0.004$) groups. Relative reactivities of i-IgG with SNA were significantly higher for control group (0.546 ± 0.165 AU, median 0.496 AU) than for E (0.263 ± 0.144 AU, median 0.250 AU, $p = 0.000000$) and NE (0.250 ± 0.115 AU, median 0.240 AU, $p = 0.000000$) groups. The ratio of MAA/SNA relative reactivities was significantly higher in control group (0.081 ± 0.048, median 0.064 for s-IgG and 0.107 ± 0.087, median 0.075, for i-IgG) than in E (0.042 ± 0.048, median 0.034, $p = 0.001$ and 0.030 ± 0.099, median 0.000, $p = 0.000000$, respectively) and NE groups (0.018 ± 0.034, median 0.001, $p = 0.000000$ for i-IgG only).

Relative reactivity of IgG with GSL-II and RCA-I. As shown in Table 1, the relative reactivities of s-IgG with GlcNAc-specific lectin GSL-II in the E (0.037 ± 0.041 AU, median 0.026 AU) and NE (0.036 ± 0.028 AU, median 0.030 AU) groups were significantly higher ($p = 0.026$ and $p = 0.022$, respectively) than those observed for the control group (0.023 ± 0.018 AU, median 0.020 AU). However, there were no significant differences between analysed groups in relative reactivities of i-IgG with GSL-II ($p > 0.05$).

The relative reactivities of s-IgG glycans with galactose-specific lectin RCA-I in the E and NE groups were significantly lower (0.331 ± 0.343 AU, median 0.292 AU, $p = 0.013$ and 0.232 ± 0.147 AU, median 0.195 AU, $p = 0.00007$, respectively) than those observed for the control group (0.468 ± 0.259 AU, median 0.358 AU). Also the expression of RCA-I-reactive IgG glycans was significantly lower in NE than E group ($p = 0.047$). The relative reactivities of i-IgG with galactose-specific RCA-I were significantly lower in E (0.341 ± 0.325 AU, median 0.221 AU, $p = 0.000000$) and NE (0.448 ± 0.443 AU, median 0.315 AU, $p = 0.00007$) groups than those measured for

Correlations between lectins relative reactivity	s-IgG	i-IgG
	Spearman rank coefficient (*r*)	Spearman rank coefficient (*r*)
RCA-I vs. GSL-II/RCA-I	$r = -0.679$ $p = 0.000000$	$r = -0.770$ $p = 0.000000$
RCA-I vs. SNA	$r = 0.743$ $p = 0.000000$	$r = 0.781$ $p = 0.000000$
RCA-I vs. MAA		$r = 0.509$ $p = 0.000000$
RCA-I vs. MAA/SNA		$r = 0.465$ $p = 0.000000$
GSL-II vs. GSL-II/RCA-I	$r = 0.770$ $p = 0.000000$	$r = 0.389$ $p = 0.00006$
GSL-II vs. MAA	$r = 0.357$ $p = 0.0002$	
GSL-II vs. MAA/SNA	$r = 0.364$ $p = 0.0002$	
SNA vs. MAA		$r = 0.538$ $p = 0.000000$
SNA vs. MAA/SNA		$r = 0.484$ $p = 0.000000$
SNA vs. GSL-II/RCA-I	$r = -0.408$ $p = 0.000000$	$r = -0.588$ $p = 0.000000$
MAA vs. MAA/SNA	$r = 0.927$ $p = 0.000000$	$r = 0.978$ $p = 0.000000$
MAA vs. GSL-II/RCA-I		$r = -0.301$ $p = 0.000000$

Table 2. The correlations between relative reactivities of IgG glycans with lectins. Serum native IgG—s- IgG; serum IgG isolates—i-IgG. MAA: *Maackia amurensis* agglutinin, recognizing sialic acid α2,3-linked; SNA: *Sambucus nigra* agglutinin, recognizing sialic acid α2,6-linked; GSL-II: *Griffonia simplicifolia* lectin II, recognizing terminal GlcNAc; RCA-I: *Ricinus communis* agglutinin I, recognizing terminal Gal. A two-tailed *p*-value of less than 0.05 was considered as significant.

samples from the control group (0.925 ± 0.419 AU, median 0.887 AU). The agalactosylation factor (AF), measured as ratio of GSL-II/RCA-I relative reactivities, was significantly lower in control group (0.054 ± 0.058, median 0.038, for s-IgG and 0.035 ± 0.028, median 0.026, for i-IgG) than in E (0.169 ± 0.200, median 0.088, $p = 0.0001$ and 0.145 ± 0.132, median 0.099, $p = 0.000001$, respectively) and NE groups (0.197 ± 0.147, median 0.182, $p = 0.00002$ and 0.135 ± 0.120, median 0.083, $p = 0.00001$, respectively).

The Spearman rank correlations, analysed between relative reactivities of lectins used with IgG glycans (s-IgG and i-IgG) are shown in Table 2 and Fig. 1S. It was a positive high correlation between relative reactivities of RCA-I and SNA ($r = 0.743$, $p = 0.00$; $r = 0.781$, $p = 0.00$) with glycans of s-IgG and i-IgG. For s-IgG a positive high correlation was observed between GSL-II relative reactivity and AF GSL-II/RCA-I ($r = 0.770$; $p = 0.00$), while a negative high correlation was observed between RCA-I relative reactivity and the AF GSL-II/RCA-I ($r = -0.679$; $p = 0.00$), which were also observed for i-IgG ($r = -0.770$; $p = 0.00$). For i-IgG a high positive correlations exist between relative reactivities with RCA-I versus MAA and with SNA versus MAA ($r = 0.509$, $p = 0.00$ and $r = 0.538$, $p = 0.00$, respectively). Full correlations were found for s-IgG and i-IgG between MAA relative reactivities and ratio MAA/SNA ($r = 0.927$, $p = 0.00$ and $r = 0.978$, $p = 0.00$, respectively). The correlations between relative reactivities of s-IgG and i-IgG glycans with lectins are presented in Table 3 and Fig. 2S. An average positive correlation in MAA reactivities was observed between glycans of s-IgG and i-IgG ($r = 0.487$, $p = 0.00$).

ROC curve analysis. The results of ROC curve analysis are showed in Fig. 3S and Table 1S. ROC curve analysis of s-IgG glycans relative reactivities with lectins, and their reactivities ratio, in endometriosis and control group, identified parameters with a sensitivity and specificity of: MAA 0.795, 0.795 (AUC 0.812—moderate clinical value); SNA 0.364, 0.842 (AUC 0.575—limited clinical value); MAA/SNA 0.659, 0.895 (AUC 0.762—moderate clinical value); RCA-I 0.864, 0.474 (AUC 0.699—limited clinical value); GSL-II 0.866, 0.474 (AUC 0.675—limited clinical value); GSL-II/RCA-I 0.750, 0.842 (AUC 0.800—moderate clinical value). For i-IgG the obtained results were following: MAA 0.886, 1 (AUC 0.970—high clinical value); SNA 0.864, 0.947 (AUC 0.943—high clinical value); MAA/SNA ratio 0.773, 1 (AUC 0.916—high clinical value); RCA-I 0.795, 1 (AUC 0.922—high clinical value); GSL-II 0.455, 0.737 (AUC 0.539—limited clinical value); GSL-II/RCA-I ratio 0.864, 0.842 (AUC 0.886—moderate clinical value). For the determination of cut-off points, the Youden index method was used. The clinical value of a laboratory test with AUC can be defined as: 0–0.5—zero, 0.5–0.7—limited, 0.7–0.9—moderate and > 0.9 high[35].

Cluster analysis. The usefulness of s-IgG and i-IgG glycans expression for differentiation of endometriosis group from group of healthy women, the relative reactivity with MAA as well as MAA/SNA and GSL-II/RCA ratio were selected from the panel of parameters examined, taking into account the results of ROC analysis

Correlations between lectins relative reactivity s-IgG vs. i-IgG	Spearman rank coefficient (r)
RCA-I vs. RCA-I	$r=0.226$ $p=0.02$
GLS-II vs. GLS-II	$r=0.440$ $p=0.000005$
GSL-II/RCA-I vs. GSL-II/RCA-I	$r=0.365$ $p=0.0002$
SNA vs. SNA	$r=0.421$ $p=0.00001$
MAA vs. MAA	$r=0.487$ $p=0.000000$
MAA/SNA vs. MAA/SNA	$r=0.449$ $p=0.000003$

Table 3. The correlations between relative reactivities of s-IgG and i-IgG glycans with lectins. Serum native IgG—s-IgG; serum IgG isolates—i-IgG. GSL-II: *Griffonia simplicifolia* lectin II, recognizing terminal GlcNAc; RCA-I: *Ricinus communis* agglutinin I, recognizing terminal Gal; MAA: *Maackia amurensis* agglutinin, recognizing sialic acid α2,3-linked; SNA: *Sambucus nigra* agglutinin, recognizing sialic acid α2,6-linked. A two-tailed p-value of less than 0.05 was considered as significant.

(AUC ≥ 0.762; Figs. 4S, 5S and Tables 2S, 3S). The analysis was performed for 59 samples, for which all selected parameters were determined. The analysis for s-IgG and i-IgG was done separately. In case of s-IgG, the first cluster could be distinguished as a homogenous group of 3 endometriosis samples (Fig. 4S, Table 2S) at 100% distance. The next group could be separated at 33% distance (Cluster 2) consists of 12 samples, including 16% of women from whole healthy group. Next cluster (Cluster 3 at 16% distance) comprised 23 subjects, 14 of which were healthy women from the control group (74% of the whole group) and 9 samples belonged to endometriosis patients (22.5% of the whole endometriosis group). Last cluster (Cluster 4) include 21 samples, 19 samples form endometriosis group (47.5% of the whole group).

In cluster analysis of i-IgG (Fig. 5S, Table 3S) first cluster could be separated at distance 92%. This homogenous group consists of 2 endometriosis samples. The next homogenous group could be separated at 69% distance (Cluster 2) in which 4 samples from the control group were gathered. Cluster 3 (homogenous, composed of endometriosis samples) and cluster 4 (gathered 3 control samples and 24 endometriosis samples) were distinguished at distance 40% and 24%, respectively. Cluster 5 include 13 samples, 12 from the control group, which make up 63% of the whole group of healthy women.

Discussion

This is the first study, to our best knowledge, in which the serum IgG sialylation degree and agalacto-glycans expression was analysed in context of advanced endometriosis. We showed that for native and isolated serum IgG the significantly decreased galactose expression, α2,3-sialylation and value of MAA/SNA ratio, as well as increased expression of agalacto-glycans (for s-IgG only) and level of agalactosylation factor GSL-II/RCA-I in patients with non-endometriosis (for MAA/SNA ratio only for i-IgG) and advanced endometriosis (not for s-IgG SNA-reactivity) in comparison to the healthy women were observed. However, for s-IgG in NE group significantly lower SNA-reactivity and galactose expression, and significantly higher agalactosylation factor GSL-II/RCA-I in comparison to E group were observed. Reduced sialylation and galactosylation is typical for inflammatory condition and abnormal glycosylation patterns of glycoproteins are associated with various diseases, such as inflammation or cancer, what was reported in many independent studies[24,36,37]. Also endometriosis is a disease accompanied by the development of inflammation[10,38]. Presence or absence in IgG glycan structure of distinct residues such as sialic acid and galactose, can alter pro- and anti-inflammatory IgG activities[20]. An absence of sialic acids and low degree of galactosylation might be linked with pro-inflammatory properties of IgG, by facilitating the formation of immune complexes and favouring the binding of IgG to activated FcγR (Fc gamma receptor)[39–41]. Moreover, Fc-linked glycans appear to modulate the activation of complement system. Whereas the classical rout of complement can be triggered by the preferential binding of C1q to fully glycosylated IgG, the lectin pathway is recruited through the recognition of agalactosylated IgG by mannose-binding lectin[42]. In opposite, the presence of terminal galactose and/or sialic acid residues on Fc glycans can be linked with anti-inflammatory properties of IgG[41]. Thus, variations in the glycan structures of IgG Fc can switch the immune system toward a pro- to anti-inflammatory response, by modulating the interaction of IgG with several immune components, including FcγR, complement factors and lectins[43].

We observed reduced IgG galactosylation in non-endometriosis women, when compared to the control group, which is probably due to the fact that NE group involved patients treated and operated for benign gynaecological conditions and some of these pathologies, e.g. tubal infertility, uterine fibroids or ovarian cysts may be accompanied by inflammation. Some studies reported that changes in IgG galactosylation are also observed in physiological conditions and it is known that they are age[44,45] and sex[46] related. In our study, we did not observe any correlations between changes in the level of IgG galactosylation and the age of patients in any of the studied groups (data not shown). Decreased levels of terminal galactose on biantennary IgG glycans may indicate that the activity of galactosyltransferases responsible for decorating N-glycans with galactose residues is impeded[25]. Glycoforms lacking terminal galactose are particularly pro-inflammatory[39,41,47], while galactosylation strongly

decreases pro-inflammatory function of IgG[41]. This is confirmed by results of our research, in which E and NE groups significantly lower reactivities of s-IgG and i-IgG with a specific lectins, compared to the healthy women, were observed. Another observation we made, is that non-endometriosis group, with benign gynecological diseases should not be used as a representative control for differentiation with endometriosis, because IgG glycosylation varies with inflammation, which can accompany a wide variety of diseases, not endometriosis only. In our study the reaction of reduced s-IgG and i-IgG with terminal galactose-specific RCA-I and terminal GlcNAc-specific GSL-II was reciprocal to each other, because terminal GlcNAc residues are exposed in glycoprotein oligosaccharides after galactose removal. These reactions were not always exactly reciprocal, what may be explained in two ways. First of them is more general and is based on the fact that measurements using the lectins it is not in practice the total amount of a specific glycotope present on a protein, but rather the amount of this glycotope currently accessible for the lectin. Unfolding of a polypeptide chain, after IgG reduction, strongly influences this feature. Secondly, it should be taken into account that some oligosaccharide chains may be present statistically also in Fab fragment of IgG[48]. Like galactose requires the terminal GlcNAc as a substrate, sialic acid requires galactose moiety[41]. Increased sialylation of IgG generally results from the increased galactosylation, because galactosylated IgG is the substrate for sialyltransferases[49]. On human glycoproteins, sialic acids may be bound α2,3 or α2,6 to galactoses and are the most exposed monosaccharides to the outer environment, participating in many biological processes including *inter alia* cancerogenesis[50]. In healthy adults mono- and disialylated glycan structures of IgG represent about 10–15% of total IgG Fc oligosaccharide structures[22]. Terminal sialic acids appear to mainly serve as a switch between IgG pro- and anti-inflammatory activity in cases of homeostasis disturbance, what is in contrast to galactosylation, which seems to represent an interface between physiological and pathological processes. This is confirmed by the results of our research, in which we observed a higher reactivity of IgG with sialic acid-specific lectins in the control group. This is in accordance with the results of Maignien et al.[51], who reported reduced α2,6 sialylation in the peritoneal fluid of women with endometriosis. Authors documented that the IgG reactivity with SNA in the endometriosis group was significantly lower when compared to the control group. In contrast, they did not observe any difference in the expression of sialic acid α2,3-linked. Authors concluded, that the mechanisms of altered sialylation during endometriosis development, especially those affecting the adhesion between endometrial cells and peritoneal mesothelial cells, are still unclear[16]. It has been reported that the glycoproteins levels are increased in peritoneal fluid, serum, and eutopic endometrium of women with endometriosis[38,52]. However, in our previous study we documented, that IgG concentration was significantly lower in severe endometriosis than in the control group of healthy women[53]. In our study, we showed a strong positive correlation between IgG RCA-I reactivity and its reactivity with lectins specific for sialic acid. RCA-I has a preference for a Galβ1,4 rather than Galβ1,3 or Galβ1,6 terminal sequences. However, there seems to be no specific requirement for the sub-terminal residue, e.g. Galβ1,4GlcNAc, Galβ1,4Glc and Galβ1,4Man have shown similar activities. The presence of sialic acid at the 3-O or 6-O positions modifies the terminal Gal, significantly reducing the binding affinity to RCA-I[54]. Structurally, the N-linked glycans of human IgGs are typically biantennary chains. The heterogeneous IgG glycans can be classified into three sets (G0, G1, and G2), depending on the number of galactose residues in the outer arms of biantennary glycans[55]. Comparing the lectins relative reactivity of s-IgG and i-IgG glycans in each of the analysed groups, they are more or less different from each other, what may be due to differences in the availability of sugar residues for lectins between native s-IgG and i-IgG isolated from serum. On the other hand the significant correlations between s-IgG and i-IgG reactivity with lectins used were observed (the highest for MAA and the lowest for RCA-I). Analysing the results obtained and the utility of them to differentiate serum samples in a way that reflects their clinical characteristics typical for endometriosis, we observed, that the values of agalactosylation factor and sialylation factor are much better than the values of relative reactivities of IgG glycans with each lectin separately, with one exclusion—MAA relative reactivity with sialic acid α2,3-linked. The above observation is in agreement with results of ROC analysis in which both for s-IgG and i-IgG cut-off point for MAA reactivity was 0.014 AU (AUC 0.812) and 0.020 AU (AUC 0.970), respectively, for MAA/SNA factor it was 0.039 (AUC 0.762) and 0.027 (AUC 0.916), respectively, and for AF it was 0.063 (AUC 0.800) and 0.055 (AUC 0.886). Although the results of the ROC analysis showed that for i-IgG also the other parameters may be taken into account as markers of endometriosis (except the value of i-IgG relative reactivity with GSL-II), we selected only three of them for the cluster analysis, those which for s-IgG had a moderate AUC value. The results of cluster analysis additionally confirm the utility of the set of three selected parameters: MAA relative reactivity, MAA/SNA ratio and agalactosylation factor values for advanced endometriosis differentiation from group of healthy women. For s-IgG of the four clusters formed, cluster 3 differ with regard to the clinical characteristics of women, gathering 74% of samples of healthy women (n = 14) and 9 samples from endometriosis group (22.5%). From 5 clusters formed for i-IgG, cluster 5 gathered 63% of samples from the control group (n = 12) and 2.5% (n = 1) of serum samples from women with endometriosis. Taking into account the thesis of our investigations, the aim of which was to check whether the analysis of sialylation and galactosylation/agalactosylation degree of s-IgG glycans, without the prior time-consuming, complicated procedure of protein isolation, may be usable as differentiating marker/markers for endometriosis, our results seem to meet this criterion. It should also be mentioned that in lectin-ELISA method, glycan-lectin reaction reflect the reactions that occur in living organisms, including the availability of sugar residues for lectins, which additionally allows to deepen the knowledge about mechanisms of these interactions.

Materials and methods

Serum samples from women with diagnosed III and IV stages of endometriosis (E; n = 40, the median age: 34 years [interquartile range (IQR) 30.5–40.5]) as well as from group of women without endometriosis (NE—non-endometriosis; n = 36, the median age: 39 years [IQR 33.5–42.0]), were collected at the Department of

Oncological Gynecology, Wroclaw Comprehensive Cancer Center, Poland. The study was conducted in agreement with the Helsinki-II-declaration and the protocol was approved by the Bioethics Human Research Committee of the Wroclaw Medical University (Permission No. KB-293/2016 and KB-719/2018). E and NE groups underwent surgical interventions, mainly laparoscopic, and after histological verification were classified to the proper group. Endometriosis women were classified on extend and severity of disease according to the revised American Fertility Society (rAFS) classification. Serum samples from healthy women (C—control group, n = 19, the median age: 39 years [IQR 35.0–48.0]), were collected at the Department of Laboratory Diagnostics, Wroclaw Medical University (positive opinion of Bioethics Committee No KB-117/2020). Non-endometriosis group was histologically confirmed with benign ovarian cyst, with severe dysplasia—CIN 3 (cervical intraepithelial neoplasia grade 3) or leiomyoma's. The control group was consisted of healthy women, with no symptoms or history connected with endometriosis, non-pregnant, without any gynecological diseases. Before starting the study, all participants gave a written and informed consent.

The profile and degree of IgG sialylation and galactosylation/agalactosylation was determined using modified solid phase enzyme-linked immunosorbent assay, lectin-ELISA described by us earlier[31–34]. The method was based on the reactivity of IgG glycan moieties with specific biotinylated lectins: *Maackia amurensis* agglutinin (MAA, recognizing sialic acid α2,3-linked) and *Sambucus nigra* agglutinin (SNA, which recognize sialic acid α2,6-linked), *Ricinus communis agglutinin I* (RCA-I) which detect the terminal galactose; *Griffonia simplicifolia* lectin II (GSL-II) detecting the terminal GlcNAc (Vector Laboratories Inc., Burlingame, CA, USA).

Lectin-ELISA. The microtiter plates were incubated with 0.01 mg/ml protein G (abcam, USA) solution in 10 mM TBS pH 7.4 for 2 h at 37 °C, next, 4 °C overnight. Then the plates were coated with native/isolated IgG diluted 10 mM TBS-T (TBS containing 0.1% Tween, pH 7.4) in an amount 500 ng IgG in 50 µl solution per well, and incubated for 3 h at 37 °C. After the incubation, the reduction with dithiothreitol (DTT) for 70 min at 37 °C was carried out. Then the plates were incubated with biotinylated lectins (90 min, 37 °C) diluted with 10 mM TBS-T as follows: MAA: 1:250, SNA: 1:2000, RCA-I: 1:500, GSL-II: 1:400. Next, the plates were incubated with phosphatase-labelled ExtrAvidin for 1 h at room temperature. After incubation the phosphatase reaction was developed with p-nitrophenyl-phosphate used as a substrate (37 °C). The reaction was stopped with 100 µl of 1 mM NaOH per well and the absorbance was measured at 405 nm, reference filter 630 nm, with Mindray-96A microplate reader. After each incubation step the plates were extensively washed with 10 mM TBS-T, pH 7.4. All samples were analysed in duplicate. Background absorbance was measured for samples in which all reagents were present, but biological sample was replaced with 10 mM TBS-T. Samples relative reactivities with lectins were expressed in absorbance units (AU). IgG concentration values in whole sera (s-IgG), necessary for calculation of IgG amount to lectin-ELISA, were estimated by us previously using turbidymetric method[53] and those in IgG isolates (i-IgG) were measured by BCA method (see below).

IgG isolation. Immunoglobulin G was isolated from serum samples using affinity chromatography on Protein A/Protein G Sepharose column, according to the procedure described earlier by Ey et al.[56]. The serum sample (0.5 mL) was diluted 1:1 with 50 mM TBS, pH 8.0, applied on the column (1 mL) and washed using starting TBS solution. IgG, retained on the column, was eluted with 0.1 M glycine/HCl, pH 2.7 and immediately neutralized with 1 M Tris to avoid IgG degradation. Elution profile was determined by measuring the absorbance at 280 nm. The fractions containing IgG were pooled, concentrated using Amicon Ultra-15 centrifugal filter unit with ultracel-100 membrane (Millipore). IgG concentration was determined spectrophotometrically on a polystyrene 96-well microtiter plate (Maxisorp, Dako, Denmark) using bicinchoninic acid (BCA) colorimetric micromethod[57]. Briefly, to the 10 µL of IgG solution, diluted with water if necessary, an aliquot of 200 µL mixture of stock A and B solutions, in the proportion 50:1, was added and the plate was incubated at 37 °C for 30 min, as a standard the bovine serum albumin (BSA) in the range 0–10 µg/well was used. The absorbances were measured at 562 nm against blank, and the protein concentrations were read from the standard curve and expressed in µg/µl.

Statistical analysis. Statistical analysis was performed using the statistical software STATISTICA 13.3PL (StatSoft). Experimental data were presented as means and standard deviations (SD), and distribution of the values within analysed groups was presented as box-whisker plots with median and interquartile (25th–75th percentile) range. According to a Shapiro–Wilk *W* test, the values did not fit normal distribution, thus the non-parametric Mann–Whitney *U* test was used to determine differences among the groups. The correlations with 95% of confidence interval were estimated according to the Spearman rank test. A two-tailed *p*-value of less than 0.05 was considered as significant. The diagnostic significance of determined parameters was analysed using receiver operating characteristic (ROC) curves. Moreover, cluster analysis was performed for glycans expression on serum native IgG and isolated IgG, only for those parameters for which the AUC values, determined in ROC analysis, simultaneously were moderate or high. In this analysis, the results are presented as a dendrogram, starting from one cluster in which all subjects (patients and controls) are gathered. Next, the subjects were clustered, with the similar in terms of the values of all analysed traits grouped together and different ones forming a separate cluster. In general, the greater distance of separation, 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 samples. The scheme of statistical analysis of results obtained in the present study we have adopted is based on our previous experience[53].

Ethics approval. 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 the

Wroclaw Medical University (Permission No. KB-293/2016 and KB-719/2018). Written informed consent was obtained from recruited patients.

Conclusion

Endometriosis diagnostics is difficult and often invasive, therefore non-invasive diagnostics methods and parameters are needed. Disease development is associated with inflammatory processes, especially in advanced stages of endometriosis, markers of which can be also detected in peripheral blood serum. In the light of above information, the analysis of glycosylation profile and degree one of those markers, IgG, may be very promising in this context. Thus, the analysis of IgG sialylation and galactosylation/agalactosylation degree could be helpful during advanced endometriosis diagnostics, and may be used as supplementary parameters for medical interview and tests. The proposed panel of parameters, the expression of MAA-reactive sialic acid α2,3-linked, values of MAA/SNA ratio and agalactosylation factor, could be taken into account as a useful clinical tool to elucidate women with high risk of endometriosis development. However, further studies are needed to evaluate its clinical utility as a panel of additional markers for endometriosis diagnostics.

Limitation of the study.

1. Lack of representative early-stage endometriosis group, what makes impossible the verification of parameters analyzed by us and their ability to evaluate as useful biomarkers for early stage of endometriosis.
2. Lack of peritoneal fluid to compare the results of IgG glycosylation analysis with those obtained for serum IgG.

Strength of the study.

1. The panel of IgG glycosylation markers proposed by us (the expression of MAA-reactive sialic acid α2,3-linked, values of MAA/SNA ratio and agalactosylation factor) may be helpful in differentiation and diagnostics of advanced stage of endometriosis.
2. The analyzed by us IgG glycosylation markers could be used as a clinical tool to differentiate women with high risk of severe endometriosis development, qualified for laparoscopy procedure.
3. We showed that the analysis of sialylation and galactosylation/agalactosylation degree of s-IgG glycans, without the prior time-consuming, complicated procedure of protein isolation, may be usable as differentiating markers for advanced endometriosis.
4. The results of our studies can be used as the basis for further studies aimed at searching for noninvasive diagnostic parameters, with potential utility in women with advanced endometriosis.
5. Our study showed that non-endometriosis group, gathering women suffering from benign gynecological diseases with accompanying inflammation, seems not to be a proper comparative group to women with advanced endometriosis, because changes in IgG glycans expression are associated with the development of inflammation. Therefore in our study as a control group we used serum samples obtained from healthy women.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

Conceptualization: E.M.K., H.K.; Collecting the study group of patients: M.J.; Methodology: E.M.K., H.K., K.S.; Validation: E.M.K., H.K., K.S.; Formal Analysis: E.M.K., K.S.; Investigation: K.S.; Data Curation: K.S.; Writing—Original Draft Preparation: E.M.K., K.S.; Writing—Review & Editing: E.M.K., K.S., H.K., M.J.; Visualization: K.S.; Supervision: E.M.K., H.K.; Project Administration: E.M.K., H.K.; Funding Acquisition: E.M.K, M.J. All authors: read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to E.M.K.

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Supplementary materials

Variability of serum IgG sialylation and galactosylation degree in women with advanced endometriosis Katarzyna Sołkiewicz, Hubert Krotkiewski, Marcin Jędryka, Ewa M. Kratz

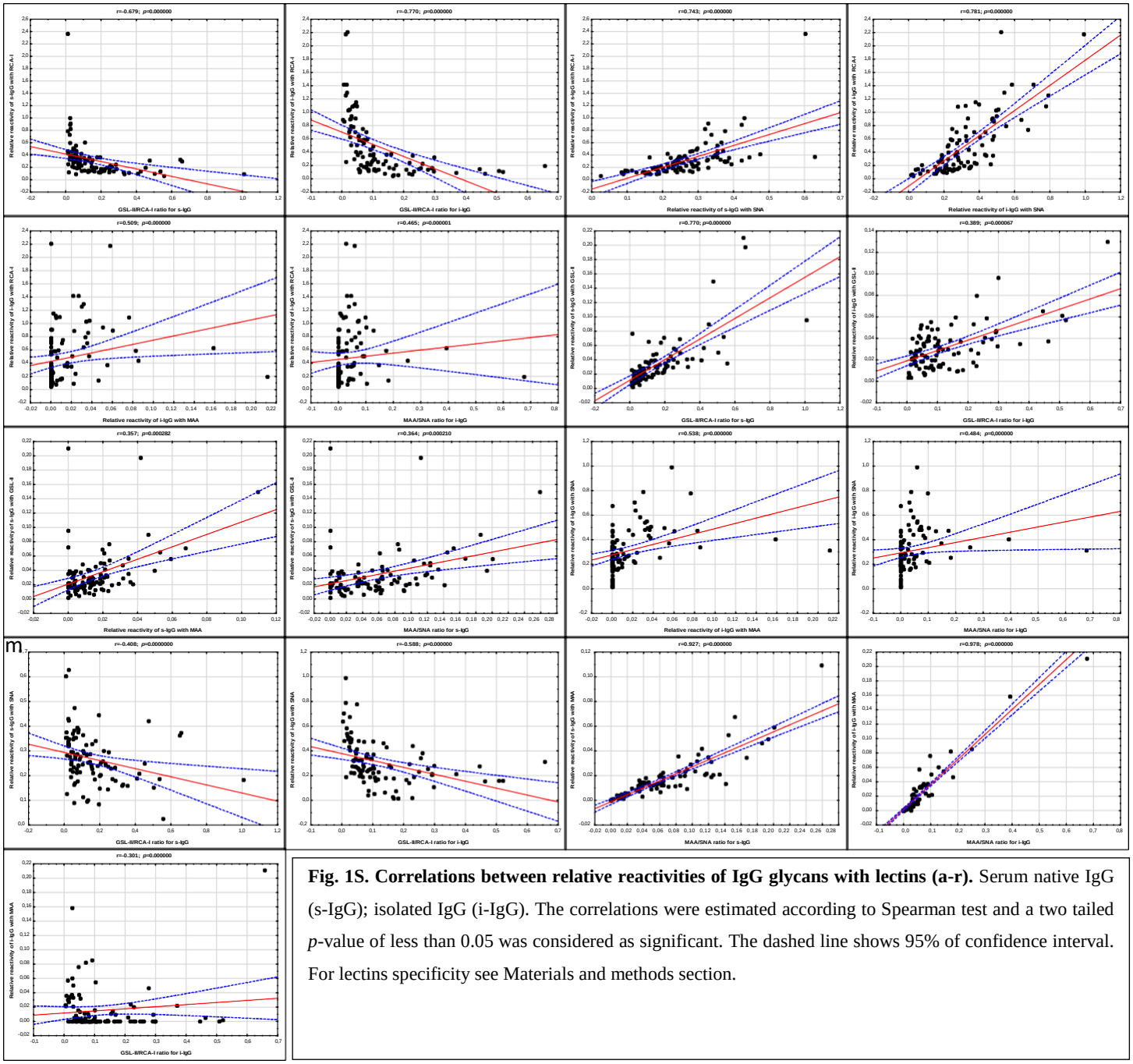

Fig. 1S. Correlations between relative reactivities of IgG glycans with lectins (a-r). Serum native IgG (s-IgG); isolated IgG (i-IgG). The correlations were estimated according to Spearman test and a two tailed *p*-value of less than 0.05 was considered as significant. The dashed line shows 95% of confidence interval. For lectins specificity see Materials and methods section.

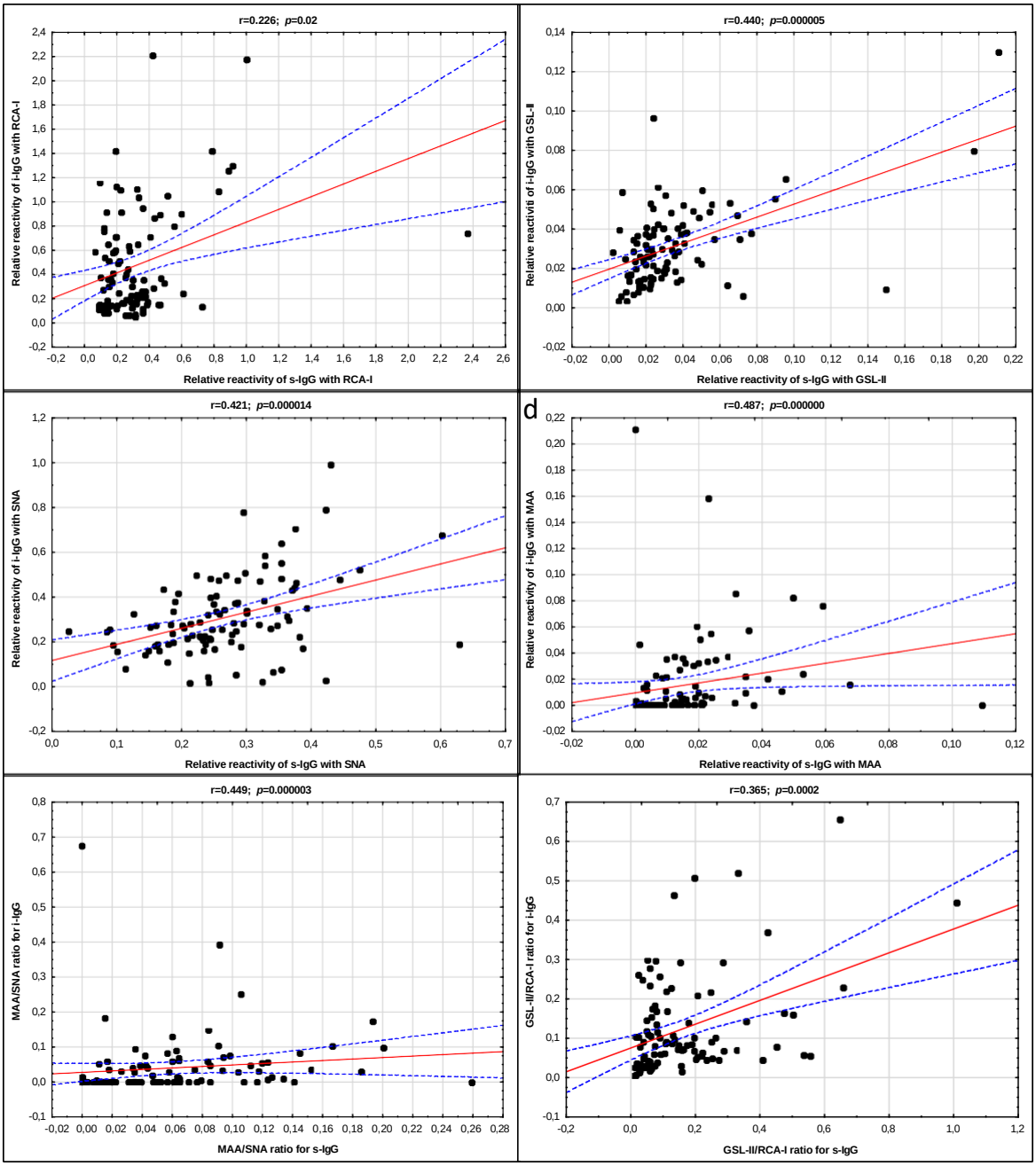

Fig. 2S. Correlations between relative reactivities of specific lectins with s-IgG and i-IgG glycans (a-f). Serum native IgG (s-IgG); isolated IgG (i-IgG). The correlations were estimated according to Spearman test and a two tailed *p*-value of less than 0.05 was considered as significant. The dashed line shows 95% of confidence interval. For lectins specificity see Materials and methods section.

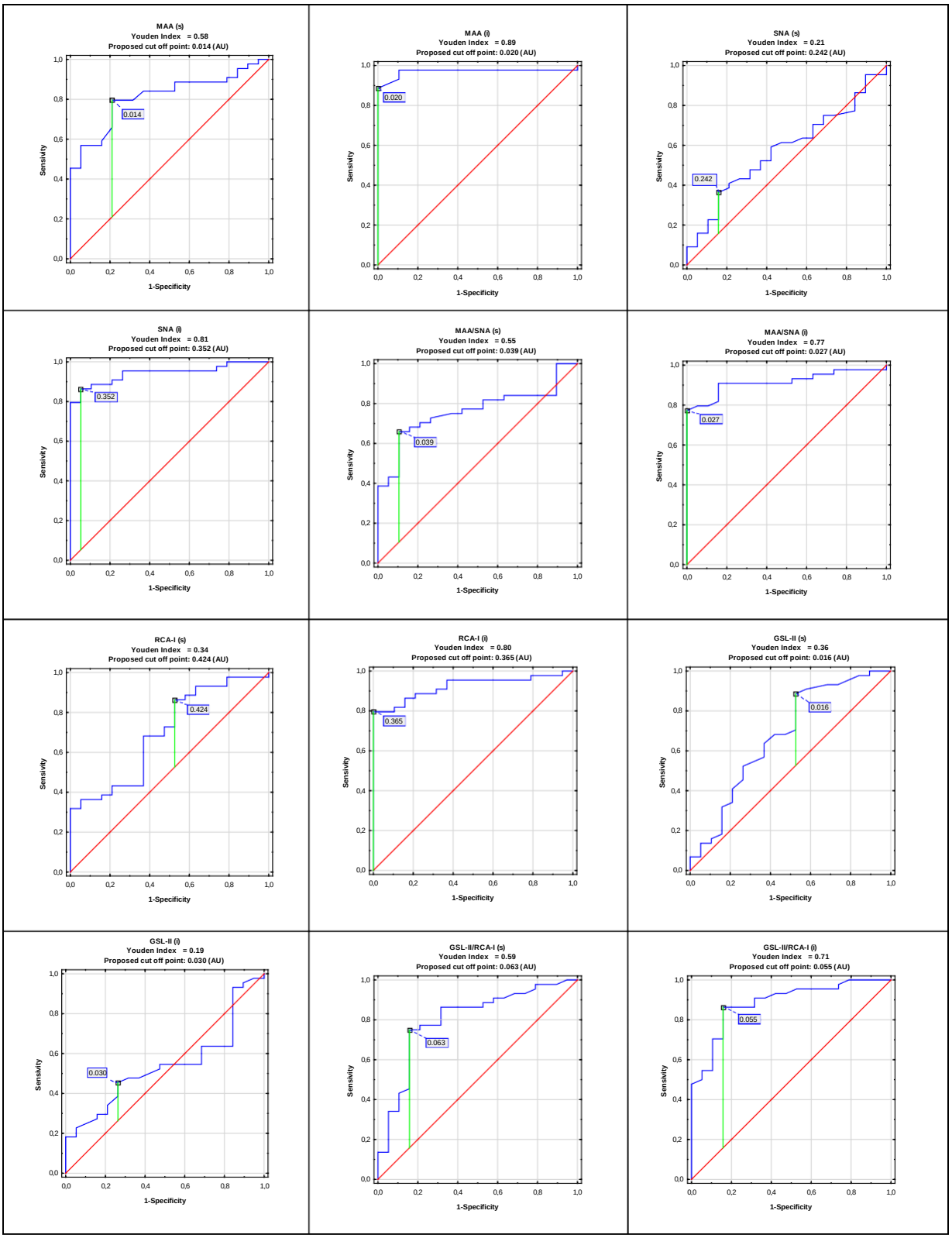

Fig. 3S. ROC curve analysis of serum native IgG (s) and isolated serum IgG (i) relative reactivity with lectins. MAA (*Maackia amurensis* agglutinin), SNA (*Sambucus nigra* agglutinin), RCA-I (*Ricinus communis* agglutinin I), GSL-II (*Griffonia simplicifolia* lectin II) and MAA/SNA ratio, GSL-II/RCA-I ratio. The analysis was done for patients with advanced endometriosis and in control group of healthy women. For lectins specificity see Materials and methods section.

Tab. 1S. ROC curve analysis of serum native IgG (s) and isolated serum IgG (i) relative reactivity with lectins

Lectins/ lectins reactivity ratio	AUC	AUC with 95% Confidence interval	Cut off point	Sensivity	Specificity	p
MAA (s) [3Sa]	**0.812**	0.705 - 0.918	0.014	0.795	0.795	0.0000
SNA (s) [3Sc]	0.575	0.428 - 0.722	0.242	0.364	0.842	0.3151
MAA/SNA (s) [3Se]	**0.762**	0.644 - 0.880	0.039	0.659	0.895	0.0000
RCA-I (s) [3Sg]	0.699	0.559- 0.838	0.424	0.864	0.474	0.0053
GSL-II (s) [3Si]	0.675	0.520 - 0.830	0.016	0.866	0.474	0.0272
GSL-II/RCA-I (s) [3Sk]	**0.800**	0.676 - 0.924	0.063	0.750	0.842	0.0000
MAA (i) [3Sb]	**0.970**	0.924 -1	0.020	0.886	1.000	0.0000
SNA (i) [3Sd]	0.943	0.887 - 0.998	0.352	0.864	0.947	0.0000
MAA/SNA (i) [3Sf]	**0.916**	0.847 - 0.986	0.027	0.773	1.000	0.0000
RCA-I (i) [3Sh]	0.922	0.856 - 0.988	0.365	0.795	1.000	0.0000
GSL-II (i) [3Sj]	0.539	0.393 - 0.686	0.030	0.455	0.737	0.5976
GSL-II/RCA-I (i) [3Sl]	**0.886**	0.801 - 0.972	0.055	0.864	0.842	0.0000

MAA (*Maackia amurensis* agglutinin), SNA (*Sambucus nigra* agglutinin), RCA-I (*Ricinus communis* agglutinin I), GSL-II (*Griffonia simplicifolia* lectin II) and MAA/SNA ratio, GSL-II/RCA-I ratio in advanced endometriosis and control group of healthy women. The analysis was done for patients with advanced endometriosis and in control group of healthy women. For lectins specificity see Materials and methods section. 3Sa-3Sl – see Figure 3S.

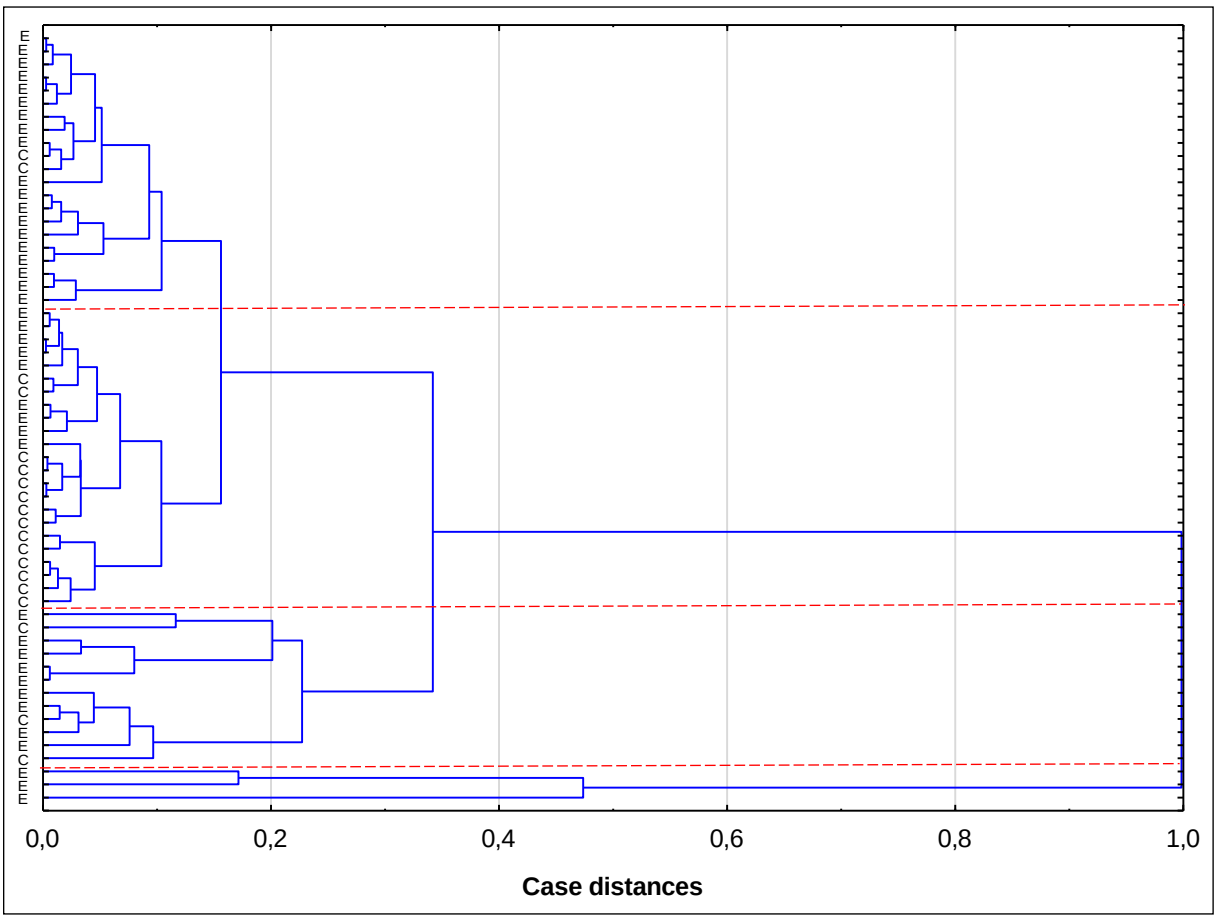

Fig. 4S. Dendrogram of cluster analysis for values of s-IgG MAA relative reactivity, MAA/SNA ratio and GSL-II/RCA-I factor. E – endometriosis, C – control group. The cluster analysis was done only for parameters for which in ROC analysis the AUC value was moderate (≥0.762). Each serum sample is represented by a vector of three features: MAA relative reactivity, MAA/SNA ratio, GSL-II/RCA-I factor.

Tab. 2S. Results of cluster analysis for values of s-IgG MAA relative reactivity, MAA/SNA ratio and GSL-II/RCA-I factor

Group / **Cluster No**	**C N=19**	**E N=40**
	The number (percentage participation in whole group)	
4	**2** (10 %)	**19** (47.5%)
3	**14** (74 %)	**9** (22.5%)
2	**3** (16%)	**9** (22.5%)
1	**0** (0%)	**3** (7.5%)

E – endometriosis, C – control group. The cluster analysis was done only for parameters for which in ROC analysis the AUC value was moderate (≥0.762). Each serum sample is represented by a vector of three features: MAA relative reactivity, MAA/SNA ratio, GSL-II/RCA-I factor.

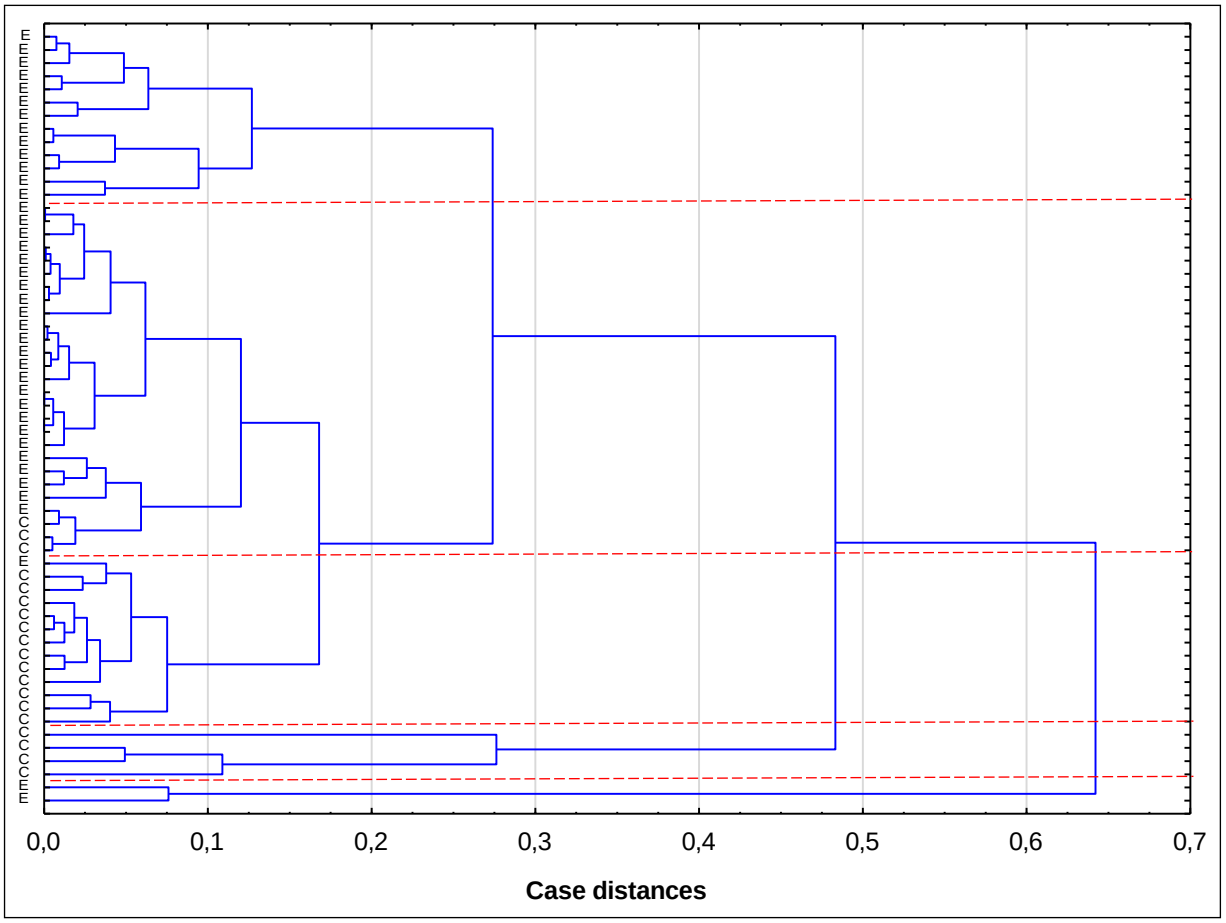

Fig. 5S. Dendrogram of cluster analysis for values of i-IgG MAA relative reactivity, MAA/SNA ratio and GSL-II/RCA-I factor. E – endometriosis, C – control group. The cluster analysis was done only for parameters for which in ROC analysis the AUC value was moderate (≥0.762). Each serum sample is represented by a vector of three features: MAA relative reactivity, MAA/SNA ratio, GSL-II/RCA-I factor.

Tab. 3S. Results of cluster analysis for values of i-IgG MAA relative reactivity, MAA/SNA ratio and GSL-II/RCA-I factor

Group / Cluster No	C N=19	E N=40
	The number (percentage participation in whole group)	
5	**12** (63%)	**1** (2.5%)
4	**3** (15%)	**24** (60%)
3	**0** (0%)	**13** (32.5%)
2	**4** (21%)	**0** (0%)
1	**0** (0%)	**2** (5%)

E – endometriosis, C – control group. The cluster analysis was done only for parameters for which in ROC analysis the AUC value was moderate (≥0.762). Each serum sample is represented by a vector of three features: MAA relative reactivity, MAA/SNA ratio, GSL-II/RCA-I factor.

7.2. Artykuł nr 2

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ORIGINAL RESEARCH

The Alterations of Serum IgG Fucosylation as a Potential Additional New Diagnostic Marker in Advanced Endometriosis

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Background: Endometriosis is an inflammatory disease leading to the growth of endometrial-like tissue outside of the uterus, which affects approximately 10% of young women of reproductive potential. The diagnosis of this disease is difficult, often invasive and time-consuming, therefore non-invasive diagnostic methods are strongly desirable in endometriosis detection. The aim of our project was to investigate whether any associations exist between the expression of serum IgG fucosylation and advanced stages of endometriosis. We were also interested in whether native serum IgG (s-IgG) fucosylation analysis, without prior IgG isolation, could provide a panel of parameters helpful in non-invasive diagnostics of advanced endometriosis.
Methods: IgG fucosylation was examined using a lectin-ELISA test with fucose-specific lectins: AAL and LCA, specific for core fucose α1,6-linked, as well as LTA and UEA which recognize α1,3- and α1,2-linked fucose, respectively.
Results: ROC curve and cluster analysis showed s-IgG reactivities with the panel of fucose-specific lectins AAL, LCA and LTA.
Conclusion: s-IgG reactivity with the panel of fucose-specific lectins AAL, LCA and LTA can be taken into account as a useful diagnostic and clinical tool to differentiate women with advanced endometriosis. Moreover, it has been shown that the analysis of native IgG fucosylation directly in serum, without prior time-consuming, expensive IgG isolation, is sufficient to distinguish advanced stages of endometriosis from a control group of healthy women.
Keywords: serum IgG, IgG fucosylation, lectin-ELISA, endometriosis

Introduction

Endometriosis, a painful, chronic inflammatory disease, is characterized by the growth of endometrial-like tissue outside of the uterus. It affects about 10% of young women of reproductive age. The symptoms of endometriosis are often nonspecific, and thus the clinical diagnosis of the disease is very difficult.[1] Laparoscopic visualization is currently the only way to definitively detect endometriosis lesions, and remains the gold standard for its diagnosis.[2–4] Endometriosis is divided into four stages based on anatomic location and disease severity: minimal, mild, moderate and severe - in accordance with the revised American Fertility Society (rAFS) classification,[5] which has been updated by the American Society for Reproductive Medicine (ASRM).[6] The etiology of endometriosis is not fully understood, and many factors may be involved in the development and progression of the disease.[7,8] The immunological aspects of endometriosis pathophysiology are thought to be related to the innate immune response, but the exact mechanisms remain unknown.[9–11] Numerous studies support

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the fact that women with endometriosis have altered immune parameters, with which may be associated decreased immuno-surveillance, reduced T-lymphocyte-mediated cytotoxicity to autologous endometrial cells, and impaired recognition of ectopic endometrial cells secondary to defective NK-cell (natural killer) activity.[12–14] Glycosylation is a common process of the post-translational modification of proteins, including antibodies, and produces a variety of glycan structures that encode a variety of information. Even slight modification of glycans by the addition or removal of a single sugar can dramatically alter a protein or antibody's structure and function, as well as interactions with the specific receptors that modulate biological responses. Immunoglobulin G (IgG) is involved in the recognition, neutralization and elimination of toxic antigens or pathogens, and is represented in the highest percentage from all immunoglobulins in blood plasma.[15] IgG molecule consists of two heavy and two light chains that together form two portions of antigen-binding Fab fragments and one portion of crystallizable constant fragment, Fc. Two N-glycans are linked to the heavy chains at Asn297 in the CH2 domain of the protein backbone (Fc part). These Fc glycans are in part located in a cavity between the two heavy chains and influence the conformation of the protein.[16,17] IgG molecules are glycoproteins, and oligosaccharides attached to the IgG Fc region are important for antibody functionality such as binding to cellular Fc receptors and complement activation. The core moiety consists of a bi-antennary heptameric structure of mannose and N-acetylglucosamine (GlcNAc), further decorated with terminal and branching residues including galactose, sialic acid, fucose, and GlcNAc. Variation in this composition influences antibody affinity to FcγR and thus antibody effector activity.[18–20] Although considerable progress has been made in understanding the structural and functional consequences of the presence or absence of various sugar moieties in IgG Fc carbohydrates, the role of their glycosylation is not yet fully understood and defined. In addition to the oligosaccharide core, more than 95% of the bi-antennary complex type structure of the final IgG glycan carries a N-acetylglucosamine on both arms,[21,22] and 85% are fucosylated.[23] In mammals, the core fucosylation (α1,6-fucosylation) is the attachment of fucose to the innermost GlcNAc at the reducing terminus of the N-glycans by α1,6-fucosyltransferase, FUT8. Fucosylation is one of the major glycan modifications and regulates a huge variety of physiological processes, including immune response, signal transduction, and cell adhesion. The absence of core fucose in IgG1 glycans has been shown to result in increased binding affinity to FcγRIIIa and FcγRIIIb, due to glycan-glycan interactions between Asn162 found only in FcγRIII and Asn297.[20,24,25] Fucosylation also plays a key role in the commitment, differentiation, and flexibility of immune cells, and also in immune processes and diseases.[26] Fucosylated glycans participate in a variety of physiological and/or pathological processes including tissue development, cell adhesion, fertilization, angiogenesis and malignancy,[27] as well as in tumor metastasis.[28] The discovery how important role fucosylation plays in immune cell development and functional regulation significantly broadened the scope of fucosylation investigations.[26] The alterations in glycans fucosylation has been observed in variety of inflammatory conditions, among which there is rheumatoid arthritis (RA),[29–33] chronic pancreatitis,[34] Crohn's disease[35] and sclerosing cholangitis.[36] Changes in IgG fucosylation have been reported eg in neonatal alloimmune thrombocytopenia, showing significantly reduced levels of fucosylated IgG, which suggests the specific regulation of IgG fucosylation and its potential role in autoimmunity.[37] In recent years, significant progress has been made in understanding the mechanisms that regulate the activity of IgG antibodies in vivo. However, to the best of our knowledge, nothing is known about the role of serum IgG fucosylation in endometriosis etiology and disease progression, or the utility of this parameter for disease diagnostics. Therefore, the aim of our present study was to investigate whether any associations exist between the expression of serum IgG fucosylation and advanced stages of endometriosis. We were also interested in whether native serum IgG fucosylation analysis, without prior IgG isolation, may reach a panel of parameters which would be helpful in the non-invasive diagnostics of advanced endometriosis.

Materials and Methods

Blood sera were collected from women with advanced endometriosis (stage III and IV) (E; n = 40, median age 34 years, interquartile range (IQR) 30.5–40.5) and from a group of women without endometriosis (NE: non-endometriosis; n = 36, median age 39 years, IQR 33.5–42.0), at the Department of Oncological Gynecology, Wroclaw Comprehensive Cancer Center, Poland. E and NE groups underwent surgical interventions, mainly laparoscopic, and after histological verification were classified to the proper group. One day after the surgery, once the diagnosis was established, the serum for the study was obtained. Women with endometriosis were classified by the extent and severity of disease according to the revised American Fertility

Society (rAFS) classification. Blood sera from healthy women (C: control group, n = 19, median age 39 years IQR 35.0–48.0), were collected at the Department of Laboratory Diagnostics, Wroclaw Medical University. The non-endometriosis group, with severe dysplasia – CIN 3 (cervical intraepithelial neoplasia grade 3) or leiomyomas, was histologically confirmed with benign ovarian cysts. The control group consisted of healthy, premenopausal and non-pregnant women, without any symptoms or history related to endometriosis. All serum samples used in this study were collected from women on any day of the menstrual cycle. The study was conducted in agreement with the Helsinki–II-declaration and the protocol was approved by the Bioethics Human Research Committee of the Wroclaw Medical University (Permission No. KB-293/2016 and KB-719/2018). Before starting the study, all participants gave written and informed consent.

The IgG fucosylation profile and degree was determined using a modified lectin-ELISA test. The method was based on the reactivity of IgG sugar moieties with four fucose-specific biotinylated lectins (Vector Laboratories Inc., Burlingame, CA, USA): 1) *Aleuria aurantia* lectin (AAL, recognizing fucose α1,6-linked to the N-acetylglucosamine core of N-glycans and with lower affinity fucoses α1,2-, α1,3- and α1,4-linked of the outer arms,[38] 2) *Lens culinaris* agglutinin (LCA, recognizing sequences containing fucosylated tri-mannose N-glycan core sites), 3) *Lotus tetragonolobus* agglutinin (LTA, specifically reacting with fucose α1,3-linked to GlcNAc, characteristic for Lewis^x oligosaccharide structures, however, it can also slightly react with fucose typical for Lewis^a and Lewis^y structures. The expression of terminal sialic acid α2,3-linked in glycoprotein glycan structures disturb the recognition by LTA of fucose α1,3-linked.[39] The fourth lectin was *Ulex europaeus* agglutinin (UEA), specific to antennary fucoses α1,2-linked to Gal and α1,3-linked to GlcNAc (Lewis^y sugar structures), but the presence of UEA-reactive fucose α1,2-linked prevents the formation of sialyl-Lewis^x glycan structures.[40]

Lectin-ELISA

The lectin-ELISA test was performed according to the procedure previously described,[41] with slight modification. In short: the microtiter plates were incubated with 0.01 mg/mL protein G solution (Abcam, United States) in 10mM TBS pH 7.4 (2h, 37°C, and next at 4°C overnight). Next, the plates were coated with native/isolated IgG diluted with 10 mM TBS-T0.1% (pH 7.4) in the amount of 500 ng IgG in 50 µL solution per well, and incubated (3h, 37°C). Additionally, in this step, for examination of LCA and AAL reactivities, the plates bound with IgG were reduced with dithiothreitol (DTT) (70 min, 37°C). The plates were incubated for 90 min at 37°C with biotinylated lectins diluted with 10mM TBS-T0.1% as follows: AAL-1:2000, LCA-1:2000, LTA-1:100, UEA-1:250. In the next step the plates were incubated with phosphatase-labelled ExtrAvidin for 1h at room temperature, and then the phosphatase reaction was developed at 37°C with a substrate, p-nitrophenyl-phosphate. The reaction was stopped with 100 µL of 1mM NaOH per well and the absorbance was measured at 405 nm with a reference filter λ=630 nm, using Mindray-96A microplate reader (Shenzhen Mindray Bio-Medical Electronics Co, China). After each incubation step the plates were extensively washed with 10 mM TBS-T0.1%, pH 7.4. All samples were analyzed in duplicate. Blank sample background absorbance was measured for samples containing all reagents, except biological sample which was replaced by 10 mM TBS-T0.1%, pH 7.4. The relative reactivities of samples' with lectins were expressed in absorbance units (AU). IgG levels, necessary for the calculation of IgG amount to lectin-ELISA, in whole sera (s-IgG), had been previously measured using the turbidimetric method,[42] and those in IgG isolates (i–IgG) were estimated by the BCA method (described below).

IgG Isolation

Immunoglobulin G was isolated from blood serum using affinity chromatography on a Protein A/Protein G Sepharose column, according to the method described earlier by Ey et al[43] and in our previous study.[41]

Statistical Analysis

Statistical analysis was performed using the statistical software STATISTICA 13.3PL (StatSoft, Poland). Obtained results were presented as means and standard deviations (SD), and distributions of the values within the analyzed groups were presented as box-whisker plots with median and interquartile (25th-75th percentile) ranges. The values of examined parameters did not fit normal distribution according to a Shapiro–Wilk *W*-test, and thus to determine differences among the studied groups the nonparametric Mann–Whitney *U*-test was used. The correlations were estimated according to the Spearman rank test with a 95%

confidence interval. A two-tailed p-value of less than 0.05 was considered as significant. The diagnostic significance of the examined parameters was analyzed using receiver operating characteristic (ROC) curves. Additionally, cluster analysis was performed for fucose expression in s-IgG and i–IgG, only for those reactivities with lectins for which the area under the curve (AUC) values, determined in ROC analysis, were simultaneously moderate or high, and differentiated the E group from the control group of healthy women. In this analysis, the results are presented as a dendrogram, beginning from one cluster in which all subjects (patients and healthy women) are gathered. The subjects were clustered, those which were similar in terms of values of all the analyzed traits were grouped together and different ones formed a separate cluster. In brief, a greater distance of separation reflects greater differences in subject characteristics. Using an Euclidean metric on the original data points were analyzed and calculated the similarities between samples with no reference to the clinical status of the samples. The order of statistical analyses performed in the present study was based on our previous experience.[41,42]

Results

The relative reactivities of serum IgG glycans with fucose-specific biotinylated lectins are presented in Table 1 as mean absorbance values and standard deviations (SD) for each analyzed group. The distributions of IgG glycans relative reactivities with lectins used, measured for the control, E and NE groups, are shown in Figure 1.

The expression of AAL-reactive core fucose in s-IgG was significantly lower in E and NE groups (0.029 ± 0.010 AU, median 0.027 AU, $p < 0.000001$ and 0.028 ± 0.014 AU, median 0.026 AU, $p < 0.000001$, respectively) than for the control group (0.056 ± 0.015 AU, median 0.054 AU). The relative reactivities of i–IgG with AAL were significantly higher for the control group (0.140 ± 0.043 AU, median 0.126 AU) than in E and NE groups (0.055 ± 0.033 AU, median 0.045 AU, $p < 0.000001$ and 0.093 ± 0.022 AU, median 0.090 AU, $p = 0.0000026$, respectively). The relative reactivities of s-IgG with LCA were significantly higher for the E group (0.587 ± 0.124 AU, median 0.563 AU) than those measured for the NE (0.461 ± 0.109 AU, median 0.452 AU, $p = 0.000020$) and control (0.467 ± 0.093 AU, median 0.467 AU, $p = 0.000677$) groups (Table 1, Figure 1).

The relative reactivities of s-IgG and i–IgG glycans with LTA specific to fucose α1,3-linked were significantly lower in the control group (0.213 ± 0.293 AU, median 0.114 AU and 0.185 ± 0.155 AU, median 0.143 AU, respectively) than in E (0.421 ± 0.149 AU, median 0.397 AU, $p = 0.000008$ and 0.603 ± 0.347 AU, median 0.503 AU, $p < 0.000001$, respectively) and NE (0.352 ± 0.152 AU, median 0.327 AU, $p = 0.00052$ and 0.540 ± 0.280 AU, median 0.462 AU, $p < 0.000001$, respectively) groups (Table 1, Figure 1).

The relative reactivity of s-IgG with UEA reactive with fucose α1,2-linked was lower in the E and NE groups (0.048 ± 0.032 AU, median 0.042 AU and 0.055 ± 0.041 AU, median 0.045 AU, respectively) than for the control group (0.067 ± 0.041 AU, median 0.054), however, these differences were insignificant ($p > 0.05$). Relative reactivities of i–IgG with UEA were significantly higher for the control group (0.193 ± 0.097 AU, median 0.172 AU) than for the E (0.012 ± 0.015 AU, median 0.008 AU,

Table 1 Relative Reactivities of Serum Native IgG and Isolated Serum IgG Glycans with Fucose-Specific Lectins

Groups	Relative Reactivity with Lectins (AU)							
	AAL (s)	AAL (i)	LCA (s)	LCA (i)	LTA (s)	LTA (i)	UEA (s)	UEA (i)
E n=40	0.029±0.010	0.055±0.033	0.587±0.124	0.540±0.151	0.421±0.149	0.603±0.347	0.048±0.032	0.012±0.015
NE n=36	0.028±0.014	0.093±0.022 p^A<0.000001	0.461 ± 0.109 p^A=0.000020	0.524±0.110	0.352±0.152	0.540±0.280	0.055±0.041	0.016±0.010 p^A=0.004
Control n=19	0.056±0.015 p^A<0.000001 p^B<0.000001	0.140±0.043 p^A<0.000001 p^B= 0.000026	0.467±0.093 p^A=0.000677	0.903±0.105 p^A<0.000001 p^B<0.000001	0.213±0.293 p^A=0.000008 p^B=0.00052	0.185±0.155 p^A<0.000001 p^B<0.000001	0.067±0.045	0.193±0.097 p^A<0.000001 p^B<0.000001

Notes: The results are expressed in absorbance units (AU) as mean values ± standard deviation (SD). Serum IgG glycan reactivities with lectins were examined by lectin-ELISA and expressed in absorbance units (AU). For lectins specificity see Materials and methods section. Significant differences versus groups: [A]with endometriosis (E), [B]non-endometriosis (NE). Control - group of healthy women. Statistically significant differences were accepted for a p-value of less than 0.05.

Abbreviations: s-IgG, serum native IgG; i–IgG, isolated serum IgG; AAL, *Aleuria aurantia* agglutinin, LCA, *Lens culinaris* agglutinin; LTA, *Lotus tetragonolobus* agglutinin; UEA, *Ulex europaeus* agglutinin.

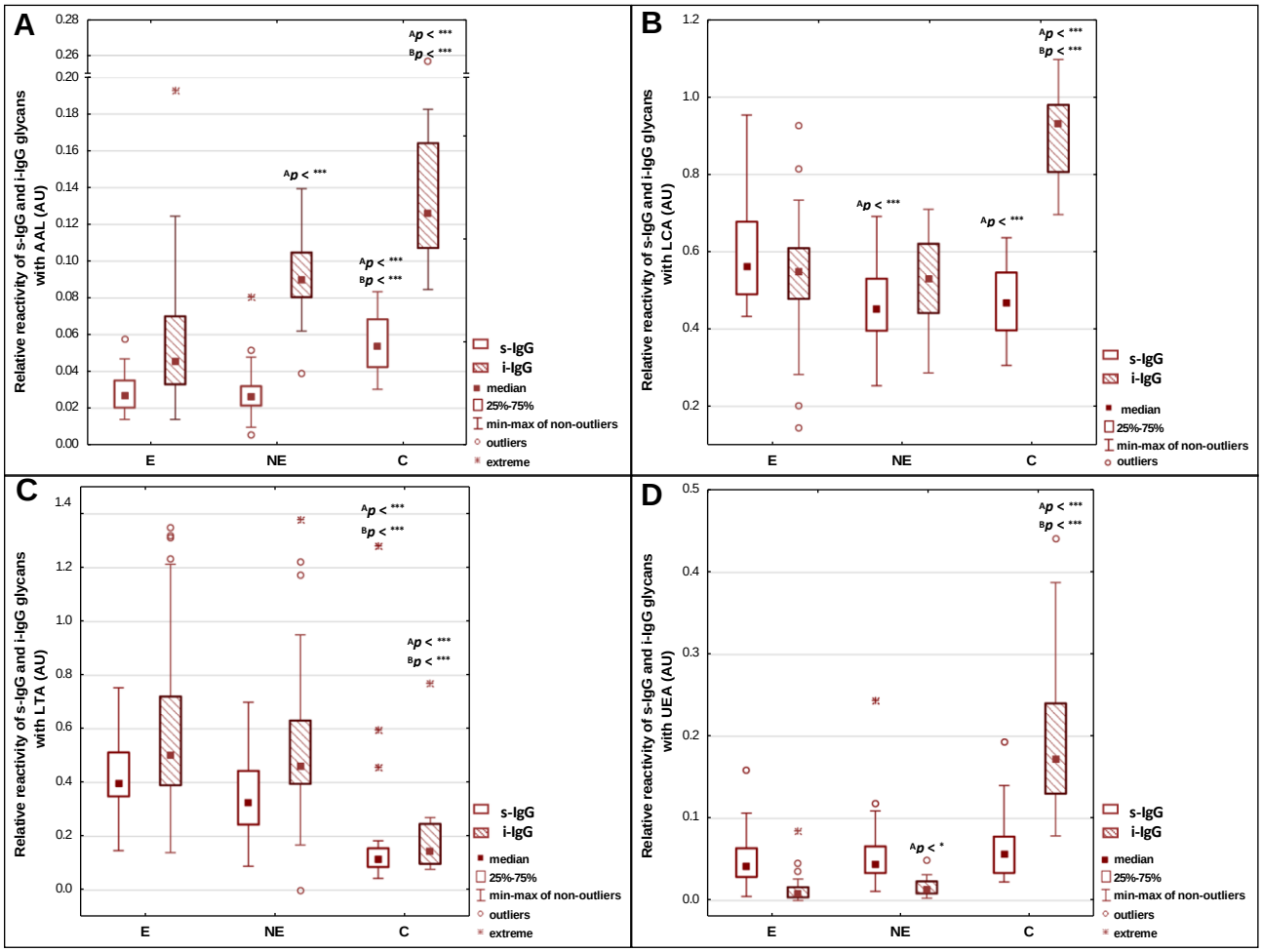

Figure 1 Relative reactivities of serum native IgG (s-IgG), and isolated serum IgG (i–IgG) glycans with fucose-specific lectins: AAL - *Aleuria aurantia* lectin, LCA - *Lens culinaris* agglutinin, LTA - *Lotus tetragonolobus* agglutinin, UEA - *Ulex europaeus* agglutinin (**A**–**D**). For lectins specificity see Materials and methods section. Significant differences versus groups: ᴬE, ᴮNE. Median is indicated as a square. A two-tailed p-value of less than 0.05 was considered as significant (*p < 0.05; ***p < 0.001).
Abbreviations: E, endometriosis; NE, non-endometriosis; C, control group of healthy women.

p < 0.000001) and NE (0.016 ± 0.010 AU, median 0.013 AU, p < 0.000001) groups (Table 1, Figure 1).

The Spearman rank test was used to analyze the correlations between relative reactivities of lectins tested with IgG glycans (s-IgG and i–IgG), and the results of the analysis are shown in Table 2 and Figure 2. A high positive correlation between the relative reactivities of AAL and LCA (r = 0.566, p < 0.000001) with glycans of i–IgG was observed. Average and weak negative correlations were also observed between the relative reactivities of s-IgG and i–IgG glycans with AAL and LTA (r = - 0.350, p = 0.0005; r = - 0.292, p = 0.004, respectively). High positive correlations exist between AAL and UEA relative reactivities with i–IgG glycans (r = 0.610, p < 0.000001). For s-IgG relative reactivities with AAL and UEA, the correlation was average and weaker than for i–IgG (r = 0.437, p = 0.000009). Average negative correlation between relative reactivities with LCA and LTA (r = - 0.352, p = 0.0004) of i–IgG glycans was observed. Positive average correlation between LCA and UEA relative reactivities (r = 0.475, p = 0.000001) with i–IgG glycans was observed. A weak negative correlation was observed between i–IgG glycans relative reactivities with LTA and UEA (r = - 0.270, p = 0.008) (Table 1).

The comparison of s-IgG and i–IgG relative reactivities with lectins used shows the presence of a high positive correlation in LTA relative reactivities examined for s-IgG and i–IgG (r = 0.583; p < 0.000001). Average positive correlations in AAL, as well as in UEA relative reactivities with IgG glycans were observed between s-IgG and i–IgG (r = 0.405; p = 0.000045 and r = 0.465; p = 0.000002, respectively). The positive correlation between relative reactivities of s-IgG and i–IgG glycans with LCA was weak (r = 0.257; p = 0.011) (Figure 2).

Table 2 The Correlations Between Relative Reactivities of IgG Glycans with Fucose-Specific Lectins

Correlations Between Lectins' Relative Reactivities	s-IgG	i–IgG
	Spearman Rank Coefficient (r)	Spearman Rank Coefficient (r)
AAL vs LCA		r=0.566 p<0.000001
AAL vs LTA	r=−0.350 p=0.0005	r=−0.292 p=0.004
AAL vs UEA	r=0.437 p=0.000009	r=0.610 p<0.000001
LCA vs LTA		r=−0.352 p=0.0004
LCA vs UEA		r=0.475 p=0.000001
LTA vs UEA		r=−0.270 p=0.008

Notes: For lectins specificity see Materials and methods section. A two-tailed p-value of less than 0.05 was considered as significant.

Abbreviations: s-IgG, serum native IgG; i–IgG, isolated serum IgG; AAL, *Aleuria aurantia* lectin; LCA, *Lens culinaris* agglutinin; LTA, *Lotus tetragonolobus* agglutinin; UEA, *Ulex europaeus* agglutinin.

ROC Curve Analysis

ROC curve analysis of relative reactivities of s-IgG glycans with fucose-specific lectins in advanced endometriosis and control groups identified parameters with a sensitivity and specificity, respectively: for AAL 0.950 and 0.737 (AUC 0.926 – high clinical value); for LCA 0.825 and 0.579 (AUC 0.775 – moderate clinical value); for LTA 0.925 and 0.842 (AUC 0.862 – moderate clinical value); for UEA 0.600 and 0.632 (AUC 0.631 – limited clinical value). For i–IgG the obtained results were the following: for AAL 0.875 and 1.0 (AUC 0.953 – high clinical value); for LCA 0.950 and 0.947 (AUC 0.978 – high clinical value); for LTA 0.825 and 0.947 (AUC 0.923 – high clinical value); for UEA 0.975 and 1.0 (AUC 0.997 – high clinical value). For the determination of cut-off points (Figure 3 and Table 3) the Youden index method was used. The clinical value of a laboratory test, based on AUC value, can be defined as: 0–0.5 – zero, 0.5–0.7 – limited, 0.7–0.9 – moderate and > 0.9 high.[44]

Cluster Analysis

The relative reactivities with AAL, LCA and LTA were selected from the panel of parameters investigated among the expression of s-IgG and i–IgG glycans used for differentiation the group of patients with endometriosis from the group of healthy women. They were taking into account the values of relative reactivities with those lectins which simultaneously meet the following criteria: they differentiate the endometriosis women from the control group of healthy women, as well as having moderate and high clinical value according to the results of ROC analysis (AUC ≥ 0.775). The analysis was performed for 59 samples, for which all three selected parameters were determined, and was conducted separately for s-IgG and i–IgG. In the case of s-IgG, the first cluster could be separated as a group of 1 control sample (Figure 4 and Table 4) at 87% distance. The next cluster, separated at 50.7% distance (Cluster 2), consists of 18 samples from which 16 samples were from women with endometriosis (40% from the whole endometriosis group). The next cluster (Cluster 3 at 46.7% distance) was separated as a homogenous group of 16 endometriosis samples (40% of the whole endometriosis group). The last cluster (Cluster 4) consists of 8 endometriosis samples and 16 samples from the control group (84% of the whole control group). For i–IgG (Figure 5 and Table 5) the first cluster was separated at distance 97.5%. This group was homogenous and consists of 9 endometriosis samples (22.5% of the whole group). The next cluster (No 2) was separated at 75% distance, in which all samples from the control group were gathered (100%). Cluster 2 and 3 (homogenous, composed of only endometriosis samples) were distinguished at distance 52%.

Discussion

While the IgG fucosylation study is not a new field in glycoimmunology, to the best of our knowledge this is the first investigation to analyse the human serum IgG fucosylation profile and degree in endometriosis using lectin-ELISA. This method, contrary to other methods used for analysis of the quantitative and qualitative composition of glycoprotein glycans, enables us to assess the interactions that occur in a living organism between monosaccharide residues of glycoprotein glycans and the corresponding ligands, which are particularly interesting and valuable in our opinion. We compared the obtained results with those for isolated serum IgG, aiming to check whether native serum IgG fucosylation analysis, without prior IgG isolation, is enough and satisfactory for the differentiation of advanced endometriosis from the control group of healthy women.

We demonstrated that for native and isolated serum IgG, the expression of AAL-reactive core fucose was

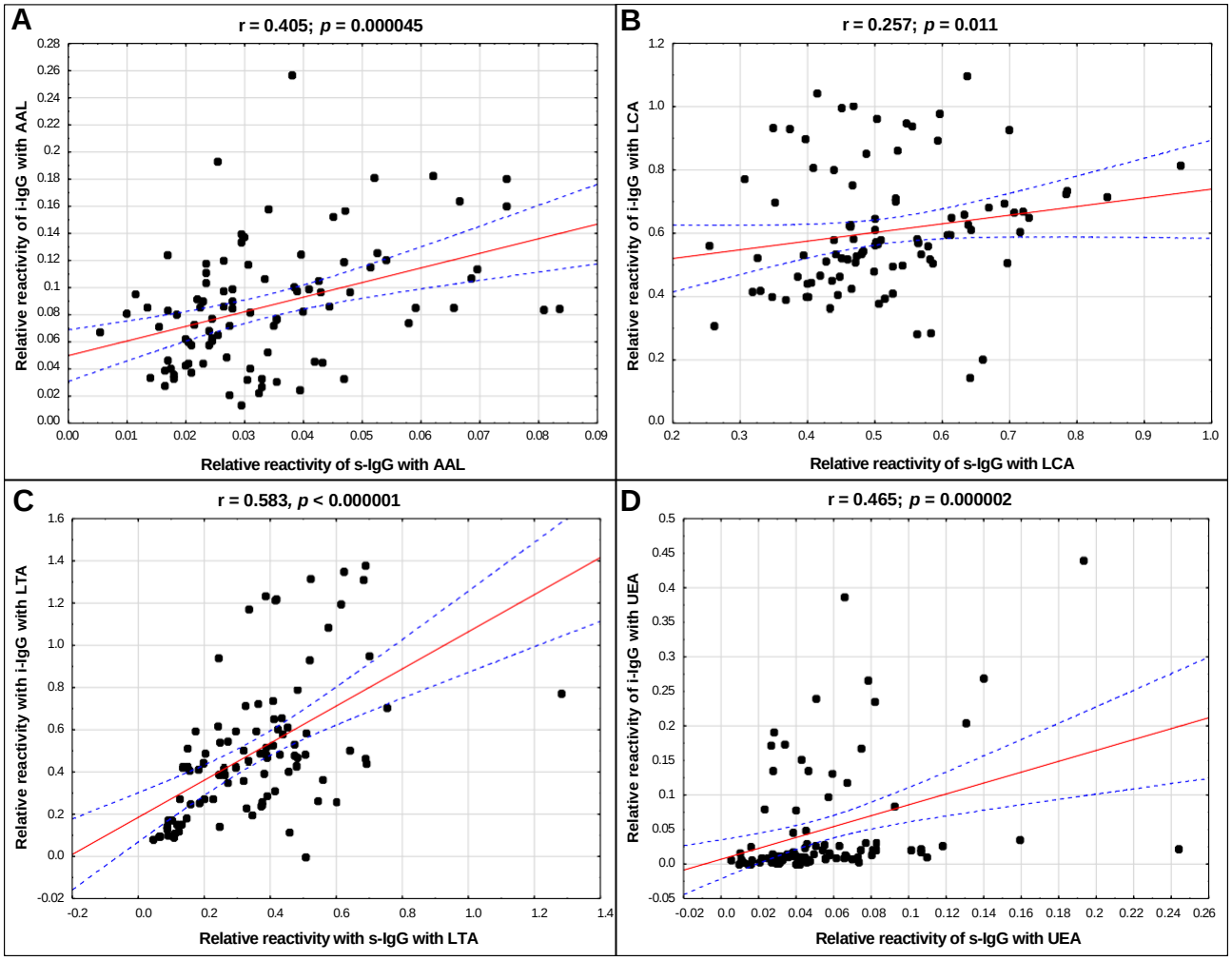

Figure 2 The correlations between relative reactivities of s-IgG and i–IgG glycans with fucose-specific lectins (**A**–**D**). For lectins' specificity see Materials and methods section. A two-tailed p-value of less than 0.05 was considered as significant.

Abbreviations: s-IgG, serum native IgG; i–IgG, isolated serum IgG; AAL, *Aleuria aurantia* lectin; LCA, *Lens culinaris* agglutinin; LTA, *Lotus tetragonolobus* agglutinin; UEA, *Ulex europaeus* agglutinin.

significantly lower in patients with non-endometriosis and advanced endometriosis when compared to the healthy women, and AAL-reactivity significantly varied between E and NE groups only for isolated IgG. In the case of the second lectin specific for core fucose, LCA, the obtained relative reactivity values were significantly different for s-IgG and i–IgG between the endometriosis group and the group of healthy women, and only for s-IgG did the expression of LCA-reactive fucose significantly differ between NE and E groups, which is particularly important considering the need to differentiate endometriosis from other gynaecological inflammatory diseases. On the other hand, only for i–IgG do the relative reactivities with LCA differentiate NE from the control group. The observed differences in AAL and LCA relative reactivities between analyzed groups of s-IgG and i–IgG may be caused by unnecessary identical specificity of both lectins, because AAL, except α1,6-linked fucose, may also detect antennary fucose α1,3-linked as a part of Lewis^x oligosaccharide structures, which could influence the obtained results. Moreover, taking into account the above information, the most plausible explanation for the observed differences in s-IgG and i–IgG reactivities with AAL and LCA may be the slightly different sugar residue availability for the lectin. Due to the fact that methods of determination based on the simplest procedures, ie without the need to carry out complicated and time-consuming isolation procedures of the glycoprotein from the biological material, are the most valuable in terms of their possible application in the diagnostics of endometriosis, and other diseases, the comparisons of reactivity with fucose-specific lectins carried out for native IgG and IgG isolated

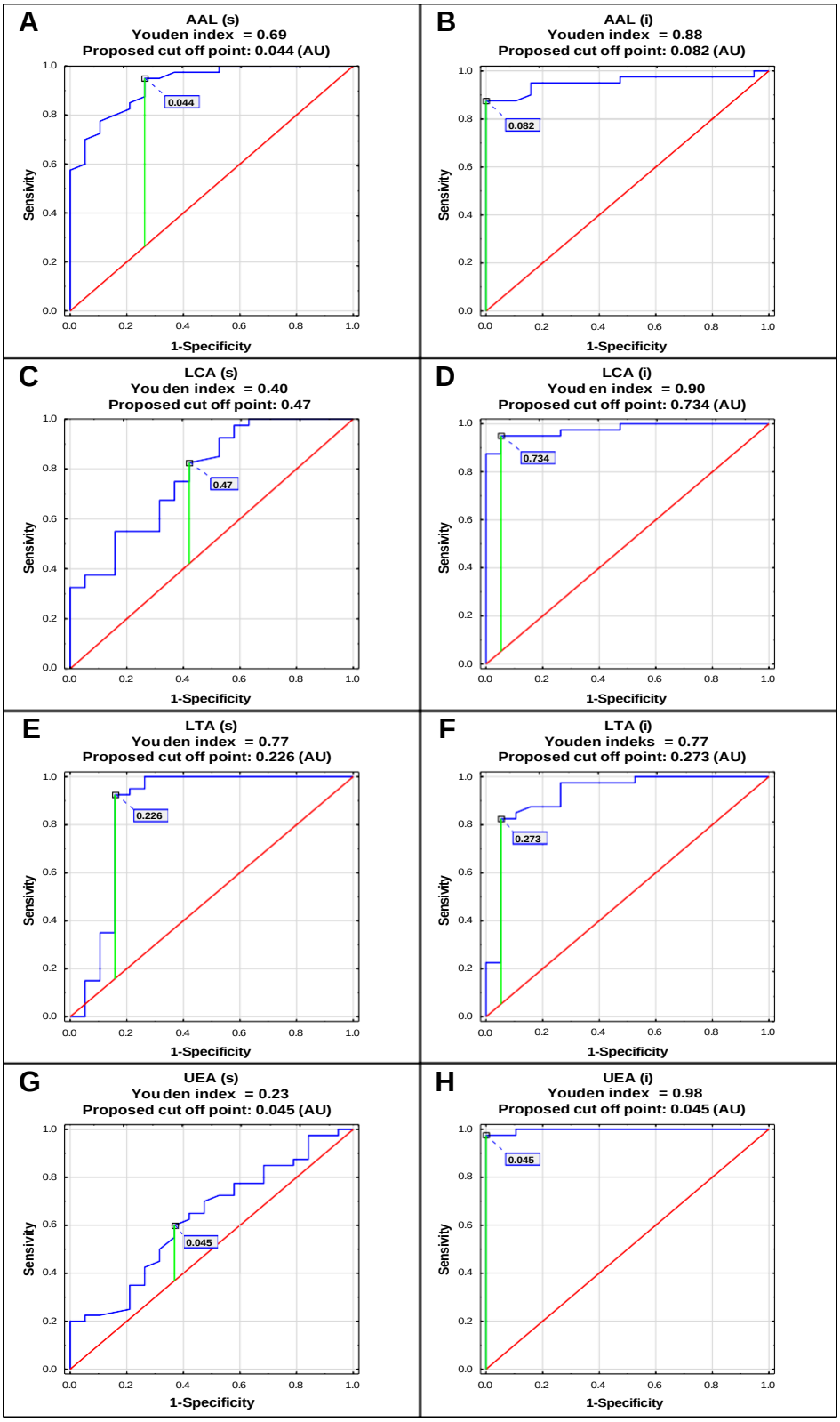

Figure 3 Receiver operating characteristic curve analysis of serum native IgG - s-IgG (s) and isolated serum IgG - i–IgG (i) relative reactivities with AAL (*Aleuria aurantia* lectin), LCA (*Lens culinaris* agglutinin), LTA (*Lotus tetragonolobus* agglutinin) and UEA (*Ulex europaeus* agglutinin) was done for women with endometriosis and healthy subjects (**A**–**H**). For lectins' specificity see Materials and methods section.

Table 3 Receiver Operating Characteristic Curve Analysis of Serum Native IgG and Isolated Serum IgG Relative Reactivities with Fucose-Specific Lectins

Reactivity with Lectin	AUC	AUC with 95% Confidence Interval	Cut-Off Point	Sensitivity	Specificity	p
s-IgG						
AAL	**0.926**	0.861–0.992	0.044	0.950	0.737	0.0000
LCA	**0.775**	0.647–0.903	0.470	0.825	0.579	0.0000
LTA	**0.862**	0.723–1	0.226	0.925	0.842	0.0000
UEA	0.631	0.480–0.782	0.045	0.600	0.632	0.0889
i–IgG						
AAL	**0.953**	0.899–1	0.082	0.875	1	0.0000
LCA	**0.978**	0.947–1	0.734	0.950	0.947	0.0000
LTA	**0.923**	0.838–1	0.273	0.825	0.947	0.0000
UEA	**0.997**	0.99–1	0.045	0.975	1	0.0000

Notes: For lectins' specificity see Materials and methods section. The analysis was done for advanced endometriosis and control group of healthy women. The values of area under the curve (AUC) ≥0.775 were bolded. AUC value can be defined as: 0–0.5 – zero, 0.5–0.7 – limited, 0.7–0.9 – moderate and > 0.9 high.
Abbreviations: s-IgG, serum native IgG; i–IgG, isolated serum IgG; AAL, *Aleuria aurantia* lectin; LCA, *Lens culinaris* agglutinin; LTA, *Lotus tetragonolobus* agglutinin; UEA, *Ulex europaeus* agglutinin.

from the sera are, in our opinion, of particular importance. The observed strong positive correlations between AAL and LCA reactivity ($r = 0.566$, $p < 0.000001$) for i–IgG only seem to confirm the hypothesis about differences in sugar moieties availability for lectins between s-IgG and i–IgG.

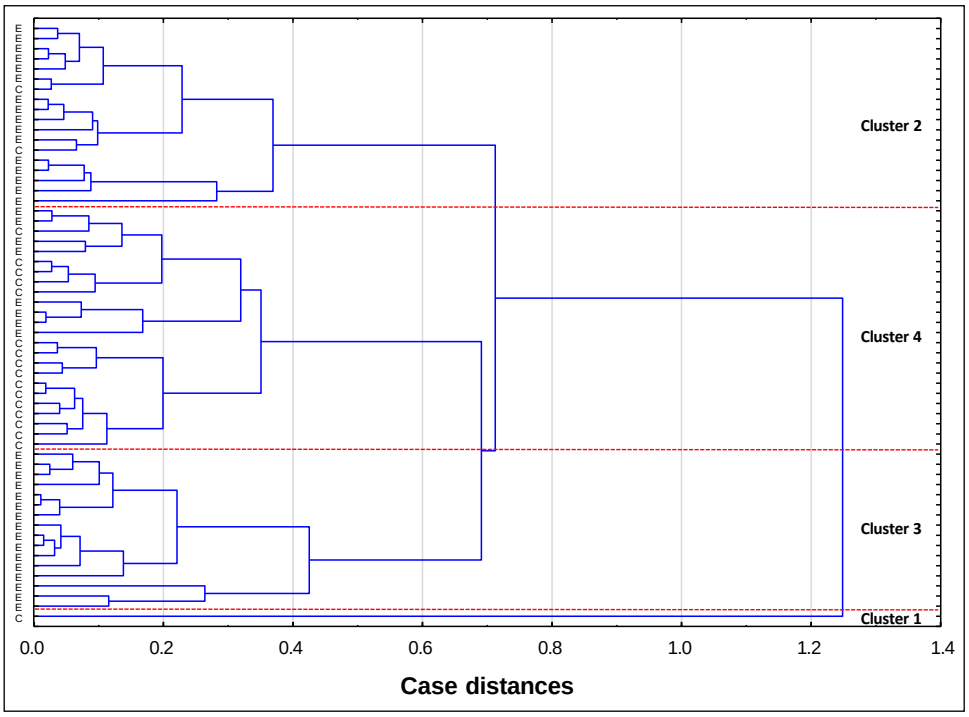

Figure 4 Dendrogram of cluster analysis for values of serum native IgG (s-IgG) relative reactivities with AAL (*Aleuria aurantia* lectin), LCA (*Lens culinaris* agglutinin) and LTA (*Lotus tetragonolobus* agglutinin). The cluster analysis was done only for parameters for which in receiver operating characteristic curve analysis the value of area under the curve was moderate or high (≥ 0.775), which significantly differentiates endometriosis patients from healthy women. Each serum sample is represented by a vector of three features: AAL, LCA and LTA relative reactivities with s-IgG.
Abbreviations: E, endometriosis; C, control group of healthy women.

Table 4 Results of Cluster Analysis for Values of Serum Native IgG Relative Reactivities with AAL, LCA and LTA

Group / Cluster No	C N=19	E N=40
	The Number of Samples (Percentage Participation in Whole Group)	
4 (n=24)	**16** (84%)	**8** (20%)
3 (n=16)	**0** (0%)	**16** (40%)
2 (n=18)	**2** (10.5%)	**16** (40%)
1 (n=1)	**1** (5.5%)	**0** (0%)

Notes: E – endometriosis, C – control group of healthy women. Cluster analysis was performed only for parameters for which in *receiver operating characteristic* curve analysis the value of area under the curve was moderate or high (≥ 0.775), and which significantly differentiated endometriosis patients from healthy women. Each serum sample is represented by a vector of three features: AAL (*Aleuria aurantia* lectin), LCA (*Lens culinaris* agglutinin) and LTA (*Lotus tetragonolobus* agglutinin) relative reactivity.

Table 5 Results of Cluster Analysis for Values of Isolated Serum IgG Relative Reactivities with AAL, LCA and LTA

Group / Cluster No.	C N=19	E N=40
	The Number of Samples (Percentage Participation in Whole Group)	
3 (n=29)	**0** (0%)	**29** (72.5%)
2 (n=21)	**19** (100%)	**2** (5%)
1 (n=9)	**0** (0%)	**9** (22.5%)

Notes: E – endometriosis, C – control group of healthy women. Cluster analysis was performed only for parameters for which in *receiver operating characteristic* curve analysis the value of area under the curve was moderate (≥ 0.775), and which significantly differentiated endometriosis patients from healthy women. Each isolated serum IgG sample is represented by a vector of three features: AAL (*Aleuria aurantia* lectin), LCA (*Lens culinaris* agglutinin) and LTA (*Lotus tetragonolobus* agglutinin) relative reactivity.

Despite the fact that endometriosis is a benign disease, accompanied by the development of inflammation,[45,46] its infiltration of peripheral organs and invasion of distant organs resembles the metastasis of malignant disease.[47–49] Fucosylation of the IgG Fc region is regulated to drive appropriate pro-inflammatory effector cell functions, and changes leading to the modification of IgG Fc glycans are key regulators of antibody activity in vivo. While Fc sialylation plays an active role in anti-inflammatory signaling and in the maturation of adaptive immune responses, fucosylation of the Fc is regulated to drive appropriate pro-inflammatory effector

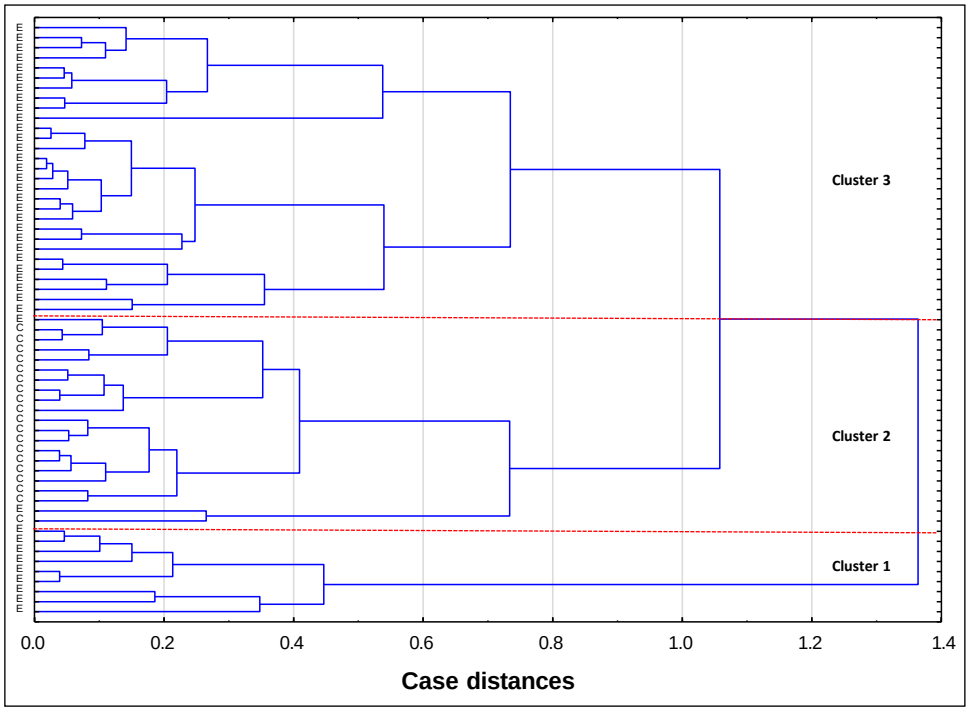

Figure 5 Dendrogram of cluster analysis for values of isolated serum IgG (i–IgG) relative reactivities with AAL (*Aleuria aurantia* lectin), LCA (*Lens culinaris* agglutinin) and LTA (*Lotus tetragonolobus* agglutinin). Cluster analysis was performed only for parameters for which in receiver operating characteristic curve analysis the value of area under the curve was higher than ≥ 0.775, and which significantly differentiated endometriosis patients from healthy women. Each serum i–IgG sample is represented by a vector of three features: AAL, LCA and LTA relative reactivity.

Abbreviations: E, endometriosis; C, control group of healthy women.

cell functions. Reduced core fucosylation is typical for inflammatory conditions and abnormal glycosylation patterns of glycoproteins are associated with variety of diseases, such as inflammation or cancer, as shown in many previous studies.[50–52] Endometriosis induces a variable amount of inflammatory reactions in the pelvic environment, depending on the stage and morphologic appearance of disease.[53,54] The inflammatory reaction associated with endometriosis has been demonstrated both in vitro and in vivo by the infiltration of immune cells and the presence of a number of primary and secondary inflammatory mediators in tissues and body fluids.[55,56] The molecular mechanism by which fucosylation affects ADCC has long remained unknown. Studies of Okazaki et al[57] showed that the afucosylated antibody binds to the FcγRIIIa receptor with greater affinity than the fucosylated version (31–21 fold). Increased affinity is achieved by engaging in additional non-covalent (enthalpy driven) interactions. Ferrara et al[20] and Mizushima et al[25] demonstrated that the fucose moiety clashes with saccharide units of the N-glycan of the receptor FcγRIIIa, thus imposing steric hindrance and thereby explaining the unfavorable interaction in the fucosylated state. This explanation is consistent with previous data showing that the N-glycan of FcγRIIIa is compatible with the afucosylated N-glycan of IgG-Fc.[58,59] In the present research we showed that the s-IgG and i–IgG glycans, except for AAL- and LCA-reactivity detecting core fucose, may also react with LTA and UEA, which may suggest the presence of α1,3-linked, and α1,2-linked antennary fucose, characteristic for Lewis^x and Lewis^y oligosaccharide structures, respectively. The relative reactivities of s-IgG and i–IgG glycans with LTA were significantly reduced in the group of healthy women in comparison to the endometriosis and non-endometriosis groups. The antennary fucoses, as part of Lewis^x/sialyl-Lewis^x and Lewis^y oligosaccharide structures, are involved in eg recognition between cells, embryo development or disease processes.[60,61] The expression of UEA-reactive glycans of s-IgG and i–IgG was very low and the significant differences between endometriosis and NE group versus control group, and between E versus NE group were observed for i–IgG only. However, as documented in many studies investigating IgG fucosylation, IgG is mainly core-fucosylated.[20,26,62] In our previous studies on IgG glycosylation we reported the presence of LTA- and UEA-reactive fucose in synovial fluid IgG glycans,[63] and for glycans of human seminal plasma IgG.[64] Flögel et al[65] and Gornic et al[66] in their study documented that serum IgG may be also decorated by UEA-reactive fucose. Zauner et al[67] reported that in IgG, fucose can be attached not only to the first GlcNAc residues in the core glycan part (core fucose) but also to the terminal Gal residues of the glycan antennas. Therefore, it was not a big surprise for us that serum IgG glycans may react also with UEA and LTA, which are specific to antennary fucose. The observed weak and strong positive correlation for s-IgG and i–IgG oligosaccharide structures, respectively, between AAL and UEA relative reactivities may suggest the simultaneous increased expression of glycans, not necessarily the same, which are decorated by core fucose as well as by UEA-reactive fucose α1,2-linked.

Based on the results of our previous study concerning sialylation of serum IgG in endometriosis[41] we also observed the presence of strong positive correlations between the specific reactivity of s-IgG and i–IgG with AAL, LCA and UEA, and their reactivity with sialo-specific lectin MAA (data not shown). The above observation is in accordance with the fact that highly sialylated IgG with a high content of fucose has a protective effect, because increased expression of fucose in IgG glycans contributes to a decrease in antibody-dependent cytotoxicity,[68–71] increases the stability of the Fc fragment of IgG[62] and, together with an increase in sialic acid expression, enhances the anti-inflammatory properties of IgG.[50-52] Comparing the lectins' relative reactivities in each of the analyzed groups for s-IgG and i–IgG glycans, they more or less differ from each other, which may be due to differences between native s-IgG and i–IgG isolated from serum in the availability of sugar residues for lectins.[41] It should be taken into account that N-glycans are present not only in the IgG Fc region. Estimates of the percentage of Fab-glycosylated IgG in healthy individuals range from ~15% to 25%, depending on the method used for glycans detection and analysis. As for the Fc region, the amounts and types of Fab glycans can vary under certain physiological and pathological conditions.[72] The observed higher level of LCA-reactive core fucose expression in i–IgG, on the contrary to s-IgG, in the group of healthy women in comparison to the E group, may be due to the fact that IgG N-glycans may be expressed and detected in Fc and Fab IgG regions as well, and the process of IgG isolation may have a positive impact on the core fucose availability for lectins, especially those which compose glycans of Fc region. This seems to be confirmed by the positive correlations observed between the relative reactivities of s-IgG and i–IgG oligosaccharides with fucose-specific lectins which, however significant, were weak, and strong only for LTA reactivity.

In our previous study[42] we reported that the values of total serum IgG concentration were significantly reduced in women with advanced stages of endometriosis in comparison to the healthy controls. We hypothesized that this phenomena may be associated with the follicular phase of the patients' menstrual cycle, as well as may be caused by the treatment of women with glucocorticoids used eg for allergies. The therapy used may eliminate the inflammatory reaction accompanying endometriosis. Unfortunately, we have no information about allergic diseases diagnosed or menstrual cycle phases in the studied groups of women.[42] As in the present study we observed that some serum samples showed extremely high IgG relative reactivity with the lectins used, we wanted to check the possible cause for these results, and we decided to analyse the concentrations of commonly known and routinely determined parameters of inflammation CA 125 and hsCRP, analyzed and discussed in our previous study.[42] We noticed that from nine samples with extremely high reactivity of IgG glycans with fucose-specific lectins, only three have CA 125 levels slightly exceeding physiological level (data not shown), and only one has a slightly increased hsCRP concentration. This additionally confirms the complexity of the processes that accompany the development of inflammation, the interrelationships of which are not fully understood, and their course may be influenced by many factors that are not always recognized. Kokot et al[42] also observed significant negative correlations between serum total IgG concentrations and the levels of CA 125 and hs-CRP. In the present study, we observed the presence of significant negative correlations between total serum IgG levels and IgG relative reactivity with LTA, specific to α1,3-linked fucose of Lewis^x oligosaccharide structures, and LCA detecting core fucose (data not shown), which indicates that decreased total serum IgG levels are accompanied by an increased degree of IgG fucosylation. On the other hand, significant positive correlations between CA 125 levels and relative reactivities of IgG glycans with LTA and LCA (data not shown) may additionally confirm that degree of IgG fucosylation is closely related to the development of inflammation.

To check the utility of the results obtained for the differentiation of s-IgG and i–IgG fucosylation in a way that reflects their clinical characteristics typical for advanced endometriosis, versus a group of healthy women, we analyzed the values of core fucose expression (AAL and LCA reactivity) and the expression of LTA- and UEA-reactive fucose, using ROC curve analysis. For s-IgG and i–IgG, the cut-off point for AAL reactivity was 0.044 AU (AUC 0.926) with sensitivity 95% and specificity 73.5%, and 0.082 AU (AUC 0.953) with sensitivity 87.5% and specificity 100%, respectively, which reflects that this parameter has a high clinical value. For LCA, the proposed cut-off point was 0.47 AU (AUC 0.775) with sensitivity 82.5% and specificity 57.9%, and 0.734 AU (AUC 0.978) with sensitivity 95% and specificity 94.7% for s-IgG and i–IgG, respectively, which proves that this parameter has moderate and high clinical value, respectively. The ROC curve analysis of LTA relative reactivity shows that for s-IgG the cut-off point was 0.226 AU (AUC 0.862, which shows a moderate clinical value) with sensitivity 92.5% and specificity 84.2%, but for i–IgG this parameter has a high clinical value and the proposed cut-off point was 0.273 AU with AUC 0.923, sensitivity 82.5% and specificity 94.7%. Although the results of the ROC analysis showed that for i-IgG, but not for s-IgG, UEA relative reactivity also has high clinical value (AUC 0.997, cut-off point 0.045 AU, sensitivity 97.5%, specificity 100%) and may be taken into account as a specific marker for endometriosis, we selected only AAL, LCA and LTA reactivity for the cluster analysis, taking into consideration only these parameters which simultaneously differentiate patients with advanced endometriosis from healthy subjects and have at least moderate clinical value, documented by ROC curve analysis. It should also be mentioned that ROC curve analysis was also performed to check the utility of s-IgG and i-IgG fucosylation determination in a way that reflects their clinical characteristics, typical for the group of women without endometriosis (NE group) but with other gynaecological diseases, versus the advanced endometriosis group and the group of healthy women, however, the results obtained showed no clinical value (data not shown). Another reason for this selection was the aim of our study, which was to check whether any changes in the profile and degree of s-IgG fucosylation could become additional diagnostic markers enabling the differentiation of women with advanced endometriosis from the control group of healthy women. Cluster analysis additionally confirmed the utility of the values of the AAL, LCA and LTA relative reactivity with IgG glycans for differentiation of advanced endometriosis patients from the group of healthy subjects. For s-IgG the fourth cluster differed with regard to the clinical characteristics of women, gathering 84% of samples from the whole group of healthy women (16 from 19) and 8 samples from the

endometriosis group (20% from the whole E group). From three clusters formed for i-IgG, cluster 2 gathered 100% of samples from the group of healthy women and only 5% of samples from women with endometriosis .

The aim of our study was to check whether the analysis of immunoglobulin G fucosylation profile and degree may be usable as markers differentiating endometriosis from the physiological state, and the results obtained in the present study seem to meet this criterion. Especially important are the findings which inform us that while for IgG isolates the differences between the E and control group were more spectacular, the IgG fucosylation analysis directly in serum, without prior IgG isolation, may also be diagnostically useful in advanced endometriosis. The lectin-ELISA method we used for this purpose, which mimics the interaction between glycan and lectin that occurs in vivo, including the availability of sugar moieties, additionally enabled us to deepen our knowledge about the molecular mechanisms of these reactions. However, we must remember that most of the lectins used do not have an absolute specificity and therefore can bind to similar carbohydrate structures with various affinities. Under unfavorable conditions, or due to the unavailability of the most preferred attachment site, lectins may bind to other structures for which they have a lower affinity.[73] Endometriosis has a profound impact on women's lives, including associated pain and infertility, which decrease the quality of their lives. For many women, the road to the diagnosis of endometriosis is long and full of obstacles, therefore any additional biomarker which can help with faster and more accurate non-invasive diagnostics of endometriosis is especially valuable, and searching for them should be a priority. An additional finding resulting from our research is the re-confirmation of one from our previous studies, that in order to be able to draw correct conclusions about the diagnostic usefulness of the parameters determined in the sera of patients with endometriosis, the control group should be composed of completely healthy women, and not non-endometriosis patients suffering from other gynaecological diseases.[41,42] Of key importance here is the inflammation accompanying both endometriosis and other female disorders, which affects the expression of many biochemical parameters, including changes in IgG glycosylation, often regardless of the disease it is associated with. The current research additionally confirms our previous observations on this topic.[41,42] Moreover, the lack of a representative group of patients with early-stage endometriosis makes it impossible the verification of analyzed in this study parameters and further evaluation whether they may be useful biomarkers the early stages development of endometriosis, which is a limitation of our study, but also a direction of future investigations.

Conclusion

Endometriosis has a profound effect on women's lives, and in many cases the path to diagnosis of endometriosis is long and full of obstacles. The relatively fast, non-invasive diagnosis of disease should be a priority, unfortunately the diagnostic process of endometriosis is difficult, expensive and often invasive, therefore the search for new patient-friendly diagnostic methods and parameters is necessary. Based on current knowledge, disease development is associated with inflammatory processes, especially in the advanced stages of endometriosis, markers of which can also be detected in peripheral blood serum. In light of the above information and the results obtained in the present study, the analysis of the profile and degree of serum IgG fucosylation may be very promising in the diagnostics of advanced endometriosis, and may be useful as an additional parameter for medical interviews and tests. The proposed panel of parameters which are the expression in IgG glycans of AAL- and LCA-reactive core fucose and α1,3-linked fucose recognized by LTA, could be taken into account as a useful clinical marker to diagnose women with advanced endometriosis, however, its clinical utility in everyday practice for disease diagnostics needs to be evaluated in further studies. It would also be interesting to check whether the determinations of the parameters we selected could be helpful in the diagnosis of the early stages of endometriosis, enabling the detection of the disease at an early stage of its development, which would be particularly valuable from the patients' point of view.

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 the Wroclaw Medical University (Permission No. KB-293/2016 and KB-719/2018). Written informed consent was obtained from recruited patients.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Disclosure

Authors declare no conflict of interests.

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7.3. Artykuł nr 3

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O-Glycosylation Changes in Serum Immunoglobulin G Are Associated with Inflammation Development in Advanced Endometriosis

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Abstract: Endometriosis is a gynecological disease, the pathogenesis of which seems to be directly related to inflammatory processes with an immune basis. Our study aimed to analyze the O-glycosylation of native serum IgG and IgG isolated from sera of women with advanced endometriosis, without endometriosis but with benign gynecological diseases, and from a control group of healthy women, in the context of its utility for differentiation of advanced endometriosis from the other two groups of women studied. For the analysis of serum IgG O-glycosylation and the expression of multi-antennary N-glycans, lectin-ELISA with lectins specific to O-glycans (MPL, VVL, and Jacalin) and highly branched N-glycans (PHA-L) was used. The relative reactivities of isolated serum IgG O-linked glycans with specific lectins as well as the MPL/VVL O-glycosylation ratio were significantly higher in patients with advanced endometriosis and those with other gynecological diseases when compared to the control group of healthy women. We also showed significantly higher expression of PHA-L-reactive multi-antennary N-glycans in isolated IgG in the advanced endometriosis and the non-endometriosis groups in comparison to the control group. Additionally, significantly higher expression of Jacalin-reactive O-glycans in isolated IgG was observed in the non-endometriosis than in the advanced endometriosis group. The results of the ROC curve and cluster analysis additionally confirmed that the lectin-based analysis of isolated serum IgG O-glycosylation and the expression of highly branched N-glycans may help distinguish women with advanced endometriosis from healthy women. Moreover, the analysis of the expression of Jacalin-reactive i-IgG O-glycans may be helpful in differentiation between women with advanced endometriosis and patients with other gynecological diseases with an inflammatory background. In the case of non-endometriosis patients, the observed differences were most probably caused by increased expression of core 3 type O-glycans.

Keywords: O-glycosylation of serum IgG; multi-antennary N-glycans in IgG; lectin-ELISA; advanced endometriosis; inflammation

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1. Introduction

Endometriosis is an inflammatory disease caused by the dissemination and proliferation of the endometrial glands outside the uterine cavity. The most common sites of occurrence of ectopic endometrial tissue are the pelvic peritoneum and pelvic organs. Moreover, endometrial ectopic tissue may also occur in organs and tissues far outside of the

pelvis. The incidence of endometriosis has been estimated to affect about 10% of women of reproductive age [1].

Despite many studies, there is still a lack of non-invasive diagnostic markers of endometriosis that could indicate the development of the disease and facilitate its diagnosis and treatment. Although chronic inflammation and high estrogen concentrations are well-established characteristics of endometriosis, the etiology of this disease remains unclear. One of the theories that could explain the development of the disease is that an alteration in the immune system in terms of immune-cell recruitment, cell adhesion, and upregulation of inflammatory processes can facilitate the implantation and survival of endometriotic lesions [2,3].

Immunoglobulins (Igs) are glycoproteins secreted by lymphocytes B during an adaptive immune response. Their characteristic glycosylation patterns may differ in number, type, and location of oligosaccharides within each Ig isotype and subclass. Sugars play specific structural roles by maintaining and modulating Igs effector functions. Abnormal glycosylation may contribute to the development of many diseases. Among immunoglobulins, immunoglobulin G (IgG) is one of the most abundant proteins found in the blood serum of healthy subjects, which accounts for approximately 10–20% of all blood serum proteins [4].

Glycosylation, an enzymatic process catalyzed by a variety of glycosyltransferases and glycosidases, is a post-translational modification of a protein that links saccharides with proteins and may be regulated by a range of B cell stimuli, including environmental factors, such as stress, age or disease [5]. This versatile posttranslational modification influences proteins' biological activity, their conformation, and the biological behavior of cells, including adhesion, molecule trafficking and clearance, receptor activation, signal transduction, endocytosis, and the interaction between a cell and its environment [6–8], including immunological and infectious disorders [7,9,10].

N-glycosylation is the most known and well-described type of glycosylation, observed in all human IgG subclasses, where the carbohydrate groups are attached to asparagine 297 (Asn 297) in the IgG CH2 domain. The N-glycans at this site can influence antibody stability [11], binding to FcγRs and complement [12], consequently modulating effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [13–16]. Structurally, human IgG N-linked glycans are typically biantennary complexes, but about 10% of endogenous human serum IgG glycoforms have intersecting GlcNAc residues. The presence of an additional GlcNAc residue increases the binding affinity to FcγRIIIa, resulting in a 10–30-fold higher ADCC activity [17]. The second N-glycosylation site is found in the VH and VL (heavy and light chain of variable regions, respectively) and has been observed in 15–25% of all serum IgG. The presence of glycans in the IgG Fab region may contribute to higher antibody stability [18] and modulate antigen binding. Human IgG3 activates complement and FcγR-mediated functions more effectively than any other immunoglobulin G subclasses [19,20]. Moreover, for IgG3, apart from N-glycans present in the Fab and Fc regions, the presence of O-linked glycans in the hinge region was also observed.

The O-GalNAc glycosylation of proteins is a multi-step process in which monosaccharides are sequentially added to a growing oligosaccharide chain, which results in the formation of a core glycan. Next, the core glycan can be branched, elongated, and finally capped with terminal glycans. The simplest O-glycan is composed of GalNAc residue linked to a serine or threonine, or less frequently to a tyrosine. The initial GalNAc residue can be elongated and branched to create eight distinct O-glycan core structures, of which cores 1, 2, 3 and 4 are the most common [21]. The core 1 structure is found on all cells and abundant on circulatory glycoproteins produced by the liver [21]. The distribution of core 2, core 3, and core 4 structures is more selective, and they are predominantly found on secreted and membrane-bound proteins from epithelial cells covering external and internal surfaces [22]. The major O-glycan cores are often elongated with longer and sometimes more complex structures shared among the different types of carbohydrates,

such as e.g., N-linked glycans. Elongation primarily involves N-acetyllactosamine (LacNAc type 2 chain Galβ1-4GlcNAc), often as repeating disaccharides (PolyLacNAc) and branches (Galβ1-4GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Galβ1-4GlcNAc), although elongation with type 1 chains (Galβ1–3GlcNAc) is also possible. The O-glycan structures are finally terminated by the addition of sialic acids, fucose, or blood type antigens (ABO and Lewis structures), which protect proteins from degradation and immune recognition, or in other situations serve as important recognition motifs for carbohydrate-binding proteins involved in some biological functions such as extravasation of immune cells to sites of inflammation [23].

In blood serum, about 10% of IgG3 polyclonal antibodies and about 13% of IgG3 monoclonal antibodies are considered to contain O-glycans. Each IgG3 molecule can contain up to three O-glycans linked to threonine residues in the triple repeat regions within the hinge region [24]. The long hinge region of IgG3 has a high degree of surface availability, which may facilitate access and detection of the O-glycans present in this region [25]. Although the function of IgG O-glycosylation is still not fully understood, the structure of the hinge region is hypothesized to be able to protect the immunoglobulin from proteolytic cleavage, and may also help maintain the extended conformation and flexibility of IgG3 [24].

O-glycosylation, like N-glycosylation, is a post-translational modification that occurs after protein synthesis and consists in attaching a sugar molecule to the oxygen atom of serine (Ser) or threonine (Thr) residues of the polypeptide chain [26,27]. Mucin-type (GalNAc type) glycosylation is the best-known type of protein O-glycosylation because of its high abundance in mucins. It is a diverse form of post-translational modification, can occur in any protein, and is initiated by the family of up to 20 GalNAc polypeptide transferases that decorate proteins with GalNAc residues (GalNAcα1-O-Ser/Thr, named Tn-antigen) [21,28]. Tn-antigen is the initial step in the O-glycosylation pathway and can be further elongated in three different ways: (1) by addition of α2,6 sialic acid (formation of sialyl-Tn-antigen), (2) by addition of galactose and formation of the oligosaccharide structure Galβ1,3GalNAcα-Ser/Thr, called T-antigen (core 1), or (3) by addition of galactose to N-acetylglucosamine (core 3) [21]. Changes in immunoglobulin glycosylation, especially in its degree, have been associated with many pathological processes, including disorders in cell adhesion, tissue development, angiogenesis, fertilization, malignancy, and tumor metastasis, as well as autoimmune diseases [9,21,29–38].

The first goal of our research was to check whether O-glycans are expressed in serum IgG in advanced endometriosis. The present study also aimed to investigate whether the profile and degree of serum IgG O-glycosylation (both for isolated serum IgG, i-IgG, and native serum IgG, s-IgG) is characteristic of an advanced stage of endometriosis and could become a diagnostic marker supporting the diagnosis of this disease, also allowing for the differentiation of advanced endometriosis from other gynecological diseases with accompanying inflammation. IgG O-glycosylation was analyzed using a modified lectin-ELISA method, as previously described [39,40], with specific biotinylated lectins reacting primarily with complete (*Maclura pomifera* lectin and Jacalin lectin) and truncated (*Vicia villosa* lectin) O-glycans.

We were also interested in whether, additionally to the presence of biantennary N-glycans, there are also highly branched N-glycans in IgG, and if so, whether the degree of their expression is characteristic of advanced endometriosis. The analysis was performed using the lectin-ELISA test with biotinylated *Phaseolus vulgaris* leucoagglutinin, which reacts specifically with multi-antennary N-glycans. This analysis aimed to answer whether the degree of expression of multi-antennary N-glycans in serum IgG makes it possible to differentiate advanced endometriosis from other gynecological inflammatory diseases. Another aspect of our research was to compare the results of analyses obtained for IgG isolated from serum and its native form, without prior immunoglobulin isolation. We wanted to check whether the glycosylation analysis of native serum IgG, without its prior isolation, would be sufficient to answer the above questions.

2. Results

The relative reactivities of s-IgG and i-IgG glycans with lectins used are presented in Table 1 as mean absorbance values and standard deviations (SD) for each analyzed group, respectively. The values of relative IgG reactivities with used lectins and trend lines, measured for E, NE, and the control group of healthy women, are shown in Figure 1. To see whether the ratio between the complete MPL-reactive and truncated VVL-reactive IgG O-glycans (T-antigen and Tn-antigen, respectively) could be of importance in differentiating the group with advanced endometriosis from the rest of the study groups, the MPL/VVL ratio was calculated based on IgG relative reactivity with these two lectins (Table 1).

The relative reactivities of s-IgG and i-IgG glycans were presented in Table 1. The relative reactivities of MPL with s-IgG O-glycans were significantly higher in the NE group (0.169 ± 0.111 AU, median 0.136) when compared to advanced endometriosis patients (0.118 ± 0.045 AU, median 0.108 AU; $p = 0.022764$) and the control group (0.103 ± 0.042 AU, median 0.102 AU; $p = 0.005500$). The relative reactivities of s-IgG O-glycans with VVL, values of the MPL/VVL ratio, reactivities with Jacalin and reactivities of s-IgG N-glycans with PHA-L were not significantly different between the studied groups ($p > 0.05$). The Spearman rank correlations, analyzed between relative reactivities of all lectins used with s-IgG glycans, are shown in Table 2 and Figure 2. Positive moderate correlations between relative reactivities of s-IgG O-glycans with MPL vs. VVL, and MPL vs. Jacalin (r = 0.566, $p = 0.000000$; r = 0.549, $p = 0.000000$, respectively) were observed.

The relative reactivities of i-IgG O-glycans with MPL in the E (0.342 ± 0.160 AU, median 0.311) and NE (0.310 ± 0.115 AU, median 0.275 AU) groups were significantly higher ($p = 0.000000$; $p = 0.000000$, respectively) when compared to the healthy women (0.020 ± 0.025 AU, median 0.007 AU). Relative reactivities of i-IgG O-glycans with VVL in the E and NE groups were significantly higher (0.061 ± 0.051 AU, median 0.043 AU; $p = 0.000000$ and 0.080 ± 0.097 AU, median 0.054 AU; $p = 0.000000$, respectively) than those observed for the control group (0.001 ± 0.002 AU, median 0.000 AU). The values of the MPL/VVL ratio were significantly lower in the control group (2.750 ± 5.546, median 0.000) than in E (7.465 ± 4.439, median 5.530; $p = 0.000000$) and NE groups (5.885 ± 3.383, median 5.685; $p = 0.000317$). The relative reactivities of i-IgG O-glycans with Jacalin in E and NE groups were significantly higher (1.025 ± 0.0.094 AU, median 1.018 AU; $p = 0.000000$ and 1.103 ± 0.1138 AU, median 1.116 AU; $p = 0.000000$, respectively) than those observed for the control group (0.106 ± 0.140 AU, median 0.064 AU). Additionally, the expression of Jacalin-reactive i-IgG O-glycans was significantly lower in the NE than the E group ($p = 0.006401$). The relative reactivities of i-IgG N-glycans with PHA-L in the E (0.340 ± 0.148 AU, median 0.314) and NE (0.402 ± 0.169 AU, median 0.381 AU) groups were significantly higher ($p = 0.000000$; $p = 0.000000$, respectively) when compared to the control group, in which the absorbances obtained were near zero (0.002 ± 0.006 AU, median 0.000 AU). Moreover, strong positive correlations were observed between the relative reactivities of i-IgG O-glycans with MPL vs. VVL and MPL vs. Jacalin (r = 0.737; $p = 0.00000$ and r = 0.832; $p = 0.00000$, respectively). Additionally, moderate positive correlations were observed between the relative reactivities of i-IgG O-glycans with VVL and Jacalin (r = 0.666; $p = 0.000000$) (Table 2; Figure 2). There were significant but weak correlations between s-IgG and i-IgG in relative reactivities with lectins specific to O-glycans (Table 3).

Table 1. Relative reactivities of serum native IgG and isolated serum IgG.

	Relative Reactivity with Lectins (AU)									
Group	***MPL (s)***	***MPL (i)***	***VVL (s)***	***VVL (i)***	***MPL/VVL Ratio (s)***	***MPL/VVL Ratio (i)***	***Jacalin (s)***	***Jacalin (i)***	***PHA-L (s)***	***PHA-L (i)***
E **n = 34**	0.118 ± 0.045	0.342 ± 0.160	0.028 ± 0.031	0.061 ± 0.051	7.361 ± 5.705	7.465 ± 4.439	0.260 ± 0.076	1.025 ± 0.094	0.197 ± 0.235	0.340 ± 0.148
NE **n = 32**	0.169 ± 0.111 p^{E} = 0.022764	0.310 ± 0.115	0.047 ± 0.076	0.080 ± 0.097	6.228 ± 4.742	5.885 ± 3.383	0.244 ± 0.082	1.103 ± 0.138 p^{E} = 0.006401	0.263 ± 0.270	0.402 ± 0.169
C **n = 19**	0.103 ± 0.042 p^{NE} = 0.005500	0.020 ± 0.025 p^{E} = 0.000000 p^{NE} = 0.000000	0.029 ± 0.022	0.001 ± 0.002 p^{E} = 0.000000 p^{NE} = 0.000000	6.227 ± 6.382	2.750 ± 5.546 p^{E} = 0.000000 p^{NE} = 0.000317	0.246 ± 0.084	0.106 ± 0.140 p^{E} = 0.000000 p^{NE} = 0.000000	0.180 ± 0.123	0.002 ± 0.006 p^{E} = 0.000000 p^{NE} = 0.000000

Significant differences versus groups: E with advanced endometriosis (E), NE non-endometriosis (NE), C—control group of healthy women. s—serum native IgG, i—isolated serum IgG. For specificity of lectins see Materials and Methods section. Significant differences were accepted for a *p*-value of less than 0.05.

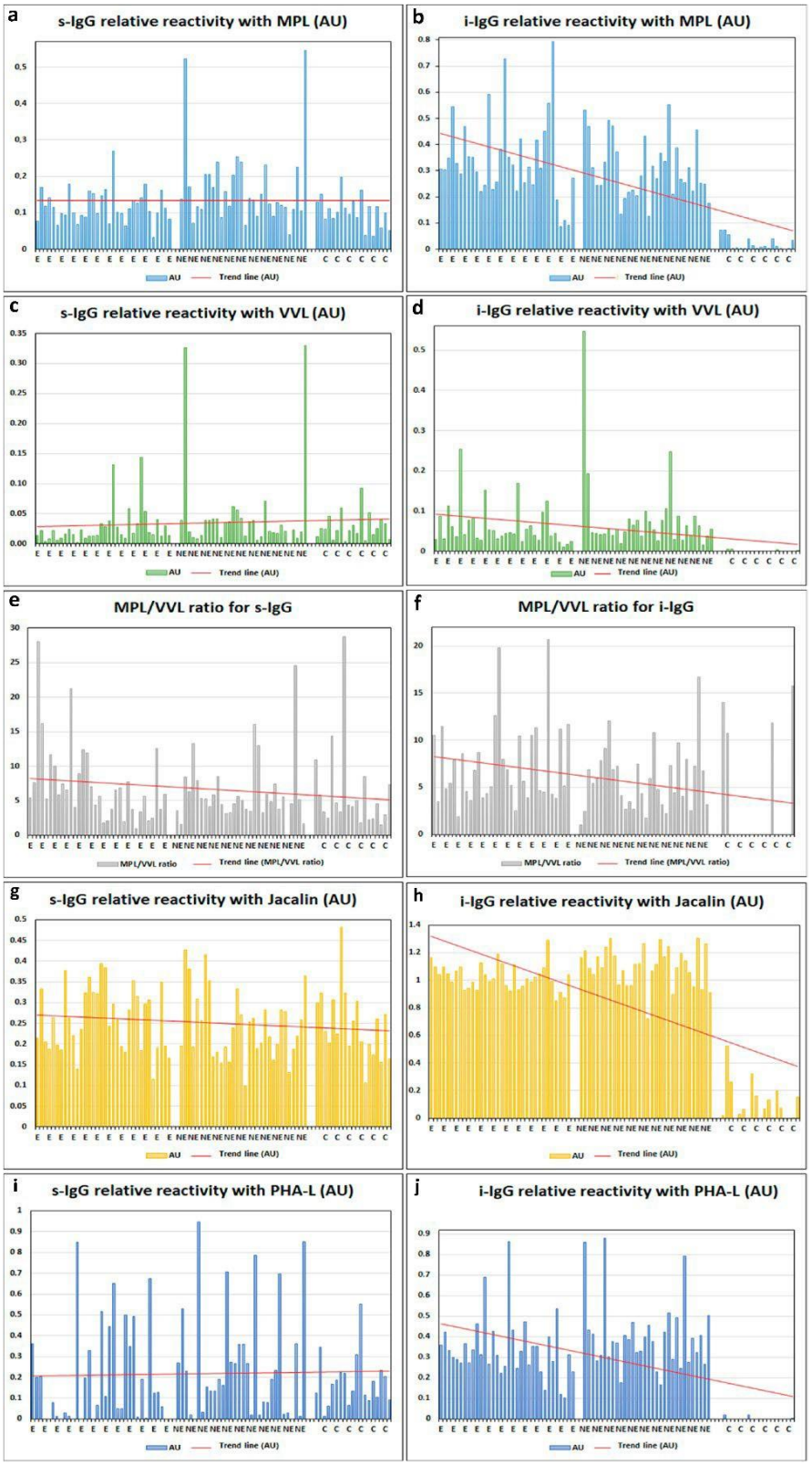


Figure 1. Relative reactivities of s-IgG and i-IgG glycans with specific lectins (**a–j**). MPL—*Maclura pomifera* lectin, VVL—*Vicia villosa* lectin, MPL/VVL—values of the ratio, Jacalin—*Artocarpus integrifolia* lectin, and PHA-L—*Phaseolus vulgaris* leucoagglutinin. For specificity of lectins see Materials and Methods section. E—advanced endometriosis, NE—non-endometriosis, and C—control group of healthy women. The trend line has been marked in red.

Table 2. Correlations between relative reactivities of s-IgG and i-IgG O-glycans with specific lectins.

	s-IgG	i-IgG
Correlations between IgG Relative Reactivities with Lectins	**Spearman Rank Coefficient (r)**	**Spearman Rank Coefficient (r)**
MPL vs. VVL	0.566 p = 0.000000	0.737 p = 0.000000
MPL vs. Jacalin	0.549 p = 0.000000	0.832 p = 0.000000
VVL vs. Jacalin	0.247 p = 0.000000	0.666 p = 0.000000

Serum native IgG—s-IgG, serum IgG isolates—i-IgG. MPL—*Maclura pomifera* lectin, VVL—*Vicia villosa* lectin, Jacalin—*Artocarpus integrifolia* lectin. A two-tailed p-value of less than 0.05 was considered significant. For specificity of lectins see Materials and Methods section.

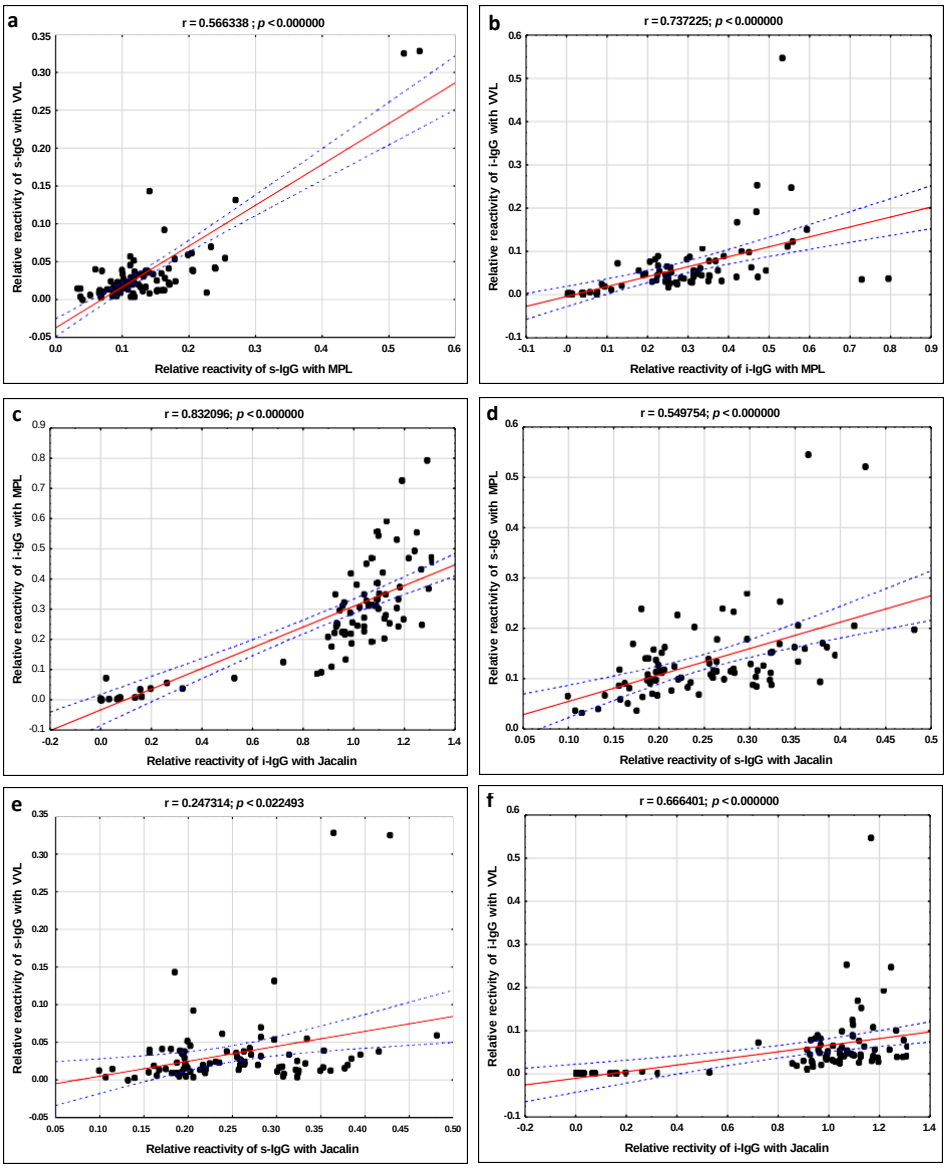


Figure 2. Correlations between relative reactivities of s-IgG and i-IgG O-glycans with specific lectins (**a**–**f**). Correlations were estimated according to a Spearman test, and a two-tailed p-value of less than 0.05 was considered significant. The 95% confidence interval is marked by dotted blue lines. MPL—*Maclura pomifera*

lectin, VVL—*Vicia villosa* lectin and Jacalin—*Artocarpus integrifolia* lectin. For specificity of lectins see Materials and Methods section.

Table 3. Correlations of relative reactivities of s-IgG and i-IgG glycans with lectins.

Correlations between Relative Reactivity of Lectins with s-IgG vs. i-IgG	Spearman Rank Coefficient (r)
MPL (s) vs. MPL (i)	0.260874 $p = 0.015890$
VVL (s) vs. VVL (i)	
MPL/VVL (s) vs. MPL/VVL (i)	0.255850 $p = 0.018109$
Jacalin (s) vs. Jacalin (i)	
PHA-L (s) vs. PHA-L (i)	0.279398 $p = 0.009610$

Serum native IgG—s-IgG, serum IgG isolates—i-IgG. MPL—Maclura pomifera lectin, VVL—Vicia villosa lectin, MPL/VVL—O-glycosylation ratio, Jacalin—Artocarpus integrifolia lectin and PHA-L—Phaseolus vulgaris leucoagglutinin. A two-tailed *p*-value of less than 0.05 was considered significant. For specificity of lectins see Materials and Methods section.

2.1. ROC Curve Analysis

ROC curve analysis of s-IgG and i-IgG glycans' relative reactivities with all four examined lectins and the values of MPL/VVL O-glycosylation ratio in advanced endometriosis and non-endometriosis patients versus a control group of healthy women identified parameters with a sensitivity and specificity shown in Figure 3, Table 4 and Figure 4, Table 5, respectively. The Youden index method was used for the determination of cut-off points. The verification of laboratory test clinical value was based on AUC value and can be defined as 0–0.5—zero, 0.5–0.7—limited, 0.7–0.9—moderate, and >0.9 high [41].

Table 4. Values of ROC curve analysis of s-IgG and i-IgG glycans' relative reactivities with lectins for women with advanced endometriosis and healthy subjects.

Lectin		AUC with 95% Confidence Interval	Cut-Off Point	Sensitivity	Specificity	*p*
			s-IgG			
MPL	0.576	0.414–0.738	0.134	0.353	0.842	0.3580
VVL	0.561	0.393–0.73	0.018	0.559	0.632	0.4771
MPL/VVL	0.611	0.479–0.744	5.265	0.647	0.684	0.1001
Jacalin	0.565	0.406–0.724	0.324	0.265	0.947	0.4242
PHA-L	0.406	0.254–0.557	0.348	0.265	0.947	0.2215
			i-IgG			
MPL	**1**	1–1	0.085	1.000	1.000	0.0000
VVL	**1**	1–1	0.01	1.000	1.000	0.0000
MPL/VVL	**0.811**	0.693–0.929	1.854	1.000	0.789	0.0000
Jacalin	**1**	1–1	0.852	1.000	1.000	0.0000
PHA-L	**1**	1–1	0.102	1.000	1.000	0.0000

MPL—*Maclura pomifera* lectin, VVL—*Vicia villosa* lectin, MPL/VVL—O-glycosylation ratio, Jacalin—*Artocarpus integrifolia* lectin and PHA-L—*Phaseolus vulgaris* leucoagglutinin. For specificity of lectins see Materials and Methods section. The analysis was performed for patients with advanced endometriosis and a control group of healthy women. Clinical utility, based on AUC value, can be defined as: 0–0.5—zero, 0.5–0.7—limited, 0.7–0.9—moderate, and >0.9—high. A two-tailed *p*-value of less than 0.05 was considered significant.

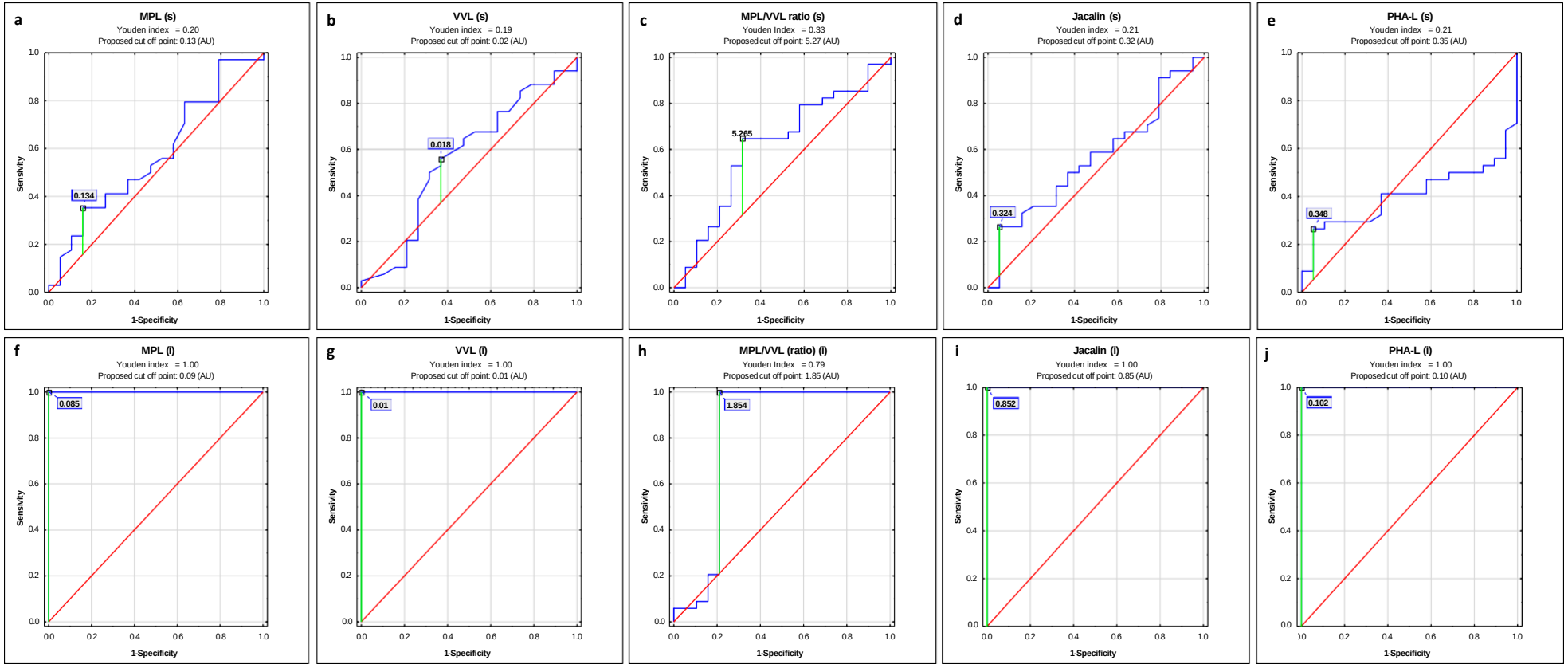


Figure 3. ROC curve analysis of s-IgG and i-IgG glycans' relative reactivities with lectins for women with advanced endometriosis and healthy subjects (**a–j**). The reference line is marked in red, the receiver operating characteristics for the parameter in blue, and the cut-off point in green, MPL—*Maclura pomifera* lectin, VVL—*Vicia villosa* lectin, MPL/VVL—O-glycosylation ratio, Jacalin—*Artocarpus integrifolia* lectin and PHA-L—*Phaseolus vulgaris* leucoagglutinin. For specificity of lectins see Materials and Methods section.

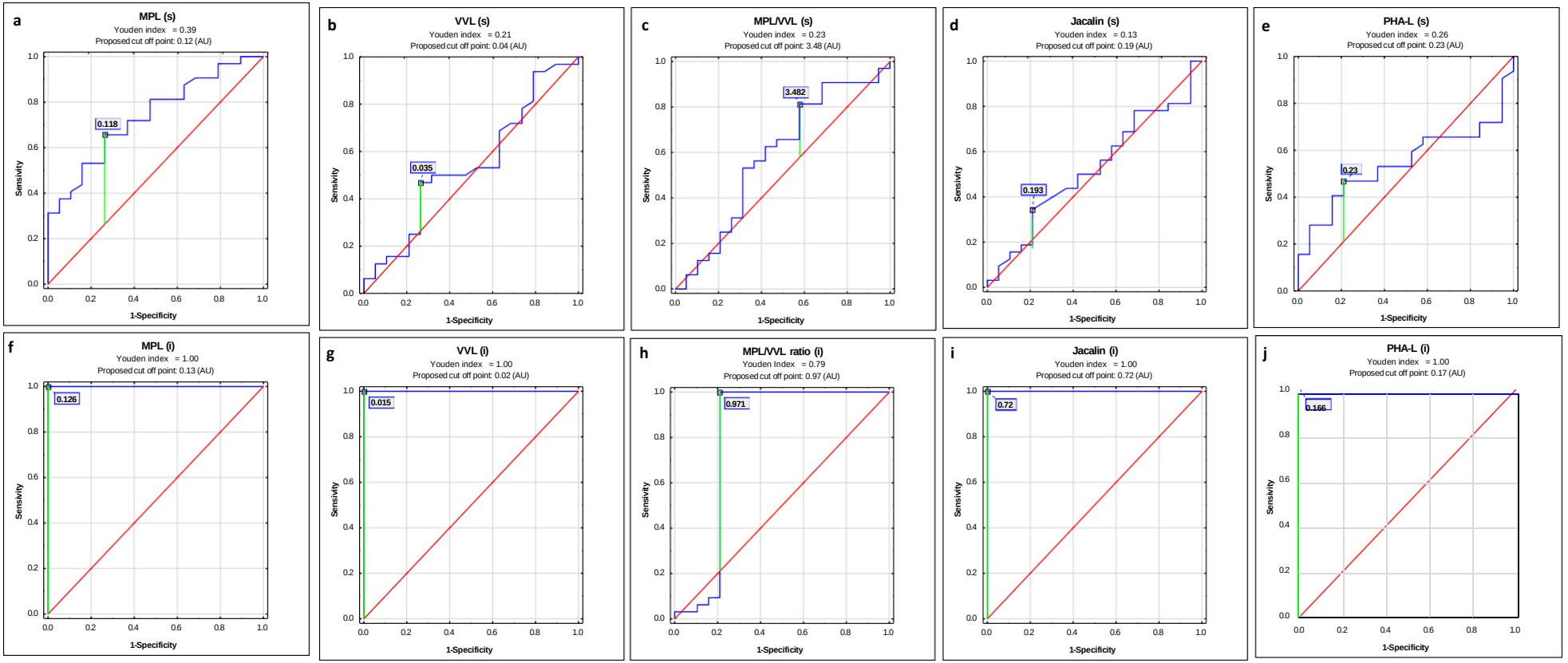


Figure 4. ROC curve analysis of s-IgG and i-IgG glycans' relative reactivities with lectins for non-endometriosis women and healthy subjects (**a–j**). The reference line is marked in red, the receiver operating characteristics for the parameter in blue, and the cut-off point in green, MPL—*Maclura pomifera* lectin, VVL—*Vicia villosa* lectin, MPL/VVL—O-glycosylation ratio, Jacalin—*Artocarpus integrifolia* lectin and PHA-L— *Phaseolus vulgaris* leucoagglutinin. For specificity of lectins see Materials and Methods section.

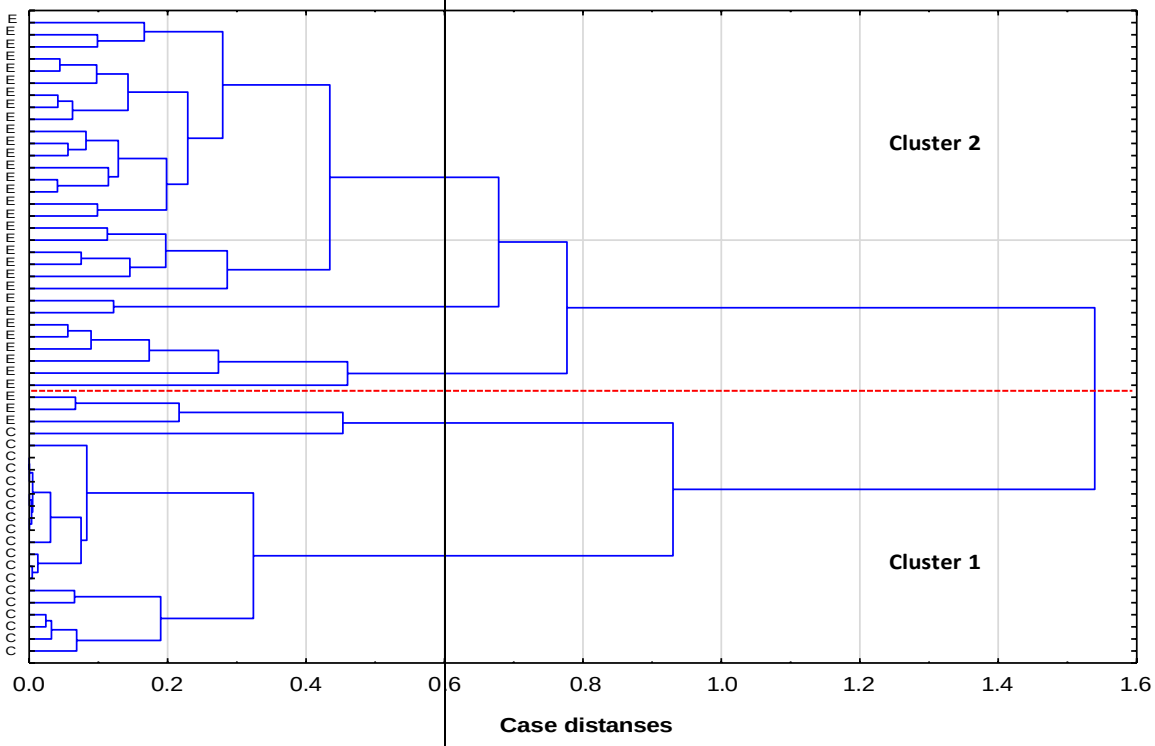
Table 5. ROC curve analysis of s-IgG and i-IgG glycans' relative reactivities with specific lectins for non-endometriosis and healthy subjects.

Lectin		AUC with 95% Confidence Interval	Cut-Off Point	Sensitivity	Specificity	*p*
s-IgG						
MPL	0.737	0.601–0.873	0.118	0.656	0.737	0.0006
VVL	0.544	0.378–0.711	0.035	0.469	0.737	0.6011
MPL/VVL	0.582	0.412–0.753	3.48	0.813	0.421	0.3450
Jacalin	0.523	0.36–0.686	0.193	0.344	0.789	0.7823
PHA-L	0.546	0.388–0.704	0.23	0.469	0.789	0.5669
i-IgG						
MPL	**1**	1–1	0.126	1.000	1.000	0.0000
VVL	**1**	1–1	0.015	1.000	1.000	0.0000
MPL/VVL	**0.801**	0.627–0.975	0.975	1.000	0.789	0.0007
Jacalin	**1**	1–1	0.72	1.000	1.000	0.0000
PHA-L	**1**	1–1	0.166	1.000	1.000	0.0000

MPL—*Maclura pomifera* lectin, VVL—*Vicia villosa* lectin, MPL/VVL—O-glycosylation ratio, Jacalin—*Artocarpus integrifolia* lectin and PHA-L—*Phaseolus vulgaris* leucoagglutinin. For specificity of lectins see Materials and Methods section. The analysis was performed for non-endometriosis and a control group of healthy women. A two-tailed *p*-value of less than 0.05 was considered significant. Clinical utility, based on AUC value, can be defined as: 0–0.5—zero, 0.5–0.7—limited, 0.7–0.9—moderate, and >0.9—high.

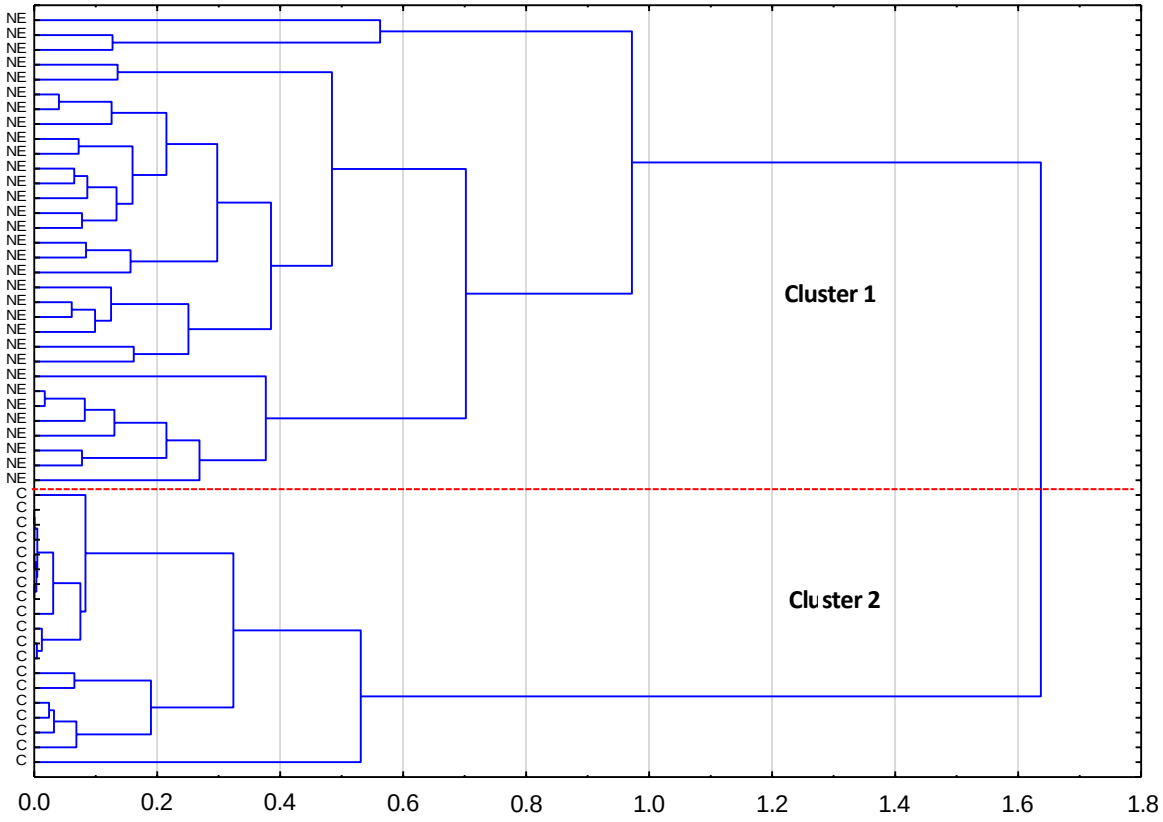
2.2. Cluster Analysis

The utility of the values of the relative reactivities of s-IgG with specific lectins for differentiation between study groups was analyzed by cluster analysis. However, due to their low clinical value, the data are not shown. The usefulness of the values of relative reactivity of i-IgG with lectins in distinguishing healthy women from patients with advanced endometriosis, as well as in differentiation between the group of healthy women and patients with non-endometriosis, was also analyzed by cluster analysis. For the analysis of the relative reactivities with the panel of lectins used, MPL, VVL, Jacalin and PHA-L meet the following criteria: they differentiate advanced endometriosis and non-endometriosis patients from the control group of healthy women as well as having high clinical value according to the results of ROC curve analysis (AUC = 1). The results obtained for a group of healthy subjects and women with advanced endometriosis are shown in Figure 5. The analysis was performed for 53 samples. The first cluster, distinguished at 53.2% distance (Cluster 1), consists of 22 samples, of which 19 were from the control group (100% of the control group) and 3 samples were from the advanced endometriosis group (4%). The next group, homogenous (Cluster 2 at 45.2% distance), was composed of advanced endometriosis samples only. Similarly, cluster analysis was performed for the non-endometriosis and control groups (Figure 6) for 51 samples. The first cluster could be separated at a distance of 55.2%. This group was homogenous and consisted of 32 non-endometriosis samples (100% of the group). Another homogenous group (Cluster 2), in which 19 samples (100%) were from the control group, could be separated at a 28% distance.



Cluster No \ Group	C n = 19	E n = 34
	The number of samples (percentage participation in whole group)	
2 (n = 30)	**0** (0%)	**30** (91%)
1 (n = 22)	**19** (100%)	**3** (9%)

Figure 5. Dendrogram of cluster analysis for relative reactivities of isolated IgG with lectins in women with advanced endometriosis and healthy subjects. Cluster analysis was performed only for parameters for which the AUC value was high (>0.9) in ROC curve analysis, and which significantly differentiated patients with advanced endometriosis from healthy women. Each sample is represented by a vector of four features: MPL, VVL, Jacalin, and PHA-L relative reactivities with i-IgG glycans. E—patients with advanced endometriosis, C—control group of healthy women. MPL—*Maclura pomifera* lectin, VVL—*Vicia villosa* lectin, Jacalin—*Artocarpus integrifolia* lectin and PHA-L—*Phaseolus vulgaris* leucoagglutinin.



Group / Cluster No	C n = 19	NE n = 32
	The number of samples (percentage participation in whole group)	
2 (n = 19)	**19** (100%)	**0** (0%)
1 (n = 32)	**0** (0%)	**32** (100%)

Figure 6. Dendrogram of cluster analysis for relative reactivities of isolated IgG with lectins in non-endometriosis women and healthy subjects. Cluster analysis was done only for parameters for which the AUC value was high (>0.9) in ROC curve analysis, and which significantly differentiated non-endometriosis patients from healthy women. Each sample is represented by a vector of four features: MPL, VVL, Jacalin, and PHA-L relative reactivities with i-IgG glycans. NE—non-endometriosis patients, C—control group of healthy women. MPL—*Maclura pomifera* lectin, VVL—*Vicia villosa* lectin, Jacalin—*Artocarpus integrifolia* lectin, and PHA-L – *Phaseolus vulgaris* leucoagglutinin.

3. Discussion

Endometriosis is considered an autoimmune disease associated with a dysfunction of natural immunity, because it fulfills most of the classification criteria for such disorders. It

is manifested by tissue damage, production of autoantibodies (against endometrium, ovary, phospholipids, and histones), and association with other autoimmune diseases [42]. In the present work, the profile and degree of O-glycosylation of human blood serum IgG were analyzed in the context of advanced endometriosis for the first time.

The first aim of our study was to check whether, additionally to biantennary N-glycans, O-glycans and highly branched N-glycans are expressed in serum IgG in advanced endometriosis. Our intention was also to check whether there are any changes in the profile and degree of O-glycans' exposition and the degree of expression of highly branched N-glycans in serum IgG, and whether the observed alterations, if present, may be used as an additional tool differentiating patients with advanced endometriosis from healthy women and patients with other gynecological diseases with accompanying inflammation. Moreover, we were also interested in whether the analysis of O-glycosylation profile and degree as well as the expression of multi-antennary N-glycans in native serum IgG (s-IgG), without prior IgG isolation, could be helpful in non-invasive diagnostics of advanced endometriosis. To our knowledge, this is the first analysis of such a type conducted in the context of advanced endometriosis diagnostics.

It should be underlined that we were first to show the presence of O-glycans in blood serum IgG in women with advanced endometriosis. The results of our study showed that the relative reactivities of isolated serum IgG O-glycans with specific lectins were significantly higher in women with advanced endometriosis and those suffering from other gynecological diseases with an inflammatory background than in the group of healthy women. It is also worthy of attention that the relative reactivity of i-IgG O-glycans with Jacalin significantly differentiated the advanced endometriosis patients from women with other gynecological diseases, which was most probably caused by increased expression of core 3 type O-glycans in the group of non-endometriosis patients. Moreover, our study also showed that the relative reactivities of serum IgG with PHA-L lectin, specific to multi-antennary N-glycans, were significantly higher in advanced endometriosis and non-endometriosis patients than in the healthy control group, indicating elevated expression of multi-branched three and tetra-antennary N-glycans in the E and NE groups. The results of cluster analysis confirmed the usefulness of the determinations of relative reactivities of i-IgG glycans with MPL, VVL, Jacalin, and PHA-L for distinguishing healthy women from women with advanced endometriosis and women without endometriosis but suffering from other gynecological diseases.

The present study has shown that the values of the MPL/VVL ratio and the relative reactivities of isolated serum IgG O-glycans with MPL and VVL, which reflect the expression of T-antigen and Tn-antigen, respectively, were significantly higher in women with advanced endometriosis than in the healthy control group. We observed a similar relationship between these groups in the relative reactivities of i-IgG O-glycans with Jacalin. Moreover, in non-endometriosis patients, the relative reactivities of i-IgG glycans with lectins specific to O-linked oligosaccharides, as well as values of MPL/VVL ratio, were significantly higher than in the control group of healthy women. Interestingly, in comparison to the group of healthy women and patients with advanced endometriosis, the presence of significantly higher relative reactivities of isolated IgG with Jacalin was observed among non-endometriosis patients. The above interrelationship is especially important from a diagnostic point of view, because we were also looking for parameters that could differentiate advanced endometriosis from other gynecological diseases. Here these parameters appear to be the degree of expression of Jacalin-reactive core 3 O-glycans in i-IgG. To confirm our observations, additional studies on a more representative number of samples in each examined groups of patients should be provided.

Jacalin is a lectin specific mainly to core 1 O-linked glycans, and binds O-glycosylated proteins. Although Jacalin's preferences for sugars that are T/Tn antigens are well understood, its specificity has not been clarified in detail [43,44]. A study by Tachibana [45] documented that apart from high Jacalin affinity for the T-antigen and Tn-antigen, this lectin also showed a significant affinity for glycans composed of core 3 oligosaccharide

structures and for sialo-T-antigen (ST), but it was unable to bind to glycans forming core 2, core 6 and sialo-Tn-antigen (STn) oligosaccharide structures. Based on the results of our previous study [36], in which we analyzed serum IgG sialylation in advanced endometriosis, we observed the presence of weak negative correlations between i-IgG glycans reactivity with Jacalin and their reactivity with sialo-specific lectins MAA and SNA (data not shown). This confirms that in the case of i-IgG, Jacalin reacts mainly with desialylated O-linked glycans, and additionally that the observed differences between non-endometriosis and advanced endometriosis groups may be caused by the expression of types of O-glycan structures other than core 1, most probably core 3, in IgG isolates. Another question is the availability of O-linked oligosaccharide structures for lectins, which, based on our observations, seems to be much greater when isolated protein is examined, especially when glycans are present in hard-to-reach places in the protein molecule.

In the case of s-IgG, the significantly higher expression of T-antigen was only observed in the non-endometriosis group when compared with advanced endometriosis patients and healthy women. However, these significances were weak. No other significant differences between examined groups were observed in relative reactivities of s-IgG O-glycans with lectins used.

Our findings indicate that in advanced endometriosis and other gynecological diseases (NE group), the expression of T- and Tn-antigens in i-IgG increases in comparison to healthy women, which is most probably caused by the accompanied inflammatory condition rather than gynecological disease. It should be also mentioned that the differences between examined groups in the expression of O-glycans when native serum IgG was analyzed are not similar to those observed for i-IgG. As we mentioned above, this may be caused by insufficient availability of O-glycans for lectins in the native protein as compared to IgG isolated from biological material, which seems to be confirmed by the observed weak correlations, or lack thereof, between the relative reactivities of s-IgG and i-IgG O-glycans with specific lectins. Due to the variety of O-glycans' functions in the human body, any changes in glycoproteins' O-glycosylation profile and degree are important, as they are associated with the development of many diseases, including tumor progression [46,47], diabetes [40], and Alzheimer's Disease [48,49]. IgG O-glycosylation, in contrast to N-glycosylation, is a poorly understood process. To date, little information is available about IgG O-glycosylation. The study provided by Plomp et al. [24] proved that there are O-glycans in the isolated serum IgG3. There are no reports on changes in IgG O-glycosylation pattern and/or degree in relation to pathological conditions. In our study on i-IgG, the expression of both T-antigen and Tn-antigen significantly increased in advanced endometriosis and non-endometriosis patients when compared with healthy women.

MPL, VVL, and Jacalin may react with complete O-glycans as well as with their truncated form. However, they have different priorities of reactivity with each of these structures. The observed significant positive correlations between relative reactivities of MPL, VVL, and Jacalin with IgG O-glycans indicate that in patients with advanced endometriosis and other gynecological diseases, together with an increase of T-antigen expression, the expression of truncated forms of O-glycans is also higher in comparison to healthy women. The above interrelationships are especially visible for IgG isolates.

Advanced endometriosis is often compared to cancer and is a known precursor to several types of ovarian cancer [50]. This could explain the significantly higher expression of PHA-L-reactive multi-antennary N-glycans in serum i-IgG in advanced endometriosis in comparison to healthy women, in whom multi-antennary N-glycans in i-IgG seem to be absent. On the other hand, in the group of non-endometriosis patients, i-IgG reactivities with PHA-L were the highest out of all three of the examined groups of women. Based on our previous examinations of serum IgG relative reactivities with sialo-specific lectins in advanced endometriosis [36], we observed the presence of weak negative correlations between i-IgG reactivities with PHA-L and its reactivities with MAA and SNA (data not shown). Taking the above observations into account, we can conclude that in advanced endometriosis and other gynecological diseases with an inflammatory background, the

observed increase in expression of multi-antennary N-glycans in serum i-IgG is accompanied by decreased sialylation. However, considering that the sialo-specific lectins we used for terminal sialic acid detection do not recognize whether sialic acid is a part of N-glycans or O-glycans, structural studies should be carried out to unambiguously define the oligosaccharide composition of IgG N- and O-glycans in inflammatory diseases, including advanced endometriosis and other gynecological diseases. It should be mentioned that in glycosylation studies with lectin-ELISA, the obtained results do not reflect the exact structure of oligosaccharides but may indicate their potential bioavailability for specific ligands. Lectins may be capable of forming less favorable bonds, and steric hindrances resulting in restricted access to the glycoprotein, especially in the native biological sample, should also be taken into account. Nevertheless, the undoubted benefit of this type of examination is that the glycosylation analysis of glycoproteins present in a native biological fluid, using lectins specific to sugar structures, reflects the sugar-ligand reaction and actual availability of the analyzed glycoepitopes in the native microenvironment, and thus also their potential for in vivo interactions [36,38,39,51]. One of our research goals was to establish whether the analysis of IgG O-glycosylation directly in the serum, without prior protein isolation, would be sufficient to differentiate the group of patients with advanced endometriosis from other study groups, and the conducted research helped us answer this question. Considering the results of the analysis of the degree of serum IgG O-glycans and highly branched N-glycans' expression presented above, the availability of oligosaccharides for the lectins used seems to be insufficient in the case of native IgG without its prior isolation. To check the usefulness of the obtained results for the clinical differentiation of all three examined groups of women, ROC curve analysis was done. In the case of s-IgG, and only for non-endometriosis patients versus a control group of healthy women, a moderate clinical value for IgG MPL-reactive O-glycans expression was demonstrated (AUC = 0.737). For the remaining parameters, the clinical value was limited. The clinical usefulness of the examined parameters for the differentiation of women with advanced endometriosis from healthy women was not demonstrated. In the case of i-IgG, we observed a maximum high clinical value (AUC = 1) for IgG relative reactivities with all four lectins used, both when comparing women with advanced endometriosis to healthy women and healthy women to a non-endometriosis group. The results of ROC curve analysis obtained for the relative reactivities of i-IgG O-glycans with Jacalin in the E and NE groups showed its limited clinical utility (AUC = 0.661; $p = 0.0106$), with a sensitivity and specificity of 0.625 and 0.694, respectively (data not shown). The conducted cluster analysis confirmed the clinical usefulness of the analysis of i-IgG glycans' relative reactivities with MPL, VVL, Jacalin, and PHA-L for the differentiation of healthy women from women with advanced endometriosis as well as women with gynecological diseases other than endometriosis. While our research has shown that both the expression of O-glycans and highly branched N-glycans in IgG may have a potential application in the diagnostics of advanced endometriosis, at the present stage of research, these conclusions mainly concern IgG isolated from serum. This makes it difficult to apply this type of determination in routine diagnostics due to the laborious and time-consuming procedure of protein isolation and purification. Nevertheless, this direction of research seems to be promising, and the development of a simple and fast protein isolation procedure would be very helpful.

4. Materials and Methods

4.1. Patient Samples

The study material was blood sera and IgG isolated from serum samples. The serum samples were derived from patients diagnosed with stages III and IV of endometriosis (E—advanced endometriosis; n = 34, mean age: 34.5 ± 7 years) and from women without endometriosis (NE—non-endometriosis; n = 32, mean age: 37.5 ± 8 years) were collected at the Department of Oncological Gynecology, Wroclaw Comprehensive Cancer Center, Poland. The study was conducted in agreement with the Helsinki II declaration and the protocol was approved by the Bioethics Human Research Committee of the Wroclaw

Medical University (Permission No. KB-293/2016 and KB-719/2018). E and NE patients underwent surgical interventions, mainly laparoscopic, and after histological verification were classified to their proper groups. Women with advanced endometriosis were classified according to extent and severity of disease according to the revised American Fertility Society (rAFS) classification. Patients from the non-endometriosis group had histologically confirmed benign ovarian cysts with severe dysplasia—CIN 3 (cervical intraepithelial neoplasia grade 3) or leiomyomas. Additionally, at the Department of Laboratory Diagnostics, Wroclaw Medical University, serum samples from healthy female volunteers were collected (C—control group; n = 19, mean age: 40.3 ± 8 years, positive opinion of Bioethics Committee No KB-117/2020). The control group of healthy women had no symptoms or history connected with endometriosis, were non-pregnant, and did not suffer from any gynecological or inflammatory diseases. Serum samples examined in this study were collected from women on any day of the menstrual cycle. Before starting the study, all participants gave written and informed consent.

4.2. IgG Isolation

Immunoglobulin G was isolated from serum samples using affinity chromatography on the Protein A/Protein G Sepharose column, according to the procedure described previously by Ey et al. [52] and Sołkiewicz et al. [36]. In short: after 1:1 dilution in 50 mM TBS, pH 8.0, the serum sample (0.5 mL) was applied to the column (1 mL) and washed with the initial TBS solution. IgG was eluted from the column with 0.1 M glycine/HCl, pH 2.7 and immediately neutralized with 1 M Tris to avoid IgG degradation. The elution profile was determined from the absorbance measurement at 280 nm. An Amicon Ultra-15 centrifuge filter with an Ultracel-100 membrane (Millipore, Merck, Germany) was used to combine and concentrate the IgG-containing fractions. IgG concentration was determined spectrophotometrically on a polystyrene 96-well microtiter plate (Maxisorp, Dako, Denmark) using the bicinchoninic acid (BCA) colorimetric micro method [53]. The IgG solution was diluted with water when necessary, and 200 mL of a 50:1 mixture of stock solutions A and B was added to 10 mL of IgG solution. In the next step, the plate was incubated for 30 min at 37 °C. Bovine serum albumin (BSA) in concentrations of 0, 2, 4, 6, 8, and 10 µg/well was used as a standard. The absorbances were measured against a blank sample at 562 nm, and a standard curve was used to read the IgG concentrations, which were expressed in µg/mL [36].

4.3. Lectin-ELISA

The profile and degree of IgG O-glycosylation were determined using the previously described method [39,40]: modified solid-phase lectin-ELISA. The expression of O-glycans was studied with biotinylated lectins: MPL (*Maclura pomifera* lectin), VVL (*Vicia villosa* lectin) and Jacalin (*Artocarpus integrifolia* lectin). Additionally, PHA-L (*Phaseolus vulgaris* leucoagglutinin), selective for tri- and/or tetra-antennary N-linked glycans, was used [54] (Vector Laboratories Inc., Burlingame, CA, USA). For specificity of lectins see Table 6.

Table 6. Specificity of the lectins used in the study.

Lectin Source	Specificity for Sugar Structures
MPL (*Maclura pomifera* lectin)	T (Galβ1,3GalNAc) and Tn antigen (single GalNAc) [55]
VVL (*Vicia villosa* lectin)	Tn antigen (single GalNAc) [56,57]
Jacalin (*Artocarpus integrifolia* lectin)	T antigen (Galβ1,3GalNAc), Tn antigen (single GalNAc), sTn antigen (NeuAcα2,6GalNAc) [45]
PHA-L (*Phaseolus vulgaris* lectin)	tri/tetra-antennary N-glycans, binds to β1,6 branches of tri- and tetra-antennary oligosaccharides [54]

Gal—galactose; GalNAc—N-Acetylgalactosamine; NeuAc—N-Acetylneuraminic Acid.

IgG concentrations in whole sera (s-IgG) and in IgG isolates (i-IgG), necessary for the calculation of IgG amount to lectin-ELISA, were determined previously using the turbidimetric method [58] as well as the BCA method [36] described above, respectively. The microtiter plates (Maxisorp, Dako, Denmark) were incubated with 0.01 mg/mL protein G (Abcam, Boston, MA, USA) solution in 10 mM TBS pH 7.4 for 2 h at 37 °C, then 4 °C overnight. The plates were then coated with native IgG diluted 10 mM TBS-T (TBS containing 0.1% Tween, pH 7.4) in an amount of 800 ng native IgG in 50 µL solution per well, and incubated for 3 h at 37 °C. For isolated IgG, the wells of microtiter plates were covered with 800 ng of isolated IgG diluted with 10 mM TBS, pH = 8.5 (total volume: 50 µL per well), and incubated for 24 h at 24 °C. After washing (TBS-T, pH = 7.5), the plates were incubated with biotinylated lectins for 60 min, 37 °C, which were diluted with 10 mM TBS-T as follows: MPL—1:1000, VVL—1:1000, Jacalin—1:5000 and PHA-L—1:250. Next, the plates were incubated with phosphatase-labeled ExtrAvidin (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at 37°C. After the incubation, the phosphatase reaction was developed with a substrate, p-nitrophenyl phosphate. The reaction was stopped with 100 µL of 1 M NaOH per well and the absorbance was read at 405 nm, reference filter 630 nm, with a Mindray-96A microplate reader (Shenzhen Mindray Bio-Medical Electronics Co., Shenzhen, China). All samples were examined in duplicate. Background absorbances were measured for samples in which all reagents were present, but the biological sample was replaced with 10 mM TBS-T. Samples relative reactivities with lectins were expressed in absorbance units (AU).

4.4. Statistical Analysis

Statistical analysis was performed with the STATISTICA 13.3PL (StatSoft Polska Sp. z o.o., Warsaw, Poland). All results are presented as mean ± SD (standard deviation), and the differences between the groups were presented as bar graphs. According to a Shapiro–Wilk W test, the values did not fit a normal distribution, thus the nonparametric Mann–Whitney U test was used to determine differences among the groups. Correlations with a 95% confidence interval between examined parameters were tested by Spearman's rank analysis. For the determination the strength of Spearman's rank correlations the following classification was used: $0.0 \leq r \leq 0.2$—lack of correlation; $0.2 < r \leq 0.4$—weak correlation; $0.4 < r \leq 0.7$—moderate correlation; $0.7 < r \leq 0.9$—strong correlation; $0.9 < r \leq 1.0$—very strong correlation. A two-tailed *p*-value of less than 0.05 was considered significant. The clinical value of determined parameters was analyzed using receiver operating characteristic (ROC) curves. Additionally, cluster analysis based on divisive hierarchical clustering was applied to estimate the diagnostic usefulness of measured parameters. For this analysis, the results are presented as a dendrogram, starting from one cluster in which all subjects (patients and controls) are gathered. In the next step, the subjects were clustered. Those with similar values of all analyzed traits were grouped together, while subjects with different ones formed a separate cluster. In summary, we observed that the greater the distance of separation, the greater were the differences in subject characteristics. Euclidean distance was used for similarity estimation. The scheme of statistical analysis of results obtained in the present study was adopted from our previous experience [36,38].

5. Conclusions

Endometriosis, due to non-specific symptoms, as well as the lack of sensitive and specific tests that would be available in routine clinical diagnostics, still remains in the group of late-diagnosed diseases. Therefore, it is extremely important to define a panel of diagnostic markers specific to this disease. However, the expression of serum IgG O-glycans is not very deeply explored. Our results indicate the presence of O-glycans in serum IgG in advanced endometriosis and other gynecological diseases, and changes in the profile and degree of serum IgG O-glycosylation between patients with gynecological diseases compared to the group of healthy women were also observed. Moreover, we have also shown that, except for the presence in IgG of typical biantennary N-glycans,

highly branched N-glycans are also expressed, mainly in advanced endometriosis and non-endometriosis groups. Significantly higher expression of multi-antennary N-glycans in blood serum IgG in women with advanced endometriosis and with other gynecological diseases compared to healthy women clearly indicates that the presence of highly branched N-glycans is linked with pathological conditions accompanied by inflammation. The results obtained in our study have allowed for the selection of parameters helpful in the diagnostics of advanced endometriosis and may be used to direct future research in this field. We believe that the results of this study will also contribute to a better understanding of the molecular mechanisms accompanying this disease and facilitate the development of specific and sensitive diagnostic markers of advanced endometriosis in the future.

5.1. Strengths of the Study

1. We are the first to show the presence of O-glycans in blood serum IgG in women with advanced endometriosis.
2. The relative reactivities of isolated serum IgG O-glycans with specific lectins were significantly higher in women with advanced endometriosis and the group of women with gynecological diseases other than endometriosis in comparison to the group of healthy women.
3. The relative reactivity of i-IgG O-glycans with Jacalin significantly differentiates the advanced endometriosis patients from women with other gynecological diseases, which was most probably caused by increased expression of core 3 type O-glycans in the case of non-endometriosis patients.
4. The relative reactivities of blood serum IgG glycans with PHA-L in advanced endometriosis and non-endometriosis patients were significantly higher than in the control group of healthy women, showing increased expression of multi-branched three and tetra-antennary N-glycans in the E and NE groups.
5. Cluster analysis confirmed the usefulness of the determinations of relative reactivities of i-IgG glycans with MPL, VVL, Jacalin, and PHA-L for distinguishing healthy women from women with advanced endometriosis and women without endometriosis but suffering from other gynecological diseases.

5.2. Limitations of the Study

1. Significant differences in relative reactivities of IgG glycans with the lectins used were observed only for IgG isolates, which was most probably caused by better bioavailability of oligosaccharides for lectins when IgG was isolated from biological material. However, given that the process of isolation and purification of the protein is long and laborious, the differences observed between the studied groups in the relative reactivities of IgG glycans with lectins used should be treated as an additional cognitive aspect that could be difficult to apply in routine diagnostics.
2. The lack of women suffering from early stages of endometriosis made it impossible to check the utility of lectin-ELISA tests used in the present study for diagnostics of the early stages of disease development.

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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 the Wroclaw Medical University (Permission No. KB-293/2016 and KB-719/2018). Written informed consent was obtained from recruited patients.

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

Data Availability Statement: Not applicable.

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

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8. OŚWIADCZENIA WSPÓŁAUTORÓW

Wrocław, dn. 15.12.2022 r.

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Oświadczam, że w pracy:

Variability of serum IgG sialylation and galactosylation degree in women with advanced endometriosis. **K. Sołkiewicz**, H. Krotkiewski, M. Jędryka, E.M. Kratz. Sci Rep. 2021 Mar 10;11(1):5586. doi: 10.1038/s41598-021-85200-x., mój udział polegał na współtworzeniu planu badań, wykonaniu części eksperymentalnej, analizie i interpretacji uzyskanych wyników, formułowaniu wniosków, zebraniu i analizie piśmiennictwa, współtworzeniu oryginalnego tekstu manuskryptu i korekcie ostatecznej formy manuskryptu.

The alterations of serum IgG fucosylation as a potential additional new diagnostic marker in advanced endometriosis. **K. Sołkiewicz**, H. Krotkiewski, M. Jędryka, A. Czekański, E.M. Kratz. J Inflamm Res. 2022 Jan 13;15:251-266. doi: 10.2147/JIR.S341906. eCollection 2022., mój udział polegał na współtworzeniu planu badań, wykonaniu części eksperymentalnej, analizie i interpretacji uzyskanych wyników, formułowaniu wniosków, zebraniu i analizie piśmiennictwa, współtworzeniu tekstu manuskryptu oraz korekcie ostatecznej formy manuskryptu.

O-glycosylation changes in serum immunoglobulin G are associated with inflammation development in advanced endometriosis. K. Sołkiewicz, M. Kacperczyk, H. Krotkiewski, M. Jędryka, E.M. Kratz. Int J Mol Sci. 2022 Jul 22;23(15):8087.doi: 10.3390/ijms23158087., mój udział polegał na współtworzeniu planu badań, wykonaniu części eksperymentalnej, analizie i interpretacji uzyskanych wyników, formułowaniu wniosków, zebraniu i analizie piśmiennictwa, współtworzeniu tekstu manuskryptu oraz korekcie ostatecznej formy manuskryptu, korespondencji z redakcją (autor korespondencyjny).

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

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The alterations of serum IgG fucosylation as a potential additional new diagnostic marker in advanced endometriosis. K. Sołkiewicz, H. Krotkiewski, M. Jędryka, A. Czekański, **E.M. Kratz**. J Inflamm Res. 2022 Jan 13;15:251-266. doi: 10.2147/JIR.S341906. eCollection 2022., mój udział polegał na opracowaniu koncepcji i założeń badań, planowaniu i nadzorze nad wykonanymi badaniami, pomocy w analizie i interpretacji wyników badań, krytycznej weryfikacji wniosków, współtworzeniu tekstu manuskryptu oraz jego ostatecznej formy, korespondencji z redakcją oraz pozyskaniu źródeł finansowania.

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Podpis 

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Variability of serum IgG sialylation and galactosylation degree in women with advanced endometriosis. K. Sołkiewicz, **H. Krotkiewski**, M. Jędryka, E.M. Kratz. Sci Rep. 2021 Mar 10;11(1):5586. doi: 10.1038/s41598-021-85200-x, mój udział polegał na: opracowaniu ogólnej koncepcji badań, konsultacji w planowaniu doświadczeń, pomocy w interpretacji wyników, korekcie ostatecznej formy manuskryptu.

The alterations of serum IgG fucosylation as a potential additional new diagnostic marker in advanced endometriosis. K. Sołkiewicz, **H. Krotkiewski**, M. Jędryka, A. Czekański, E.M. Kratz. J Inflamm Res. 2022 Jan 13;15:251-266. doi: 10.2147/JIR.S341906. eCollection 2022., mój udział polegał na: ustaleniu koncepcji i założeń badań związanych z izolacją IgG, konsultowaniu metodologii wykonywanych badań, korekcie ostatecznej formy manuskryptu.

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Variability of serum IgG sialylation and galactosylation degree in women with advanced endometriosis. K. Sołkiewicz, H. Krotkiewski, **M. Jędryka**, E.M. Kratz. Sci Rep. 2021 Mar 10;11(1):5586. doi: 10.1038/s41598-021-85200-x, mój udział polegał na polegał na wyselekcjonowaniu pacjentek do grup badanych, pobraniu i zabezpieczeniu materiału biologicznego do badań, korekcie ostatecznej wersji manuskryptu, pozyskaniu źródeł finansowania.

The alterations of serum IgG fucosylation as a potential additional new diagnostic marker in advanced endometriosis. K. Sołkiewicz, H. Krotkiewski, **M. Jędryka**, A. Czekański, E.M. Kratz. J Inflamm Res. 2022 Jan 13;15:251-266. doi: 10.2147/JIR.S341906. eCollection 2022., mój udział polegał na wyselekcjonowaniu pacjentek do grup badanych, pobraniu i zabezpieczeniu materiału biologicznego do badań, korekcie ostatecznej wersji manuskryptu.

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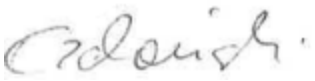
OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

The alterations of serum IgG fucosylation as a potential additional new diagnostic marker in advanced endometriosis. K. Sołkiewicz, H. Krotkiewski, M. Jędryka, **A. Czekański**, E.M. Kratz.
J Inflamm Res. 2022 Jan 13;15:251-266. doi: 10.2147/JIR.S341906. eCollection 2022., mój udział polegał na współudziale w wyselekcjonowaniu pacjentek do grup badanych, współudziale w pobraniu i zabezpieczeniu materiału biologicznego do badań.

Podpis



Wrocław, dn. 14.12.2022 r.

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

O-glycosylation changes in serum immunoglobulin G are associated with inflammation development in advanced endometriosis. K. Sołkiewicz, **M. Kacperczyk**, H. Krotkiewski, M. Jędryka, E.M. Kratz. Int J Mol Sci. 2022 Jul 22;23(15):8087.doi: 10.3390/ijms23158087., mój udział polegał na współudziale w wykonaniu części oznaczeń.

Podpis

Uniwersytet Medyczny we Wrocławiu
ZAKŁAD
DIAGNOSTYKI LABORATORYJNEJ
asystent
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9. OPINIE KOMISJI BIOETYCZNEJ

Badania zostały przeprowadzone zgodnie z deklaracją Helsinki-II, a protokół został zatwierdzony przez Komisję Bioetyczną przy Uniwersytecie Medycznym we Wrocławiu (Zezwolenie nr KB-293/2016 i KB-719/2018), (pozytywna opinia Komisji Bioetycznej nr KB- 297/2022).

KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu
ul. Pasteura 1; 50-367 WROCŁAW

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 293/2016

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 78/XV R/2014 z dnia 26 listopada 2014 r. oraz działająca w trybie przewidzianym rozporządzeniem Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. (Dz.U. nr 47, poz. 480) na podstawie ustawy o zawodzie lekarza z dnia 5 grudnia 1996 r. (Dz.U. nr 28 z 1997 r. poz. 152 z późniejszymi zmianami) w składzie:

prof. dr hab. Maciej Bagłaj (chirurgia, pediatria)
prof. dr hab. Karol Bal (filozofia)
dr hab. Jacek Daroszewski (endokrynologia, diabetologia)
prof. dr hab. Krzysztof Grabowski (chirurgia)
dr Henryk Kaczkowski (chirurgia szczękowa, chirurgia stomatologiczna)
mgr Irena Knabel-Krzyszowska (farmacja)
prof. dr hab. Jan Kolasa (prawo)
prof. dr hab. Jerzy Liebhart (choroby wewnętrzne, alergologia)
ks. dr hab. Piotr Mrzygłód (duchowny)
prof. dr hab. Krystyna Orzechowska-Juzwenko (farmakologia kliniczna, choroby wewnętrzne)
prof. dr hab. Zbigniew Rudkowski (pediatria)
dr hab. Sławomir Sidorowicz (psychiatria)
Danuta Tarkowska (położnictwo)
dr hab. Andrzej Wojnar (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)

pod przewodnictwem
prof. dr hab. Jana Kornafela (ginekologia i położnictwo, onkologia)

Przestrzegając w działalności zasad Good Clinical Practice oraz zasad Deklaracji Helsińskiej, po zapoznaniu się z projektem badawczym pt.

„Badanie galaktozylacji surowiczej IgG, uzyskanej od pacjentek z endometriozą"

zgłoszonym przez **dr Marcina Jędrykę** zatrudnionego w Klinice Onkologii Ginekologicznej Katedry Onkologii Uniwersytetu Medycznego we Wrocławiu oraz **prof. dr hab. Huberta Krotkiewskiego** zatrudnionego w Instytucie Immunologii i Terapii Doświadczalnej PAN we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła wyrazić zgodę na przeprowadzenie badania w Instytucie Immunologii i Terapii Doświadczalnej PAN we Wrocławiu oraz w Klinice Onkologii Ginekologicznej Katedry Onkologii Uniwersytetu Medycznego we Wrocławiu pod nadzorem dr Marcina Jędryki i w Oddziale Ginekologii Onkologicznej Dolnośląskiego Centrum Onkologii we Wrocławiu **pod warunkiem zachowania anonimowości uzyskanych danych.**

Pouczenie: W ciągu 14 dni od otrzymania decyzji wnioskodawcy przysługuje prawo odwołania do Komisji Odwoławczej za pośrednictwem Komisji Bioetycznej UM we Wrocławiu

Opinia powyższa dotyczy: projektów badawczych będących podstawą działalności statutowej

z up. Przewodniczącego
Komisji Bioetycznej przy
Uniwersytecie Medycznym we Wrocławiu

prof. dr hab. Krzysztof Grabowski

Wrocław, dnia 22 czerwca 2016 r.

KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu
ul. Pasteura 1; 50-367 WROCŁAW

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 719/2018

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 133/XV R/2017 z dnia 21 grudnia 2017 r. oraz działająca w trybie przewidzianym rozporządzeniem Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. (Dz.U. nr 47, poz. 480) na podstawie ustawy o zawodzie lekarza z dnia 5 grudnia 1996 r. (Dz.U. nr 28 z 1997 r. poz. 152 z późniejszymi zmianami) w składzie:

dr hab. Jacek Daroszewski (endokrynologia, diabetologia)
prof. dr hab. Krzysztof Grabowski (chirurgia)
dr Henryk Kaczkowski (chirurgia szczękowa, chirurgia stomatologiczna)
mgr Irena Knabel-Krzyszowska (farmacja)
prof. dr hab. Jerzy Liebhart (choroby wewnętrzne, alergologia)
ks. dr hab. Piotr Mrzygłód (duchowny)
mgr Luiza Műller (prawo)
dr hab. Sławomir Sidorowicz (psychiatria)
dr hab. Leszek Szenborn (pediatria, choroby zakaźne)
Danuta Tarkowska (pielęgniarstwo)
prof. dr hab. Anna Wiela-Hojeńska (farmakologia kliniczna)
dr hab. Andrzej Wojnar (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)
dr hab. Jacek Zieliński (filozofia)

pod przewodnictwem
prof. dr hab. Jana Kornafela (ginekologia i położnictwo, onkologia)

Przestrzegając w działalności zasad Good Clinical Practice oraz zasad Deklaracji Helsińskiej, po zapoznaniu się z wnioskiem zgłoszonym przez **dr Marcina Jędrykę** zatrudnionego w Klinice Onkologii Ginekologicznej Katedry Onkologii Uniwersytetu Medycznego we Wrocławiu oraz **prof. dr hab. Huberta Krotkiewskiego** zatrudnionego w Instytucie Immunologii i Terapii Doświadczalnej PAN we Wrocławiu

do projektu badawczego pt.

„Badanie galaktozylacji surowiczej IgG, uzyskanej od pacjentek z endometriozą"

w tajnym głosowaniu postanowiła **wyrazić zgodę** na dołączenie do zespołu badawczego dr hab. Ewy Kratz i mgr Katarzyny Sołkiewicz z Zakładu Diagnostyki Wydziału Farmaceutycznego z Oddziałem Analityki Medycznej Uniwersytetu Medycznego we Wrocławiu.

Komisja Bioetyczna wyraziła zgodę na prowadzenie badania w Instytucie Immunologii i Terapii Doświadczalnej PAN we Wrocławiu oraz w Klinice Onkologii Ginekologicznej Katedry Onkologii Uniwersytetu Medycznego we Wrocławiu i w Oddziale Ginekologii Onkologicznej Dolnośląskiego Centrum Onkologii we Wrocławiu pod nadzorem dr Marcina Jędryki **pod warunkiem zachowania anonimowości uzyskanych danych**.

Pouczenie: W ciągu 14 dni od otrzymania decyzji wnioskodawcy przysługuje prawo odwołania do Komisji Odwoławczej za pośrednictwem Komisji Bioetycznej UM we Wrocławiu.

Opinia powyższa dotyczy: projektu badawczego będącego podstawą działalności statutowej
Projekt ten otrzymał pozytywną opinię Komisji Bioetycznej Nr: KB -293/2016

Wrocław, dnia 4 grudnia 2018 r.

BW

Uniwersytet Medyczny we Wrocławiu
KOMISJA BIOETYCZNA
przewodniczący
prof. dr hab. Jan Kornafel

KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 297/2022

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 278/XVI R/2020 z dnia 21 grudnia 2020 r. oraz działająca w trybie przewidzianym rozporządzeniem Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. (Dz.U. nr 47, poz. 480) na podstawie ustawy o zawodzie lekarza z dnia 5 grudnia 1996 r. (Dz.U. nr 514 z 2020 r.) w składzie:

dr Joanna Birecka (psychiatria)

dr Beata Freier (onkologia)

dr hab. Tomasz Fuchs (ginekologia, położnictwo)

prof. dr hab. Dariusz Janczak (chirurgia naczyniowa, transplantologia)

dr hab. Krzysztof Kaliszewski, prof. UMW (chirurgia endokrynologiczna)

dr prawa Andrzej Malicki (prawo)

dr hab. Marcin Mączyński, prof. UMW (farmacja)

Urszula Olechowska (pielęgniarstwo)

prof. dr hab. Leszek Szenborn (pediatria, choroby zakaźne)

prof. dr hab. Andrzej Szuba (choroby wewnętrzne, angiologia

ks. prof. Andrzej Tomko (duchowny)

prof. dr hab. Mieszko Więckiewicz (stomatologia)

dr hab. Andrzej Wojnar, prof. nadzw. (histopatologia, dermatologia) przedstawiciel

Dolnośląskiej Izby Lekarskiej)

dr hab. Jacek Zieliński (filozofia)

pod przewodnictwem
prof. dr hab. Jerzego Rudnickiego (chirurgia, proktologia)

Przestrzegając w działalności zasad Good Clinical Practice oraz zasad Deklaracji Helsińskiej, po zapoznaniu się z projektem badawczym pt.:

„Analiza profilu i stopnia glikozylacji surowiczej immunoglobuliny G u kobiet z zaawansowaną endometriozą"

zgłoszonym przez **mgr Katarzynę Sołkiewicz,** pracownika Katedry i Zakładu Diagnostyki Laboratoryjnej Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła **wyrazić zgodę** na przeprowadzenie badania

w Katedrze i Zakładzie Diagnostyki Laboratoryjnej Uniwersytetu Medycznego we Wrocławiu oraz Instytucie Immunologii i Terapii Doświadczalnej PAN we Wrocławiu, pod nadzorem dr hab. Ewy Marii Kratz, prof. UMW i prof. dr hab. Huberta Krotkiewskiego, **pod warunkiem zachowania anonimowości uzyskanych danych**.

Uwaga: Badanie to zostało objęte ubezpieczeniem odpowiedzialności cywilnej Uniwersytetu Medycznego we Wrocławiu z tytułu prowadzonej działalności.

Pouczenie: W ciągu 14 dni od otrzymania decyzji wnioskodawcy przysługuje prawo odwołania do Komisji Odwoławczej za pośrednictwem Komisji Bioetycznej UM we Wrocławiu.

Opinia powyższa dotyczy projektu badawczego będącego podstawą rozprawy doktorskiej.

Przewodniczący Komisji Bioetycznej
przy Uniwersytecie Medycznym

prof. dr hab. Jerzy Rudnicki

Wrocław, dnia 27 kwietnia 2022 r.

10. ŹRÓDŁA FINANSOWANIA

Badania, których wyniki przedstawiono w w/w publikacjach oryginalnych zostały sfinansowane z subwencji Ministra Zdrowia wg systemu SIMPLE Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu o następujących numerach:

I publikacja

SUB.D270.20.010 Katedra Diagnostyki Laboratoryjnej, Zakład Diagnostyki Laboratoryjnej

SUB.C280.21.023 Katedra Onkologii i Kliniki Onkologii Ginekologicznej

II publikacja

SUB.D270.21.096 Katedra Diagnostyki Laboratoryjnej, Zakład Diagnostyki Laboratoryjnej

III publikacja

SUB.D270.21.096; SUBZ.D270.22.047 Katedra Diagnostyki Laboratoryjnej, Zakład Diagnostyki Laboratoryjnej

11. CAŁKOWITY DOROBEK NAUKOWY

Wrocław, 16.12.2022 r.

Katarzyna Sołkiewicz

Wykaz publikacji

1. Publikacje w czasopismach naukowych

1.1 Publikacje w czasopiśmie z IF

Lp	Opis bibliograficzny	IF	Punkty
1	Pawlik-Sobecka Lilla, **Sołkiewicz Katarzyna**, Kokot Izabela, Kiraga Aleksandra, Płaczkowska Sylwia, Schlichtinger Agnieszka Matylda, Kratz Ewa Maria: The influence of serum sample storage conditions on selected laboratory parameters related to oxidative stress: a preliminary study, Diagnostics, 2020, vol. 10, nr 1, art.51 [17 s.], DOI:10.3390/diagnostics10010051	3,706	70
2	Kratz Ewa M., Żurawska-Płaksej Ewa, **Sołkiewicz Katarzyna**, Kokot Izabela, Faundez R., Piwowar Agnieszka: Investigation of seminal plasma chitotriosidase-1 and leukocyte elastase as potential markers for 'silent' inflammation of the reproductive tract of the infertile male - a pilot study, Journal of Physiology and Pharmacology, 2020, vol. 71, nr 3, s. 343-349, DOI:10.26402/jpp.2020.3.04	3,011	100
3	Kratz Ewa Maria, **Sołkiewicz Katarzyna**, Kubis-Kubiak Adriana, Piwowar Agnieszka: Sirtuins as important factors in pathological states and the role of their molecular activity modulators, International Journal of Molecular Sciences, 2021, vol. 22, nr 2, art.630 [31 s.], DOI:10.3390/ijms22020630	6,208	140
4	Kokot Izabela, Piwowar Agnieszka, Jędryka Marcin, **Sołkiewicz Katarzyna**, Kratz Ewa Maria: Diagnostic significance of selected serum inflammatory markers in women with advanced endometriosis, International Journal of Molecular Sciences, 2021, vol. 22, nr 5, art.2295 [20 s.], DOI:10.3390/ijms22052295	6,208	140
5	Kratz Ewa Maria, **Sołkiewicz Katarzyna**, Kaczmarek Agnieszka, Piwowar Agnieszka: Sirtuiny - enzymy o wielokierunkowej aktywności katalitycznej, Postępy Higieny i Medycyny Doświadczalnej, 2021, vol. 75, s. 152-174, DOI:10.5604/01.3001.0014.7866	0,357	40
6	**Sołkiewicz Katarzyna**, Krotkiewski Hubert, Jędryka Marcin, Kratz Ewa M.: Variability of serum IgG sialylation and galactosylation degree in women with advanced endometriosis, Scientific Reports, 2021, vol. 11, art.5586 [10 s.], DOI:10.1038/s41598-021-85200-x	4,997	140
7	Płaczkowska Sylwia, **Sołkiewicz Katarzyna**, Bednarz-Misa Iwona, Kratz Ewa Maria: Atherogenic plasma index or non-high-density lipoproteins as markers best reflecting age-related high concentrations of small dense low-density lipoproteins, International Journal of Molecular Sciences, 2022, vol. 23, nr 9, art.5089 [13 s.], DOI:10.3390/ijms23095089	6,208*	140

1

Lp	Opis bibliograficzny	IF	Punkty
8	**Sołkiewicz Katarzyna**, Kacperczyk Monika, Krotkiewski Hubert, Jędryka Marcin, Kratz Ewa Maria: O-glycosylation changes in serum immunoglobulin G are associated with inflammation development in advanced endometriosis, International Journal of Molecular Sciences, 2022, vol. 23, nr 15, art.8087 [22 s.], DOI:10.3390/ijms23158087	6,208*	140
9	**Sołkiewicz Katarzyna**, Krotkiewski Hubert, Jędryka Marcin, Czekański Andrzej, Kratz Ewa Maria: The alterations of serum IgG fucosylation as a potential additional new diagnostic marker in advanced endometriosis, Journal of Inflammation Research, 2022, vol. 15, s. 251-266, DOI:10.2147/JIR.S341906	4,631*	140

**IF 2021*

1.2 Publikacje w czasopiśmie bez IF

Lp.	Opis bibliograficzny	Punkty
1	**Sołkiewicz Katarzyna**, Pawlik-Sobecka Lilla, Płaczkowska Sylwia, Piwowar Agnieszka: Erytropoetyna a utrata krwi w wybranych zabiegach ortopedycznych, Polski Merkuriusz Lekarski, 2018, vol. 45, nr 268, s. 141-149	7

2. Monografie naukowe

2.1 Książka autorska

Lp.	Opis bibliograficzny	Punkty
1	Chmielewski Piotr, **Sołkiewicz Katarzyna**, Borysławski Krzysztof, Strzelec Bartłomiej: Variability in the frequency of ABO and Rh blood groups in Lower Silesia (Poland): the role of natural selection and genetic drift, Wrocław 2017, DN Publisher, 128 s., (Monographs of Physical Anthropology, nr Vol.6), [Publikacja w wydawnictwie spoza listy MNiSW]	20

2.2 Książka redagowana -

2.3 Rozdziały

Lp.	Opis bibliograficzny	Punkty
1	Kiraga Aleksandra, Pawlik-Sobecka Lilla, Kokot Izabela, **Sołkiewicz Katarzyna**, Płaczkowska Sylwia: Porównanie dwóch metod oceny potencjału antyoksydacyjnego krwi, W: Nauka, badania i doniesienia naukowe 2018 : nauki przyrodnicze i medyczne, (red.) Tobiasz Wysoczański, Świebodzice 2018, Idea Knowledge Future, s. 152-160, ISBN 978-83-945311-7-1, [Publikacja w wydawnictwie spoza listy MNiSW]	5
2	Babst Agata, Kokot Izabela, **Sołkiewicz Katarzyna**, Pawlik-Sobecka Lilla, Płaczkowska Sylwia: Zróżnicowanie potencjału antyoksydacyjnego oraz stężenia oksydacyjnie modyfikowanych białek i lipidów w zależności od płci u ludzi młodych, W: Nauka, badania i doniesienia naukowe 2018 : nauki przyrodnicze i medyczne, (red.) Tobiasz Wysoczański, Świebodzice 2018, Idea Knowledge Future, s. 7-16, ISBN 978-83-945311-7-1, [Publikacja w wydawnictwie spoza listy MNiSW]	5

2

3. Abstrakty

Lp	Opis bibliograficzny
1	Kokot Izabela, Płaczkowska Sylwia, **Sołkiewicz Katarzyna**, Sokolik Renata, Korman Lucyna, Pawlik-Sobecka Lilla: Całkowity status antyoksydacyjny u kobiet chorujących na ŁZS i RZS - badanie pilotażowe, Diagnostyka Laboratoryjna, 2017, vol. 53, nr suppl.1, 52 poz.P-07, [XIX Zjazd Polskiego Towarzystwa Diagnostyki Laboratoryjnej. Kraków, 3-6 września 2017 r. Streszczenia]
2	Płaczkowska Sylwia, Kokot Izabela, **Sołkiewicz Katarzyna**, Pawlik-Sobecka Lilla, Piwowar Agnieszka: Insulinooporność i cechy zespołu metabolicznego u ludzi młodych, Diagnostyka Laboratoryjna, 2017, vol. 53, nr suppl.1, 83 poz.P-72, [XIX Zjazd Polskiego Towarzystwa Diagnostyki Laboratoryjnej. Kraków, 3-6 września 2017 r. Streszczenia]
3	Babst Agata, Jaroszewska Karolina, Kokot Izabela, **Sołkiewicz Katarzyna**, Pawlik-Sobecka Lilla, Płaczkowska Sylwia: Nieinwazyjna diagnostyka wad genetycznych płodu z wykorzystaniem próbek krwi matki, W: II Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 3 kwietnia 2017 r. Książka abstraktów 2017, 18 poz.P1
4	Kiraga Aleksandra, Filipek Aneta, Kokot Izabela, **Sołkiewicz Katarzyna**, Pawlik-Sobecka Lilla, Płaczkowska Sylwia: Diagnostyka molekularna alergii IgE zależnych, W: II Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 3 kwietnia 2017 r. Książka abstraktów 2017, 28 poz.P12
5	Kokot Izabela, Płaczkowska Sylwia, **Sołkiewicz Katarzyna**, Pawlik-Sobecka Lilla: Kapilaroskopia jako nieinwazyjna metoda diagnostyczna w chorobach reumatycznych, W: II Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 3 kwietnia 2017 r. Książka abstraktów 2017, 29 poz.P13
6	Pawlik-Sobecka Lilla, Kokot Izabela, **Sołkiewicz Katarzyna**, Płaczkowska Sylwia: Ocena insulinooporności i insulinowrażliwosci w oparciu o pośrednie metody laboratoryjne, W: II Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 3 kwietnia 2017 r. Książka abstraktów 2017, 39 poz.P23
7	**Sołkiewicz Katarzyna**, Płaczkowska Sylwia, Kokot Izabela, Pawlik-Sobecka Lilla: Nowoczesne systemy pobierania krwi żylnej do badań diagnostycznych, W: II Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 3 kwietnia 2017 r. Książka abstraktów 2017, 45 poz.P29
8	**Sołkiewicz Katarzyna**, Kratz Ewa Maria: Immunoglobulin G glycosylation in endometriosis, W: 3rd Wroclaw Scientific Meetings. Wrocław, 1st-2nd March 2019, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Wężgowiec, Wrocław 2019, Wydawnictwo Naukowe TYGIEL sp. z o.o., 161 poz.P107, ISBN 978-83-65932-64-8
9	Kokot Izabela, Piwowar Agnieszka, Kratz Ewa Maria, Płaczkowska Sylwia, **Sołkiewicz Katarzyna**, Pawlik-Sobecka Lilla, Sokolik Renata: Effect of disease activity on parameters of oxidative-antioxidant balance in rheumatoid arthritis patients, W: 3rd Wroclaw Scientific Meetings. Wrocław, 1st-2nd March 2019, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Wężgowiec, Wrocław 2019, Wydawnictwo Naukowe TYGIEL sp. z o.o., 96 poz.P42, ISBN 978-83-65932-64-8
10	**Sołkiewicz Katarzyna**, Krotkiewski Hubert, Jędryka Marcin, Kratz Ewa Maria: Variability of serum immunoglobulin G degree of galactosylation in women with endometriosis - pilot study, W: 4th International Wroclaw Scientific Meetings. Wrocław, 09-10 October 2020, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Wężgowiec, Wrocław 2020, Wydawnictwo Naukowe TYGIEL sp. z o.o., s. 219-220, ISBN 978-83-66489-37-0

Lp	Opis bibliograficzny
11	**Sołkiewicz Katarzyna**, Kaczmarek Agnieszka, Piwowar Agnieszka, Kratz Ewa Maria: Sirtuiny jako multipotencjalne markery diagnostyczne, W: II. WSML - Wrocławskie Spotkanie Medycyny Laboratoryjnej. Wrocław, 26 marca 2021. Program konferencji i książka abstraktów 2021, 26 poz.P16, [[Dostęp 7.04.2021]. Dostępny w: https://medtube.pl/uploads/a/4/03252a48ceca6bebd6b26b609a92fb950fe3.pdf]
12	Kokot Izabela, **Sołkiewicz Katarzyna**, Dymicka-Piekarska Violetta, Kratz Ewa Maria: Variability of the degree of serum IgG sialylation and galactosylation in patients with SARS-CoV-2 infection, W: Proceedings of the 15th Bratislava Symposium on Saccharides. Smolenice Castle, Slovakia, June 20 - 24, 2022, Smolenice 2022, 105 poz.P26, ISBN 978-80-971156-8-5
13	**Sołkiewicz Katarzyna**, Kacperczyk Monika, Krotkiewski Hubert, Jędryka Marcin, Kratz Ewa: The changes of O-glycosylation and the expression of highly-branched N-glycans in serum immunoglobulin G in advanced endometriosis, W: Proceedings of the 15th Bratislava Symposium on Saccharides. Smolenice Castle, Slovakia, June 20 - 24, 2022, Smolenice 2022, 106 poz.P27, ISBN 978-80-971156-8-5

Łączny impact factor: 41,534

	Liczba punktów MNiSW
do roku 2018	37,0
od roku 2019	1050
Razem:	**1087,0**