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ROZPRAWA DOKTORSKA

Badania potencjalnych biomarkerów biochemicznych zaburzających szlaki metaboliczne w trisomii 21 pary chromosomów

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Spis treści

1. WYKAZ PUBLIKACJI STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

Praca przeglądowa:

1. Angelika Buczyńska; Iwona Sidorkiewicz; Anna Trochimiuk; Sławomir Ławicki; Adam Jacek Krętowski; Monika Zbucka-Krętowska
   Novel approaches to an integrated route for Trisomy 21 evaluation
   Biomolecules, 2021, 11(9), 1328.
   Doi: 10.3390/biom11091328
   IF = 4.879; MNiSW = 100

Prace oryginalne:

1. Angelika Buczyńska; Iwona Sidorkiewicz; Sławomir Ławicki; Adam Jacek Krętowski; Monika Zbucka-Krętowska
   The Significance of Apolipoprotein E Measurement in the Screening of Fetal Down Syndrome
   Journal of Clinical Medicine, 2020, 9(12), 3995.
   Doi: 10.3390/jcm9123995
   IF = 4,241; MNiSW = 140

2. Angelika Buczyńska; Iwona Sidorkiewicz; Sławomir Ławicki; Adam Jacek Krętowski; Monika Zbucka-Krętowska
   Prenatal Screening of Trisomy 21: Could Oxidative Stress Markers Play a Role?
   Journal of Clinical Medicine, 2021, 10(11), 2382.
   Doi: 10.3390/jcm10112382
   IF = 4,241; MNiSW = 140

2. ZESTAWIENIE PUBLIKACJI DOKTORANTA

Rodzaj publikacji	Liczba	Impact Factor	Punktacja MNiSW
Prace włączone do rozprawy doktorskiej	3	13,36	380
Prace, które nie zostały włączone do rozprawy doktorskiej	9	29,47	840
Streszczenia zjazdowe	7	0	0
Razem	**19**	**42,83**	**1220**

3. WYKAZ SKRÓTÓW

A1AT- alfa-1-antytrypsyna

AGE – *ang. advanced glycation end products,* końcowe produkty glikacji

AUC- *ang. area under the curve,* pole pod krzywą

cbDNA – *ang. cell-based DNA*, DNA uzyskane z komórek

ELISA – *ang. enzyme-linked immunosorbent assay*, metoda immunoenzymatyczna

ffDNA – *ang. free fetal DNA*, wolnokrążące, płodowe DNA

IMA – *ang. ischemia-modified albumin*, albumina modyfikowana niedotlenieniem

NIPT –*ang. Non Invasive Prenatal Testing*, nieinwazyjne badania przesiewowe

OR – *ang. odds ratio*, iloraz szans

OSDP DNA/RNA – *ang. oxidative stress damage product*, produkty oksydacyjnego rozpadu DNA/RNA

ROC- *ang. receiver operating characteristic curve*, krzywa charakterystyki

ROS – *ang. reactive oxygen species,* reaktywne formy tlenu

T21 – trisomia 21 chromosomu

USG – badanie ultrasonograficzne

4. WSTĘP DOTYCZĄCY TEMATYKI PRACY DOKTORSKIEJ

Trisomia 21 pary chromosomu (T21) to najczęściej występująca aneuploidia u płodu [1], która fenotypowo (zespół Downa) występuje jako zespół wrodzonych wad (m.in. serca, układu pokarmowego, kostnego, moczowo-płciowego) z towarzyszącym upośledzeniem umysłowym [2]. W 2006 roku *National Institute of Child Health and Human Development* oszacował częstotliwość występowania T21 na 1:800 do 1:1000 żywych urodzeń. Dodatkowo, występowanie tej aneuploidii w populacji jest niezależne od przynależności do grupy etnicznej czy społecznej [3].

Obecnie, w celu oszacowania ryzyka wystąpienia aneuploidii u płodu stosuje się nieinwazyjne badania biochemiczne połączone z badaniem ultrasonograficznym (USG), gdzie czułość badania przekracza 90% [4,5]. Następnie, w celu potwierdzenia (diagnostyki) występowania T21 u płodu stosuje się badania inwazyjne, takie jak amniopunkcja lub biopsja kosmówki, które charakteryzują się wyższą swoistością niż badania nieinwazyjne, jednak wiążą się z 0,5% ryzykiem poronienia [6]. Przełomowym momentem diagnostyki nieinwazyjnej (NIPT) okazało się wprowadzenie testów genetycznych, polegających na badaniu wolnokrążącego, płodowego DNA (ffDNA) w krwi matki [7,8]. Dane literaturowe wskazują na 99,8% czułości tego testu, jednak w dalszym ciągu metoda ta nie została uznana za metodę diagnostyczną. Ponadto, wykonanie tego badania jest związane z poniesieniem wysokich kosztów oraz zapewnieniem wysokospecjalistycznej aparatury [9].

Zastosowanie nowych biochemicznych markerów przesiewowych może skutkować zwiększeniem czułości i swoistości nieinwazyjnych

badań prenatalnych oraz ograniczeniem nieuzasadnionego stosowania procedur inwazyjnych przy jednoczesnym zmniejszeniu ryzyka poronienia w połączeniu z zastosowaniem badań inwazyjnych [10]. Dane literaturowe podkreślają związek aberracji chromosomowych płodu z zaburzeniami procesów zależnych od potencjału procesów oksydacyjno-antyoksydacyjnych [11]. Obecność dodatkowego chromosomu 21 powoduje zaburzenie szeregu szlaków metabolicznych, skutkujących wystąpieniem wad wrodzonych u płodu [12,13]. Dodatkowo, biorąc pod uwagę fakt, że kluczowe geny szlaku stresu oksydacyjnego są zmapowane na chromosomie 21, należałoby odnosić się do znaczenia stresu oksydacyjnego nie tylko w patogenezie T21, ale także w diagnostyce prenatalnej [14]. Liczne dane literaturowe podkreślają, iż wskutek wystąpienia aberracji u płodu dochodzi do wzrostu stężenia markerów stresu oksydacyjnego oraz wystąpienia zaburzeń w procesach antyoksydacyjnych, odpowiedzialnych za przeciwdziałanie wystąpieniu oksydacyjnych uszkodzeń struktur biologicznych [14,15]. Nadmierna produkcja reaktywnych form tlenu (ROS) lub wyczerpanie endogennych rezerw antyoksydantów mogą stanowić przyczynę powstania stresu oksydacyjnego w przebiegu T21. Reaktywne formy tlenu mogą wiązać się z białkami, lipidami oraz materiałem genetycznym, powodując zniszczenie struktur komórkowych i zaburzenie integralności tkanki biologicznej [16]. Zana i in. w swojej pracy sugerują, że wolne rodniki osłabiają intensywność systemów naprawczych materiału genetycznego, tym samym zwiększając prawdopodobieństwo powielania powstałych mutacji [17]. W czasie prowadzonych badań zauważono, że podwyższony poziom stresu oksydacyjnego w patogenezie T21 może powodować utlenianie wielonienasyconych kwasów tłuszczowych, a tym samym wywoływać

efekty niszczące błony komórkowe. Sugeruje się, że proces utleniania jest jedną z głównych przyczyn zaburzeń poznawczych obserwowanych w tej chorobie [18]. Badania wykazały również, że podwyższony poziom stresu oksydacyjnego powoduje uszkodzenie DNA, reorganizację cytoszkieletu i chromatyny, defekty w szlaku apoptozy komórek i nieprawidłowe funkcjonowanie punktów kontrolnych cyklu komórkowego. Wobec powyższych danych parametry stresu oksydacyjnego mogłyby pełnić rolę wczesnych biomarkerów przesiewowych stosowanych w celu oszacowania ryzyka wystąpienia T21 u płodu [10,18,19].

Celem rozprawy doktorskiej była ocena przydatności oznaczania wybranych parametrów stresu oksydacyjnego w badaniach prenatalnych. W badaniu poddano ocenie użyteczność przesiewowego oznaczania następujących białek: produkty oksydacyjnego rozpadu DNA/RNA (OSDP), a także innych powszechnie stosowanych markerów stresu oksydacyjnego (albumina modyfikowana niedokrwieniem (IMA) i produkty końcowe zaawansowanej glikacji (AGE)) oraz nowe białka antyoksydacyjne –asprosin i alfa-1-antytrypsyna (A1AT) – oraz apolipoproteinę E (ApoE) i witaminę D.

Na dodatkową uwagę zasługuje fakt, iż, pomimo że defekt genetyczny w przebiegu zespołu Downa – trisomia 21 pary chromosomów - jest znany, etiologia powstających nieprawidłowości nie jest do końca zrozumiała [20]. Można przypuszczać, że poznanie biochemicznych mechanizmów odpowiedzialnych za pojawienie się obrazu klinicznego oraz ich wczesne rozpoznanie w okresie płodowym umożliwi regulację zmienionych szlaków patogenetycznych, co mogłoby potencjalnie zwiększyć szansę na prawidłowy rozwój płodu [21].

5. OMÓWIENIE PRAC SKŁADAJĄCYCH SIĘ NA ROZPRAWĘ DOKTORSKĄ

5.1. Cele pracy

Celem rozprawy doktorskiej było zbadanie potencjalnych wczesnych biomarkerów biochemicznych trisomii 21 pary chromosomów powodujących zaburzenia szlaków metabolicznych. Cel pracy został zrealizowany poprzez:

1. Ocenę przydatności pomiaru stężenia Apolipoproteiny E, alfa-1- antytrypsyny (A1AT) oraz białka asprosin w diagnostyce przesiewowej T21.

2. Ocenę przydatności pomiaru stężenia wybranych markerów stresu oksydacyjnego: produktów oksydacyjnego rozpadu DNA/RNA, albuminy modyfikowanej niedotlenieniem (IMA), końcowych produktów glikacji (AGE) w diagnostyce przesiewowej T21.

3. Przeprowadzenie analizy w/w markerów w aspekcie patogenezy wystąpienia zaburzeń rozwoju płodu z rozpoznanym zespołem Downa.

5.2. Praca przeglądowa stanowiąca podsumowanie tematyki poruszanej w rozprawie doktorskiej

- Praca przeglądowa pt.: „Novel approaches in an integrated route of Trisomy 21 evaluation”

5.2.1. Materiał i metody

Ocenę literatury przeprowadzono z wykorzystaniem bazy PubMed wg kryteriów i szablonów strategicznych PRISMA. Wykorzystano artykuły medyczne opublikowane w latach 2000-2021. Współczynnik oddziaływania (IF) analizowanych czasopism wahał się od 1,14 do 70,67. Aby ocenić użyteczność diagnostyczną proponowanych nowych markerów z potencjalnym zastosowaniem w prenatalnych badaniach przesiewowych, uwzględniono wartości pod krzywą charakterystyki (AUC) na podstawie krzywych charakterystyki (ROC).

5.2.2. Wnioski

Wprowadzenie nieinwazyjnych prenatalnych badań przesiewowych z wykorzystaniem wolnokrążącego DNA płodowego (ffDNA) było momentem przełomowym w czasie nieustannego rozwoju dziedziny prenatalnych badaniach przesiewowych. Dalsze postępy w rozwoju prenatalnych badań przesiewowych mogą doprowadzić do poprawy dokładności metod biochemicznych wykorzystywanych do tej pory. Chociaż czułość metod omicznych w badaniach prenatalnych jest dużo wyższa niż dotychczas stosowanych metod, nadal istnieje wiele wyzwań związanych z ich wprowadzeniem do rutynowej diagnostyki. Niemniej jednak, metody omiczne mogą być stosowane do szybkiej i dokładnej analizy dużych ilości próbek. Niestety badania te pozostają relatywnie drogie, co ogranicza wprowadzenie ich do rutynowej diagnostyki, zwłaszcza w krajach słabiej i średnio rozwiniętych. Metoda ELISA może być użyteczną metodą, umożliwiającą wprowadzenie wyników uzyskanych metodami omicznymi do codziennej rutynowej diagnostyki, z jednoczesnym ujednoliceniem stosowanych procedur.

Przypuszcza się, iż dalsza walidacja wyników z wykorzystaniem metody ELISA umożliwi wydobycie zalet wyników uzyskanych metodami omicznymi, zapewniając szybką i dokładną detekcję T21. W związku z tym nadal istnieje potrzeba kontynuacji badań, opartych na metodach omicznych w celu poprawy wartości diagnostycznej przeprowadzanych prenatalnych badań przesiewowych.

Dodatkowo badania przeprowadzane w celu poszukiwania nowych markerów T21 mogą prowadzić do odkrycia potencjalnych celów terapeutycznych. Podkreślając złożoność zaburzeń występujących podczas rozwoju T21, zastosowanie zintegrowanych metod omicznych do zbadania patogenezy tej aneuploidii może być najskuteczniejszym sposobem wprowadzenia odpowiedniego leczenia prenatalnego T21 w przyszłości.

5.3. Publikacje oryginalne

- „The Significance of Apolipoprotein E Measurement in the Screening of Fetal Down Syndrome"
- „Prenatal Screening of Trisomy 21: Could Oxidative Stress Markers Play a Role?"

5.3.1. Materiał

Materiał potrzebny do badania został zbiobankowany w ramach wcześniej prowadzonych projektów naukowych, na które zostało wydane pozwolenie Komisji Bioetycznej Uniwersytetu Medycznego w Białymstoku (R-I-002/36/2014).

Pacjentki zostały zakwalifikowane do grupy badanej oraz grupy kontrolnej na podstawie wyników kariotypowania uzyskanych w trakcie

amniopunkcji wykonanej w Klinice Rozrodczości i Endokrynologii Ginekologicznej Uniwersytetu Medycznego w Białymstoku. Na potrzeby tego badania zostało zakwalifikowanych 20 pacjentek z rozpoznanym T21 u płodu (stanowiące grupę badaną) oraz 20 pacjentek z prawidłowym kariotypem (grupa kontrolna). Pacjentki zostały włączone do badania na podstawie kryteriów włączenia (rozpoznany T21 u płodu) i wyłączenia (niestosowanie terapii hormonalnej, przewlekłe choroby oraz nieprawidłowy przebieg ciąży).

5.3.2. Zgoda Komisji Bioetycznej

Protokół eksperymentalny przygotowany na potrzeby przeprowadzenia badań zawartych w rozprawie doktorskiej został zatwierdzony przez Komisję Bioetyczną Uniwersytetu Medycznego w Białymstoku (APK/002/351/2020).

5.3.3. Metody

5.3.3.1. Materiał badany

Materiał biologiczny (osocze oraz płyn owodniowy) pozyskany od pacjentek był przechowywany w temperaturze -80°C.

5.3.3.2. Oznaczenia laboratoryjne

Stężenia IMA, AGE, A1AT i białka asprosin oznaczono z wykorzystaniem testów immunoenzymatycznych (ELISA) (Cloud-Clone Corp., Wuhan, Chiny; odpowiednio CEA825Hu, CEB353Ge, SEB697Hu i SEA332Hu) zgodnie z instrukcjami dostarczonymi przez producenta. Stężenia DNA/RNA OSDP oznaczono również przy użyciu zestawu ELISA (DNA/RNA Oxidative Damage (High Sensitivity),

Cayman Chemicals, Ann Arbor, Michigan, MI, USA, 589320). Zestaw ten umożliwiał jednoczesną detekcję OSDP DNA/RNA, takich jak 8-hydroksyguanozyna (8-OHG), 8-hydroksy-2'-deoksyguanozyna (8- OHdG) i 8-hydroksyguanina. Całkowity pomiar witaminy D oceniono metodą chemiluminescencji z wykorzystaniem aparatu Cobas E411 firmy Roche (07464215). Stężenie 25-OH witaminy D zostało oznaczone metodą ELISA (Gentaur, Sopot, Polska, KAP1971). Próbki i kontrole były randomizowane, a następnie mierzone w tej samej serii, przy użyciu metody ślepej analizy.

5.3.3.3. Analiza statystyczna

Wyniki badań poddano analizie statystycznej z użyciem programu GraphPad PRISM v.9.0. (GraphPad Software Inc., San Diego, CA) z użyciem klasycznych metod statystycznych. Różnice uznano za istotne statystycznie przy $p<0,05$. W celu określenia użyteczności diagnostycznej badanych parametrów wykreślono krzywe charakterystyk (ROC), gdzie pole pod krzywą (AUC) oraz wyliczony iloraz szans (OR), będący miarą związku między ekspozycją a wynikiem, poddano analizie.

5.3.4. Wyniki

5.3.4.1. Porównanie stężeń markerów w osoczu i płynie owodniowym pomiędzy kobietami w ciąży z rozpoznaną T21 u płodu oraz ciężarnymi z euploidalnym płodem

Przeprowadzone badania udowodniły istotnie wyższe stężenie ApoE u kobiet z rozpoznanym T21 u płodu w porównaniu do grupy kontrolnej ($p < 0,001$). Analizując parametry związane z oznaczeniami markerów stresu oksydacyjnego wykazano, że stężenia OSDP DNA/RNA były istotnie wyższe w próbkach płynu owodniowego pozyskanych od kobiet w ciąży z rozpoznaną T21 u płodu w porównaniu do grupy kontrolnej ($p < 0,05$). Nie zaobserwowano istotnej różnicy w stężeniach OSDP DNA/RNA w osoczu pomiędzy grupą badaną i kontrolną. W grupie pacjentek z T21 u płodu wykazano istotnie niższe stężenia AGE zarówno w próbkach osocza, jak i płynu owodniowego w porównaniu z wynikami uzyskanymi w grupie kontrolnej ($p < 0,001$). Dodatkowo stężenia IMA w osoczu były niższe w grupie badanej w porównaniu z grupą kontrolną ($p<0,0001$).

Analizując parametry antyoksydacyjne oceniane w grupie badanej, całkowite stężenia witaminy D w osoczu były istotnie niższe w porównaniu z grupą kontrolną ($p < 0,05$). Aby potwierdzić niedobór witaminy D, zmierzono stężenie 25-OH witaminy D w próbkach osocza i płynu owodniowego. Nie wykazano istotnych różnic między uzyskanymi oznaczeniami w grupie badanej i kontrolnej, jednakże, w obu grupach zaobserwowano niższe niż zalecane stężenie 25-OH witaminy D (powyżej 30 ng/ml).

Stężenia białka asprosin w grupie badanej były istotnie wyższe zarówno w próbkach osocza, jak i płynu owodniowego w porównaniu z grupą kontrolną ($p < 0,001$). Ponadto, stwierdzono, że stężenia A1AT były istotnie niższe w próbkach płynu owodniowego uzyskanego w grupie pacjentek z rozpoznaną T21 u płodu niż w grupie kontrolnej pacjentek z ciążami euploidalnymi ($p < 0,001$). W trakcie analizy wyników otrzymanych stężeń A1AT w osoczu, nie zaobserwowano istotnej różnicy między grupą badaną a kontrolną.

5.3.4.2. Wzajemna zależność między badanymi markerami

Wśród parametrów biochemicznych zmierzonych w grupie kontrolnej zaobserwowano dodatnie korelacje między następującymi oznaczeniami: stężenie całkowitej witaminy D w osoczu, a 25-OH witaminą D w osoczu ($r = 0,85$; $p < 0,001$) oraz między całkowitą witaminą D w osoczu, a A1AT w płynie owodniowym ($r = 0,59$; $p < 0,05$). Dodatkowo zaobserwowano dodatnie korelacje między 25-OH witamina D i A1AT w grupie kontrolnej ($r = 0,47$; $p < 0,05$) zmierzonej w płynie owodniowym oraz między stężeniem białka asprosin, a A1AT ($r = 0,45$; $p < 0,05$) zmierzonej w płynie owodniowym. Ponadto, wykazano dodatnią korelację między A1AT zmierzonej w płynie owodniowym, a stężeniem OSDP DNA/RNA mierzonym w osoczu ($r = 0,44$; $p < 0,05$). Wykazano również ujemną korelację między A1AT oznaczoną w płynie owodniowym, a osoczowym stężeniem OSDP DNA, oznaczonej w grupie kontrolnej ($r = -0,45$; $p < 0,05$). W odniesieniu do grupy badanej zaobserwowano silne dodatnie korelacje w osoczu między całkowitą witaminą D a witaminą D 25-OH ($r = 0,80$; $p < 0,001$) oraz między całkowitą witaminą D a IMA ($r = 0,45$; $p < 0,05$)). Dodatkowo, w grupie badanej zaobserwowano dodatnią korelację między stężeniem IMA

zmierzonym w płynie owodniowym, a poziomem 25-OH witaminą D oznaczoną również w tym materiale ($r = 0,52$; $p < 0,05$). Wykazano również ujemną korelację między pomiarami stężenia asprosinu w płynie owodniowym, a stężeniem 25-OH witaminy D w osoczu ($r = -0,54$ $p < 0,05$), a także pomiędzy oznaczeniami AGE i A1AT mierzonymi w osoczu($r = -0,60$; $p < 0,05$). Zaobserwowano również ujemną korelację między OSDP DNA/RNA, a A1AT w płynie owodniowym ($r = -0,54$; $p < 0,05$). Nie zaobserwowano istotnej korelacji między osoczem, a płynem owodniowym dla pozostałych parametrów ani w grupie kontrolnej, jak i badanej.

5.3.4.3. Użyteczność przesiewowa badanych parametrów

W czasie trwania badania przeanalizowano, jakie parametry spośród badanych mają wpływ na wzrost ilorazu szans (OR) wystąpienia T21.. Stwierdzono zależności pomiędzy występowaniem T21, a stężeniem asprosinu w płynie owodniowym (OR=22,78), AGE (OR=2,11), IMA (OR=0,18) oraz w osoczu (OR=8,20) i A1AT (OR=5,75) ($p < 0,05$). W celu określenia przydatności diagnostycznej badanych parametrów (wartość AUC), będących przedstawionych za pomocą zależności między czułością, a swoistością, przygotowano wykresy ROC. Wartości graniczne zostały ustalone za pomocą indeksu Youdena. Największą czułość charakteryzowało oznaczanie stężenia białka asprosin w osoczu i płynie owodniowym, stężenia AGE w płynie owodniowym oraz ApoE w osoczu(odpowiednio AUC=1,00; 0,95; 0,95; 0,98) (Tabela1).

Tabela 1. Wartość diagnostyczna badanych markerów w badaniach przesiewowych T21.

Marker	Jednostka	AUC	Punkt odcięcia	Czułość	Swoistość
ApoE PS	ng/mL	0.98	>1.37	80%	100%
Asprosin PS	ng/mL	0.97	>12.70	100%	85%
Asprosin AF	ng/mL	0.83	>12.91	95%	65%
AGE PS	ng/mL	0.85	<11.00	81%	80%
AGE AF	ng/mL	0.96	<4.184	95%	90%
A1AT PS	mg/L	0.53	<2.341	81%	33%
A1AT AF	mg/L	0.87	<0.3180	76%	86%
DNA/RNA OSDP PS	pg/mL	0.51	<40.30	80%	40%
DNA/RNA OSDP AF	pg/mL	0.73	>31.76	84%	58%

A1AT - alfa-1-antytrypsyna; AF – płyn owodniowy; ApoE- apolipoproteina E; AGE – końcowe produkty glikacji; AUC - pole pod krzywą charakterystyk (ROC); OSDP, produkty oksydacyjnego rozpadu DNA/RNA; PS - osocze.

5.3.5. Wnioski

Podczas przeprowadzonych badań wykazano użyteczność diagnostyczną oznaczenia nowych białek w prenatalnych badaniach przesiewowych T21, a osoczowe stężenie białka asprosin, ApoE, IMA, AGE wykazały najwyższe wartości czułości i swoistości. Dodatkowo, uzyskane wyniki potwierdziły potencjalne zastosowanie markerów stresu oksydacyjnego w badaniach przesiewowych T21.

Badanie wybranych białek obecnych w płynie owodniowym i osoczu ciężarnych z potwierdzoną T21 u płodu dostarczyło informacji na temat występujących zaburzeń szlaków metabolicznych skutkujących powstaniem defektów narządowych u płodu. Potencjalne źródło stresu oksydacyjnego w ciąży z T21 objawia się w szczególności w łożysku, ale pochodzi również z komórek matki i/lub płodu oraz czynników zewnętrznych. Ponadto, w czasie badań zauważono obniżone stężenie A1AT w płynie owodniowym pozyskanym od kobiet ciężarnych z T21 u płodu. Potencjalnie, obniżenie stężenia A1AT może powodować wystąpienie szeregu wad rozwojowych spowodowanych niedoborem A1AT, będącej jednym z najsilniejszych modulatorów układu odpornościowego. Skutki niedoboru A1AT mogą również wynikać ze zwiększonego poziomu stresu oksydacyjnego, który również został potwierdzony w przeprowadzonych badaniach na podstawie oznaczenia stężenia produktów oksydacyjnego rozpadu DNA/RNA w płynie owodniowym. Podczas analizy wyników wykazano ujemną korelację między OSDP DNA/RNA, a A1AT, gdzie istotnie zwiększony poziom stresu oksydacyjnego mógłby skutkować odpowiednio proporcjonalnym obniżeniem stężenia A1AT zaobserwowanym w płynie owodniowym. Z tego względu można dodatkowo przypuszczać, iż organizm ciężarnej

nie jest w stanie zneutralizować zwiększonego poziomu stresu oksydacyjnego spowodowanego potwierdzoną T21 u płodu. Niezbędne są dalsze badania nad mechanizmami transferu potencjalnych markerów między przedziałem matczynym i płodowym, aby określić związek między parametrami badanymi w płynie owodniowym a oznaczanymi w matczynym osoczu. Ponadto, obniżone stężenie A1AT wraz z obniżonym stężeniem witaminy D oraz zwiększonym stężeniem białka asprosin i DNA/RNA OSDP są związane zaburzeń wynikających z deregulacji wielu ścieżek metabolicznych związanych z rozwojem T21 u płodu. Na podstawie niniejszego badania uzasadnione jest przypuszczenie, że stres oksydacyjny występuje głównie w płynie owodniowym, gdzie bezpośrednio związany jest z rozwijającą się T21 powodującą szereg zaburzeń metabolicznych skutkujących wystąpieniem wad narządowych u płodu. Sugeruje to, że organizm matki jest niewydajny w przezwyciężaniu niedoborów antyoksydacyjnych spowodowanych przez wzrost poziomu stresu oksydacyjnego spowodowanego poprzez wystąpienie T21. W związku z powyższym, zasadnym wydają się dalsze badania oceniające potencjalny pozytywny wpływ zastosowania antyoksydantów w prenatalnej interwencji medycznej w przebiegu T21. Potencjalny korzystny wpływ podawania przeciwutleniaczy podczas rozwoju T21 może zmniejszyć dysfunkcje poznawcze i neuronalne. Obliczenia OR wykazały, że zwiększone stężenie białka asprosin oznaczanego w osoczu i płynie owodniowym wraz z obniżonym stężeniem A1AT mierzonej w płynie owodniowym oraz stężeniem IMA, zwiększało ryzyko wystąpienia T21 u płodu. W przeprowadzonym badaniu potwierdzono użyteczność przesiewową oznaczania ApoE, białka Asprosin oraz wybranych markerów stresu oksydacyjnego w celu oszacowania ryzyka wystąpienia T21.

Dodanie powyższych potencjalnych nowych markerów T21 w celu przeprowadzenia kalkulacji ryzyka wystąpienia T21 może umożliwić poprawę wartości diagnostycznych stosowanych dotychczas testów nieinwazyjnych.

6. KOPIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

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Review

Novel Approaches to an Integrated Route for Trisomy 21 Evaluation

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Abstract: Trisomy 21 (T21) is one of the most commonly occurring genetic disorders, caused by the partial or complete triplication of chromosome 21. Despite the significant progress in the diagnostic tools applied for prenatal screening, commonly used methods are still imprecise and involve invasive diagnostic procedures that are related to a maternal risk of miscarriage. In this case, novel prenatal biomarkers are still being evaluated using highly specialized techniques, which could increase the diagnostic usefulness of biochemical prenatal screening for T21. From the other hand, the T21's pathogenesis, caused by the improper division of genetic material, disrupting many metabolic pathways, could be further evaluated with the use of omics methods, which could result in bringing relevant insights for the evaluation of potential medical targets. Accord- ingly, a literature search was undertaken to collect novel information about prenatal screening for Down syndrome with the use of advanced technology, with a particular emphasis on the evaluation of novel screening biomarkers and the discovery of potential medical targets. These meta-analyses are focused on novel approaches designed with the use of omics techniques, rep- resenting the most rapidly developing and promising field in research today. Considering the limitations and progress of these methods, the use of omics techniques in evaluating T21 pathogene- sis could bring beneficial results in prenatal screening, simultaneously uncovering novel potentialmedical targets.

Keywords: trisomy 21; metabolomics; genomics; prenatal screening

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1. Introduction

Trisomy 21 (T21), also known as Down syndrome, is one of the most frequently occurring chromosomal aberrations, appearing in 1 in 319 to 1 in 1000 live births [1]. The most frequently diagnosed duplication of chromosome 21 as a result of the abnormal nondisjunction of chromosomes occurs in an estimated 95% of cases, and the remaining 5% are associated with translocation and somatic mosaicism [1–3]. T21 patients struggle with physical and mental disabilities and many others comorbidities, such as heart defects, thyroid disease, leukemia, cancers, Alzheimer's disease, and others [1,3–5]. The clinical manifestation of an additional chromosome 21 determines the well-recognized phenotype, which includes an altered facial appearance (flatness of the bridge of the nose, midfacial hypoplasia, and a tendency to protrude the tongue) and musculoskeletal features (inflammatory arthritis, scoliosis, and patellar instability) [1,3,6].

The prenatal screening for T21 is currently based on noninvasive methods, which enable the estimation of the risk of its occurrence, and invasive techniques, mainly used to verify the presence of chromosomal aberrations. Serum screening and ultrasound are used to identify women whose pregnancies are at a high risk of chromosomal abnormalities. Positive screening results can lead to the need to undergo invasive procedures, such as amniocentesis or chronic villus sampling (CVS), where the 21-trisomic karyotype can be confirmed. CVS involves the aspiration of placental tissue, and amniocentesis involves the collection of amniotic fluid. Although invasive techniques are characterized by high diagnostic specificity, they are also associated with a 1% risk of miscarriage [6,7]. On the other hand, material collected using invasive techniques, such as amniotic fluid, is still useful for research [8]. The breakthrough moment in prenatal diagnosis was the development of noninvasive cell-free fetal DNA (cffDNA) evaluation; however, its high cost has limited the introduction of this test into routine management. There has been significant development of diagnostic tools for prenatal diagnosis, but the number of patients undergoing invasive tests remains constant [7,8]. Therefore, it is still important to find a cost-effective and noninvasive screening method for biomarker discovery characterized by high sensitivity and specificity, which would provide certain benefits, subsequently leading to the evaluation of novel medical targets. In this case, the application of novel biochemical screening markers, determined using specific novel techniques, may lead to a reduction in unnecessary invasive procedures [9].

Clearly, there is still a need to evaluate the insufficiencies in metabolic pathways involved in the patomechanism of T21. Bioinformatics has enabled comprehensive multi-omics and clinical data integration for insightful interpretation. In this review, we outline considerations of omics methods applied to experimental design and general frameworks for the integration of omics data in T21 research, along with analytic strategies, and speculate about future multi-omics approaches. Information about T21 prenatal screening received with use of advanced technology, with a particular emphasis on the evaluation of novel screening biomarkers and the discovery of potential novel medical targets, was collected. We hope that this study will also provide novel insights to improve the management of complications related to the genetic, metabolomic, and proteomic disturbances observed in T21 development, while also providing possible insights into the role of prenatal screening.

2. Materials and Methods

The literature evaluation was conducted using the PubMed database following the PRISMA and EQUATOR network guidelines [10–13]. We considered medical papers published in 2000–2021. The papers were independently selected and reviewed. The impact factors of the journals used in this study ranged from 1.14 to 70.67. Articles with irrelevant conclusion statements or inappropriate study methods, inadequate reporting, or dissemination of incomplete reports were excluded from the study (Figure 1).

To assess the diagnostic tools for prenatal screening markers, the receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC) were taken into consideration [14].

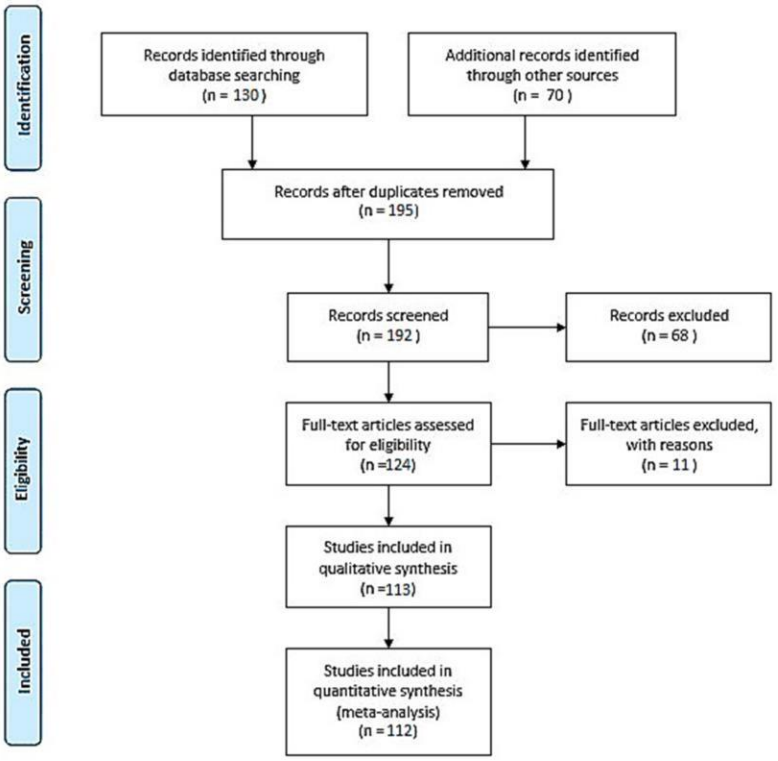

Figure 1. PRISMA flow diagram of meta-analysis process performed during research [13].

3. Current Recommendation for Down Syndrome Screening

The screening for T21 is currently based on noninvasive methods using serum biomarkers and ultrasound examination. First-trimester aneuploidy screening is performed during the 11th to 13th weeks of gestation and includes the measurement of nuchal translucency by ultrasound and maternal serum-free beta-human chorionic gonadotrophin (β hCG) and pregnancy-associated plasma protein A (PAPP-A) [15].

Lately, the second-trimester screening for T21 has been primarily assigned to lower levels of maternal serum alpha fetoprotein (MSAFP) and unconjugated estriol with elevated β hCG and inhibin a concentrations. An increased concentration of MSAFP was associated with open spina bifida in the fetus [16]. The most reliable serum biomarkers— β hCG, alpha-fetoprotein (AFP), unconjugated estriol (E3), and PAPP-A—were associated with 5–10% rates of false positives (Table 1) [2,7,17–19].

Table 1. Biochemical prenatal screening markers [10,20–24].

Pregnancy Period	Ultrasound	Biochemical Test	Sensitivity	Specificity
First trimester (11–13 weeks)	+ (NT)	PAPP-A and free β hCG	85–90%	82–87%
Second trimester (18–24 weeks)	+	β hCG + uE3 + AFP + inhibin A	69–92%	81–96%
First or second trimester	+	PAPP-A, AFP, uE3, total hCG	88%	90–95%
First or second trimester	+	PAPP-A, inhibin A, AFP, uE3, free β hCG/total hCG	85%	90–95%

AFP, alpha-fetoprotein; β hCG, chorionic gonadotropin beta subunit; hCG, chorionic gonadotropin; uE3, unconjugated estradiol; NT, nuchal translucency; PAPP-A, pregnancy-associated plasma protein A.

In the last decade, the first-trimester prenatal screening replaced the performance of second-trimester measurements. It was proved that first-trimester prenatal screening biochemical tests, when combined with the ultrasound marker of fetal NT thickness, are more reliable, thus detecting more than 90% of the cases [15,25].

Due to the rapid development of promising untargeted omics evaluations using advanced technology for the comprehensive comparative analysis of genomes, knowledge

of the metabolome and proteasome could enhance the diagnostic use of prenatal screening. These methods are characterized by high sensitivity, specificity, and reproducibility. Moreover, these studies enable the dissemination of knowledge about the disturbances of metabolic pathways involved in T21 development, which could reveal unanticipated metabolic perturbations and lead to the discovery of novel medical targets. The detection of fetal cells and fetal DNA circulating in maternal blood has increased the role of prenatal screening [26,27]. The progressive integration of different omics methods in T21 pathogenesis could elucidate potential causative changes that lead to this disease or determine the treatment targets that could be studied in further clinical trials.

4. Prenatal Genetic Diagnosis of Down Syndrome

Modern genetics started with research by the Augustinian friar Gregor Johann Mendel, published in 1866, when the theory of Mendelian inheritance was established [28]. The next generation and the rapid development of science led to the discovery of DNA as the structure of chromosomes in 1950 [29]. In 2003, the successful completion of the Human Genome Project, with 99% of the genome sequenced at a 99.99% accuracy, was a breakthrough in genomic development. In the past few decades, many biologists have focused on large-scale genetics projects based on clinical diagnosis and medical intervention possibilities for many diseases [30]. The basic method used to determine trisomy 21 is amniotic fluid chromosome analysis [31]. Amniocentesis is usually performed between the 15th and 18th gestational weeks. Although invasive techniques are associated with a risk of miscarriage, the removal of amniotic fluid itself causes no harm to the developing fetus and is optimal for obtaining fetal cells for culture. Metaphase chromosome analysis or chromosomal microarray analysis (CMA) is almost always performed on amniotic fluid samples [32].

In order to avoid invasive prenatal procedures, there is a need for new T21 biomarkers that have high sensitivity and specificity. Thus, genetic tests could be implemented for noninvasive T21 screening panels. Since the presence of fetal DNA in maternal plasma and serum was established by Lo et al. in 1997 using genome sequencing techniques, rapid progress has been observed in prenatal genetic testing [23]. Gene identification is important for understanding the pathophysiology of diseases and improving diagnosis, prevention, and treatment. The complete sequencing of chromosome 21 provided a basis for the identification of candidate genes for T21 phenotype manifestations. The mechanisms by which an extra copy of chromosome 21 produces the phenotypes of T21 are complex. Next-generation sequencing (NGS) technology is not limited to gene chip technology, so the identification of novel genes is less time-consuming and expensive. Moreover, post-data analysis has been improved by the establishment of huge public data repositories.

NGS technology facilitates a high detection rate and a low percentage of false positive T21 results [24]. There are several genes located on chromosome 21 associated with T21 phenotypes. The genes that have been implicated in T21 development include Cu/Zn superoxide dismutase (SOD1), amyloid precursor protein (APP), Ets-2 transcription factors, Down syndrome critical region 1 (DSCR1) stress-inducible factor, beta-site APP cleav- ing enzyme (BACE), and S100 [33,34]. The Down syndrome critical region (DSCR) is a chromosome 21 segment purported to contain genes responsible for many features of T21 [35]. The DSCR hypothesis predicts that genes in this region are sufficient to produce T21 phenotypes. Studies should evaluate the association of DSCR with T21 diagnosis. The involvement of other genes can be elucidated with advances in omics methods. Sequencing methods are characterized by higher accuracy than chromosome analysis and can detect gene variation effectively. Moreover, this type of prenatal screening is not dependent on gestational age [25]. Compared with traditional first-generation sequencing technology, the sequencing of the human genome initiated the discovery of the T21 patomechanism, leading to a growing understanding of the genetic determinants of this disease, resulting in noninvasive prenatal screening (NIPT) [24].

Rapid improvements in genetic technologies led to the evolution of the prenatal screening of cffDNA, which is more sensitive and specific than biochemical screening methods. From early pregnancy, cffDNA is present in maternal blood, the majority of which originates from the mother herself, but with relevant fetal components contributing approximately 10–20% of the total. Most cffDNA is derived from villous cells; its concentration increases with increasing gestational age, and it is rapidly cleared from the maternal circulation within hours of delivery, making it pregnancy-specific [36]. This measurement is highly specific with regard to representing the entire fetal genotype [28]. The rapid development of massively parallel sequencing (MPS) technology made it feasible to use maternal plasma cffDNA to detect trisomy 21. However, relying on complex and expensive MPS techniques hinders the use of cffDNA as a common screening procedure [37]. With noninvasive tests of maternal blood (fetal and maternal) circulating free DNA (cfDNA) and cffDNA (originating from placenta), results that are discordant with the fetal karyotype can arise from the detection of maternal chromosomal rearrangements or mosaicism, maternal malignancy, or confined placental mosaicism. However, false negatives can occur in cases of decreased concentrations or inconsistent laboratory techniques. Moreover, NIPT is not considered as a diagnostic tool in less economically developed countries, and the confirmation of positive results by invasive testing is still required [29]. Various factors affect the accuracy of ccfDNA results, including confined placental mosaicism, the contribution of maternal DNA, and technical or statistical issues [38].

Currently, the NIPT field is dominated by the cffDNA approach. However, cell-based NIPT (cbNIPT) has been proposed as a superior alternative to overcome the challenges associated with cffDNA [39]. Trophoblasts, granulocytes, lymphocytes, stem cells, and nucleated red blood cells (nRBC) have been identified in maternal blood as a source of cell-based DNA (cbDNA) [40–42]. Intact fetal cells harvested from the maternal circulation represent the uncontaminated fetal DNA which enable to avoid the issues associated with using fragmented cffDNA. A study by Vossaert et al. has demonstrated no significant correlation between maternal age, body mass index, and trophoblast yield of single circulating trophoblast testing, which proves the advantage over cffDNA testing [43].

One of the most limiting factors in cbDNA procedure is to isolate the rare fetal cell from maternal circulation. Fingerprinting by short tandem repeat analysis, fetal cell enrichment, and staining, cell sorting based on physical characteristics, antigens, and proteins have been proposed as useful methods to obtain information regarding the cellular origin [44]. Recently, automated fetal nRBC and extravillous trophoblast capture systems have been validated in the genetic diagnosis [45,46]. Nevertheless, insufficient clinical trials able to provide evidence demonstrating a robustness of cbDNA and its diagnostic value in fetal aneuploidy diagnosis still restrain its clinical implementation [47].

Altered RNA expression is observable for many but not all of the genes mapped on chromosome 21 and for a larger number of genes located on other chromosomes. Epigenetics refers to the regulation of gene expression through microRNA synthesis, DNA methylation, and histone modification processes. Data in the literature suggest that DNA methylation, as a mechanism regulating gene expression, plays an important role in the pathogenesis of T21 [48,49]. DNA methylation is a chemical modification of the fifth carbon of a cytosine base to form 5-methylcytosine (5-mc) catalyzed by DNA methyltransferases. Studying the baseline epigenetic effects on chromosome 21 is a useful approach for evaluating novel therapies. Studies focused on the epigenome-wide evaluation of T21 have identified 1052 differentially methylated regions associated with this disease, including significant hypermethylation regions of RUNX family transcription factor 1 (RUNX1) and *Fli-1* proto-oncogene (FLI1), the main regulators of hematopoiesis [48]. Furthermore, it was proved that reduced neuron-restrictive silencer factor/RE1-silencing transcription factor (NRSF/REST) expression with the simultaneous upregulation of DYRK1A (mapped on chromosome 21q22.13) and protocadherin gamma cluster (PCDHG) gene expression observed during early T21 development may contribute to insufficient neural circuit formation in the developing brain. The upregulation of DNMT3L (on chromosome 21q22.4)

could additionally lead to de novo methylation during neurodevelopment, resulting in DNMT3A and DNMT3B downregulation in the brains of T21 fetuses [50]. The epigenetic signature of T21 is mainly enriched in genes responsible for hematopoiesis, morphogenesis, and development, and the regulation of the chromatin structure in neurons [51]. This observation may provide useful novel biomarkers for T21 brain development and potential novel medical targets for prenatal therapeutic interventions.

Analyses of novel markers in T21 screening have mainly focused on noncoding nucleic acids such as microRNA (miRNA). A miRNA is a single, non-coding RNA molecule that can be obtained from the maternal compartment as a useful diagnostic tool to identify fetal T21 occurrence [22,28]. MiRNAs can affect protein expression by interfering with RNA translation or promoting mRNA degradation [32,33]. Combined with DNA methylation, miRNA provides a means to evaluate changes in gene activation and expression, and to understand the impact on gene clusters that affect particular pathways [34,35]. Data in the literature prove that T21 is associated with multiple patterns of deregulation in maternal plasma miRNA expression, such as that of let-7c, miRNA-99a, miRNA-125b, miRNA- 155, miRNA-802, miRNA-3118, miRNA-3156, miRNA-3196, miRNA-3648, miRNA-3687, miRNA-4327, miRNA-4759, and mir-99a [32,34–36]. This differential expression is related to the occurrence of neuropathology, leukemia, hematopoiesis, congenital heart defects,and autism during T21 development [32,33,37,38]. Moreover, altered expression of miR-1973, miR-3196, and miR-138 related to T21 comorbidities has also been reported [38–40]. Deregulated expression of miR-138-5b and miRNA-155 has a significant impact on hippocampal tissues from T21 fetuses, and the downregulation of this target may be involved in intellectual disability and neurological deficiency [35,38,41]. Zbucka-Krętowska et al. revealed 13 miRNAs differentially expressed—six miRNAs upregulated (hsa-miR-15a, hsa-let-7d, hsa-miR-142, hsa-miR-23a, hsa-miR-199 and hsa-miR-191) and seven downregulated (hsa-miR-1290, hsa-miR-1915, hsa-miR30e, hsa-miR-1260, hsa-miR-483, hsa-miR-548 and hsa-miR-590)—in maternal plasma obtained from T21 pregnancies, which were considered to make up a potential noninvasive second-trimester prenatal screening panel [35]. The study was conducted on 12 patients with fetal DS and 12 patients with uncomplicated pregnancies considered as the control group, using NanoString technology, with the determination of the expression levels of 800 miRNAs. Prenatal biomarkers play an essential role in early diagnosis, prediction and clinical management [28]. Since the pathophysiology of T21 is extremely complicated, determining the disturbed metabolic pathways is highly recommended. It can be hypothesized that assessing the trisomy 21-induced overexpression of chromosome 21-derived miRNAs will become the standard of T21 diagnostics in the future [52]. Moreover, a study performed by Erturk et al. showed that the suggested variation in miR-155 expression commonly observed with miR-802 assessed in T21 tissues was associated with immunological complications, in particular, with the upregulation of CD4+ T cells. For this study, 56 patients underwent invasive prenatal testing, 23 of which were carrying fetuses affected by Down syndrome, and 33 control cases were included for comparison. All the biological material was collected during the 17th and 18th weeks of gestation, and the miRNA expression levels were measured using real-time RT-PCR. In this case, differentiation into Th-1 lymphocytes, leading to a reduced number of embryonic B cells and extra-follicular B cells, was observed. The deregulation of microRNA expression may be the reason for the reduced synthesis of high-affinity IgG antibodies observed in T21 patients [30,34]. These results could also be analyzed using novel medical target approaches.

Undeniably, genomics is still a new field in science. As mentioned, cffDNA has had a significant influence on the evolution of prenatal T21 screening, and the promise of miRNA is under constant examination. On the other hand, genetic tests are characterized by limitations in sensitivity because only a subset of causative mutations can be identified simultaneously. Furthermore, the sensitivity of the test may be influenced by ethnicity. The detection of global changes in miRNA expression and the subsequent interpretation of such data may additionally be dependent on the specific platform used [53]. Novel

genetic technologies could enhance the diagnostic sensitivity. Genomic instability, aneuploidy and other polymorphism-based variations that originate in the female germline and contribute to developmental defects during T21 development can be determined through investigations based on sequencing and epigenetic techniques. However, the high cost and complex nature of post-data metanalyses currently limit the worldwide implementation of this procedure, especially in underdeveloped and moderately developed countries [52,54]. Molecular genetic techniques augment chromosome analysis, broadening the range of identifiable genetic abnormalities, and may accelerate the clinical management of patients [55].

5. Metabolomic Profiles as Down Syndrome Markers

Metabolomic methods are mainly based on nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) [56]. NMR and MS are powerful analytical techniques used to quantify unknown/known biological materials, identify unknown compounds in samples and elucidate the structure and chemical properties of different molecules, with the subsequent evaluation of concentrations. NMR is characterized by the chemical shift of protons (H-1 NMR) or carbon (13C-NMR) atoms. The shift depends on the range of atoms in the subject atom's vicinity. A mass spectrometer generates multiple ions from the sample under investigation, followed by separation based on their specific mass-to-charge ratio (m/z). Thus, records of the relative abundance of each ion type are established [57]. In comparison with genomics, metabolomics techniques can be used to analyze pleiotropic molecular metabolites obtained from biological compartments, the quantitative determination of which could be considered in screening novel biochemical and, in the future, evaluating treatment follow-up markers [58]. The results obtained from maternal plasma and amniotic fluid evaluation are reliable and have been validated for the discovery of novel T21 screening biomarkers [58–60].

Following a study performed by Bahado-Singh et al. using NMR-based metabolomics, 11 maternal serum novel metabolites (2-hydroxybutyrat, 3-hydroxybutyrate, 2-hydroxyisov alerate, acetamide, acetone, carnitine, dimethylamine, lactate, methionine, pyruvate and L-methylhistidine) were revealed as being significantly different between T21 and euploid pregnancies, and, more importantly, three of the examined molecules (3-hydroxybutyrate, 3-hydroxyisovalerate and 2-hydroxybutyrate) were reported to have increased concentrations during the first trimester of pregnancy. These metabolites are produced as a result of complementary mechanisms, and are involved in myelination and in the prevention of increased levels of oxidative stress, which are confirmed in T21 pathogenesis. Furthermore, 3-hydroxybutyrate is a ketone, which is an important substrate for phospholipid and sphingolipid synthesis. Accordingly, both phospholipids and sphingolipids are required for neuronal transition processes and myelination.

Regarding sphingolipid pathways, Charkiewicz et al. proved the second-trimester screening utility of measuring selected sphingolipids in the maternal plasma and amniotic fluid [61]. A significant increase in the levels of two ceramides, C22-Cer (AUC = 0.814) and C24:1-Cer (AUC = 0.729), in the T21 pregnancies was observed. On the other hand, decreases in the concentrations of seven ceramides were reported: C16-Cer (AUC = 0.857), C18-Cer (AUC = 0.968), C18:1-C (AUC = 0.897), C20-Cer (AUC = 0.960), C22-Cer (AUC = 0.873), C24:1-Cer (AUC = 0.905), and C24-Cer (AUC = 0.802) [61]. The study was conducted on samples from 10 pregnancies with confirmed Down syndrome between the 15th and 18th gestational weeks using ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC/MS/MS).

In another extended study, Parfieniuk et al. performed plasma metabolomics using liquid chromatography–mass spectrometry (LC-MS), using samples obtained from 12 pregnancies with confirmed fetal T21, and 15 pregnant women with euploid fetus consisted as a control group, being between the 15th and 18th gestational weeks, and reported a significant decrease in five maternal metabolites: butyryl-L-carnitine, palmitic amide, linoleamide, oleamide, and piperine. The combination of linoleamide and piperine was

reported to have higher sensitivity and specificity in the screening of T21 aberrations. Palmitic amide, linoleamide, and oleamide are also known as fatty acid amides (FAAs) and have been described as molecules able to block gap junction communication in glial cells, with a relevant impact on memory processes, the stimulation of Ca^{2+} release, and the activation of serotonin and endocannabinoid receptors [59]. Piperine, an exogenous alkaloid, is characterized by anti-inflammatory, antioxidant, antipyretic, antidiarrheal, and gastro- and neuroprotective properties. Therefore, the observed decreased piperine level could be another reliable biomarker of insufficient nervous system development in T21 fetuses (Table 2) [62].

Table 2. Comprehensive list of discriminating metabolites that can serve as reliable biomarkers in T21 prenatal screening ($p < 0.05$) [58,63].

Biological Sample	Significant Deregulated Metabolites in T21 Prenatal Screening
maternal blood	2-hydroxybutyrate, alanine, citric acid, phenylalanine, 3-methyl histidine, proline, benzoic acid, glyceric acid, mannose, myristic acid, stearic acid
maternal serum	2-hydroxybutyrate, 3- hydroxybutyrate, acetone, glycerol, glycine, isobutyrate, ornithine, phenylalanine, succinate, methylhistidine, arginine, 12-hydroxybutyrate, carnitine, lactate, pyruvate, dimethylamine, methionine
maternal plasma	butyryl-L-carnitine, palmitic amide, linoleamide, oleamide, piperine, proline, methanol, creatinine
maternal urine	dihydrouracil, methanol, β -hydroxybutyrate
amniotic fluid	methylhistidine, hexanoylcarnitine, diacetylspermine, and p-cresol sulfate

A study conducted by Nemutlu et al. used a metabolomic platform, gas chromatography–mass spectrometry (GC-MS), and liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-qTOF-MS) to find possible metabolites differentiating between healthy/normal and T21 pregnancies that could confidently be used for T21 screening. This study noted significant alterations in the concentrations of l-threonic acid, beta-alanine, oxalic acid, creatinine, alpha-tocopherol, cholesterol, uracil, and 2-piperidone, associated with an increased risk of T21 occurrence [64]. All of these metabolites previously showed vital roles in fetal development, such as beta-alanine (an antioxidant), which is the building block of carnosine and has been associated with extended muscular endurance in pregnancy. Alpha-tocopherol, a naturally occurring form of vitamin E (antioxidant), participates in lipid metabolism and regulates oxidative stress status, which is essential for proper fetal (brain) development. Furthermore, uracil has neuroprotective properties as a substrate of uracil-DNA glycosylase and uridine phosphorylase enzymes. These enzymes also elimi- nate mediators of oxidative stress, providing protection against brain neurodegeneration. The decreased plasma levels of uracil observed in T21 pregnancies could potentially be associated with fetal neurodegeneration and increased oxidative stress and lipid peroxidation [64].

In summary, metabolomics has been considered as a powerful tool for identifyingnovel T21 screening biomarkers. However, one should bear in mind the relevant differences in the patients' genotypes, medical histories, disease development, ethnicities, and diets and ages, which might affect the metabolome and directly influence the obtained results. Additionally, as metabolomics evaluation is usually based on different platforms, analytical protocols with different sample preparation methods and data analysis techniques may also contribute to controversial and divergent outcomes [65,66]. Furthermore, metabolomics studies require highly trained personnel and huge financial investment, which may not be feasible at the clinical level for the purpose of T21 screening. However, these methods are widely used in the determination of disturbed metabolic pathways, constituting a founda- tion for research in the development of novel screening markers, and are more valuable in evaluating possible medical targets. It is still impossible to designate a uniform T21 treatment; however, efforts are ongoing to devise personalized therapy for T21 treatment using state-of-the-art omics (genomic, proteomic, and metabolomic) approaches. Last but

not least, metabolomics is a novel, useful tool for determining insufficiencies in metabolic pathways in T21 and T21 pregnancies, with potential for the evaluation of novel medical targets. However, there is still a need to find cost-effective techniques for validation.

6. Proteomics and Down Syndrome Screening

The proteome describes the protein component expressed in cells and tissues. By using proteomic techniques, isoforms and protein post-translational variants can also be evaluated. In addition, post-translational modifications such as phosphorylation, ubiquitination (which modulates protein activity and mediates signal transduction), and proteolytic cleavage can be determined. Current proteomics use MS with LC-MS-MS and matrix-assisted laser desorption/ionization (MALDI) equipment [67]. The MALDI method is based on an ionization technique that uses a laser energy-absorbing matrix to create ions from large molecules with minimal fragmentation.

Proteomics could also enable the detection of novel and more affordable T21 biomarkers [68,69]. Accordingly, Charkiewicz et al. suggested that imbalance in the level of circulating proteins in maternal blood can stimulate an immune response producing au- toantibodies. In this study, 190 amniocenteses were performed, and 10 patients with confirmed fetal Down syndrome (15th–18th weeks of gestation) were found. Statistical analysis of the expression of 9000 autoantibodies in T21 pregnancies, revealed using a protein microarray, which allows for the simultaneous determination of 9000 proteins per sample, showed that the expression of 213 autoantibodies was significantly different when compared with that in euploid pregnancies. Moreover, this panel could potentially be used in prenatal T21 screening, based on the specification of the predictive value (specificity and sensitivity) equal to 100%, 0% classification errors, and 0% cross-validation errors [70].

Following the evaluation of disturbed immune response, Laudanski et al. indicated that chemokine measurement could also be relevant in prenatal T21 screening. Based on a protein microarray, that study reported that seven women with fetal DS in the 15th–18th weeks of gestation had increased plasma concentrations of one chemokine, CXCL7 (NAP-2), and decreased plasma concentrations of four chemokines, hemofiltrate CC chemokine 4 (HCC-4), interleukin 28A (IL-28A), interleukin 31 (IL-31), and monocyte chemotactic protein 2 (MCP-2). The MCP-2 measurement was characterized by the highest diagnostic value, based on AUC = 0.830 [71]. Research performed by Zbucka-Kretowska et al. demonstrated significant increases in the T21 maternal plasma concentrations of four angiogenic factors (transforming growth factor beta 1 (TGFb1), angiostatin, chemokine (C-C motif) ligand 1 (I-309), transforming growth factor beta 3 (TGFb3), and vascular en- dothelial growth factor D (VEGF-D)), and one antiangiogenic (angiostatin), and decreases in the concentrations of 14 angiogenic factors (leptin, angiopoietin 1 (ANG-1), angiostatin, epidermal growth factor (EGF), interleukin 1-beta (IL-1b), interleukin 4 (IL-4), interleukin 12p40 (IL-12p40), MCP-2, matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-9 (MMP-9), platelet endothelial cell adhesion molecule 1 (PECAM-1), transforming growth factor alpha (TGF alpha), vascular endothelial growth factor 2 (VEGFR2), and vascular endothelial growth factor 3 (VEGFR3)). The study used protein microarrays, which enable the simultaneous determination of 60 angiogenic factors per sample [72]. It was conducted on 20 patients with T21 fetuses and a control group of 28 healthy patients with uncom- plicated pregnancies in women who delivered healthy newborns at term. The biological material was collected during the 15th–18th weeks of gestation. Based on bioinformatic analysis, these disturbances were associated with tissue remodeling, bone formation during embryogenesis, and the insufficient immune system activity observed during T21 fetus development [72,73].

Proteomics can also be used to determine disturbed metabolic pathways. Many stud- ies have reported the overexpression of several plasma proteins as a result of trisomy 21 development [74–77]. In evaluating duplicated chromosome 21 genes, several antiangiogenic factor genes were mapped. These gene abnormalities were the basis for subsequent research in the field of disturbed protein expression, resulting in specific changes in T21

pregnancy proteinograms. Several proteins have been shown to be differentially expressed in T21 maternal serum [68,78]. A study by Kolialexi et al., using Western blotting, found that the plasma transthyretin (THY), ceruloplasmin (CERU), afamin (AFAM), alpha-1-microglobulin (AMBP), apolipoprotein E (APOE), serum amyloid P-component (SAMP), and histidine-rich glycoprotein (HRG) concentrations were upregulated, with a simultaneous decreased concentration of clusterin (CLUS) [79]. In this study, plasma obtained from eight women carrying DS fetuses and twelve with non-DS fetuses was analyzed using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Three proteins (AFAM, CERU, and TTHY) are involved in carrying factors, such as fat-soluble vitamin E, copper, a thyroid hormone, thyroxine (T4), and retinol-binding protein bound to retinol. These results were also associated with poor pregnancy outcomes [80]. These proteins are necessary for proper hormone synthesis, antioxidant defense, and cell development. CLUS, an acute phase protein, is involved in diseases related to oxidative stress [81]. In this case, the disturbed protein profile observed in the maternal compartment resulting from T21 pregnancy could be related to the many comorbidities observed in T21 fetuses [79,82,83].

Sui et al. reported increased levels of seven proteins (oxoglutarate dehydrogenase L (OGDHL), serum amyloid P component (SAP), ApoE, nucleosome assembly protein 1-like 1 (NAP1L1), thymosin beta 10 (T β 10), complement factor B, and endoplasmic reticulum oxidoreductase 1 alpha (ERO1L)) in maternal plasma and umbilical cord blood obtained from T21 pregnancies [83]. The study was conducted on maternal peripheral blood (eight with fetal DS and eight with normal fetuses) using Western blotting. OGDHL is a functionally active isoenzyme of oxoglutarate dehydrogenase (OGDH) present in brain tissue, the main malfunction of which is related to neurodegeneration [83]. SAP can interfere with lipoprotein metabolism by activating and regulating amyloid formation [83]. NAP1L1 has a prominent role in the early development of cardiac or stem cells that differentiate into myocardial cells [83]. T β 10 is related to cell proliferation, cell morphology, cell migration, and endocytosis and participates in cytoskeleton assembly. It can be hypothesized that the overexpression of SAP, NAP1L1, and T β 10 proteins is associated with cognitive impairment in T21 individuals and the early development of Alzheimer's disease (AD) observed in early-stage T21 development [83,84].

In summary, the application of proteomic technologies to the evaluation of biological compartments creates novel possibilities for elucidating the patomechanism and discovering novel drug targets and early disease markers. At the same time, proteomic results showing how sets of proteins interact with environmental factors are constantly changing [85]. Furthermore, the concentrations of many proteins depend on their locations in biological compartments and the phase of the cell cycle, which can also be interrupted by many diseases [68,69]. However, the extensive software required for utilizing proteomic data and the need for highly proficient technicians substantially increase the cost. Moreover, quality control has not yet been developed, so the clinical requirements are not met [86]. It should be emphasized that proteomics has already contributed to significant progress being made in determining insufficient biological pathways in T21 aneuploidy.

7. Single-Protein Determination

Single proteins can be measured using different methods, but the enzyme-linked immunosorbent assay (ELISA) is the one most often used. This is a plate-based method used in a wide range of diagnostic laboratories around the world, designed for the sensitive and quantified measurement of soluble substances such as peptides, proteins, antibodies, steroids, and glycoproteins [87]. ELISA can be used in many settings, including the clinical diagnosis of human diseases. Based on its cost-effectiveness and uncomplicated protocols, not involving complicated sample pre-treatment, this method is an important part of medical care and scientific research [88]. This method could also be a useful tool for meeting the challenge of introducing results obtained with omics-based methods into daily routine diagnostics while also validating procedures. Unfortunately, it cannot

be ignored that traditional ELISA is time-consuming and imprecise, on account of the evaluation of one variable (substance), compared with metabolomics or proteomics, in which the entire metabolome and proteome can be studied simultaneously. In this case, the multiplex ELISA-based method could be a corresponding modification to meet these requirements [89]. However, single biomarkers are not likely able to serve as the best diagnostic or prognostic markers for T21 due to their limited discriminatory power. On the other hand, biomarker panels comprising multiple measured analytes provide high sensitivity and specificity for distinguishing T21 from euploid pregnancies [90].

ELISA is still widely used in research on improving the utility of recommended prenatal screening. A study performed by Chambers et al. revealed that the additional assessment of β hCG with its cognate receptor (hCG-sLHCGR) increased the diagnostic usefulness of single-protein prenatal measurements. A comparison of the assessed methods for prenatal screening with received AUC values is presented in Table 3.

Table 3. Utility of T21 prenatal screening panels.

T21 Screening Panel	AUC Based on ROC Curves
β hCG + PAPP-A	0.918
PAPP-A + NT	0.922
PAPP-A + hCG-sLHCGR	0.920
β hCG + hCG-sLHCGR	0.856
β hCG + NT	0.753
hCG-sLHCGR + NT	0.888
NT + PAPP-A + β hCG	0.940
NT + PAPP-A + hCG-sLHCGR	0.928
hCGsLHCGR + NT + PAPP-A + β hCG	0.966

AUC, area under the receiver operating characteristic (ROC) curve; β hCG, chorionic gonadotropin beta subunit; hCG-sLHCGR, human chorionic gonadotropin with its cognate receptor LH/hCG-R or LHCGR; NT, nuchal translucency; PAPP-A, pregnancy-associated plasma protein A.

To date, several proteins have been determined by novel T21 prenatal screening applications. It is suggested that proteins related to lipid metabolism may be of great importance in the T21 patomechanism, and therefore in diagnostics. Furthermore, the relationship between maternal ApoE and fetal T21 occurrence was previously suggested through polymorphism evaluation [91]. Studies suggest that an increased maternal frequency of the APOE4 allele should be considered a risk factor of T21 occurrence [92,93]. Moreover, APOE ε 4 is associated with a worse prognosis in early development for individuals with DS [94]. Following the evaluation of ApoE polymorphism, the screening utility of ApoE measurement in second-trimester T21 screening was analyzed. Considering this preliminary study, the plasma concentration of ApoE was significantly higher in the T21 pregnancy group than in euploid pregnancies. Furthermore, the screening utility was proved by AUC = 0.978, with the cut-off point set at 1.37 mg/mL. This T21 screening marker was characterized by 80% sensitivity and 100% specificity [9].

Following the evaluation of disturbed metabolic pathways in T21 pregnancies, where the lipid pathway is inherently connected to the carbohydrate pathway, the novel insulin-resistance marker protein asprosin and advanced glycation end products (AGEs) were evaluated for T21 prenatal screening. The beneficial role of asprosin measurement in prenatal screening was characterized by 100% sensitivity, 85% specificity, and AUC = 0.965. The AGE assessment showed 80% specificity and 81% sensitivity for screening. Furthermore, the SOD-2 genes mapped on chromosome 21 and the impact of oxidative stress on T21 development have been studied [95–99]. Accordingly, oxidative stress markers were evaluated for T21 prenatal screening. It was proved that measuring the products of DNA/RNA damage induced by oxidative stress could be a novel tool in T21 prenatal screening. Moreover, the anti-inflammatory protein α -1-antitrypsin (A1AT), which has antioxidative properties, was also suggested as a novel T21 screening marker [100]. Interestingly, the level of A1AT was found to be downregulated in T21 aneuploidy. The results

suggest that the decrease in A1AT concentration combined with aggravated inflammation processes and oxidative stress observed in T21 pregnancies may negatively impact multiple comorbidities and the occurrence of fetal malformations. The proposed novel T21 screening markers are characterized in Table 4 [101].

Table 4. Diagnostic utility of tested novel screening markers.

Marker	Unit	AUC	Cut-Off Value	Sensitivity	Specificity
ApoE	ng/mL	0.978	>1.37	80%	100%
Asprosin PS	ng/mL	0.970	>12.70	100%	85%
Asprosin AF	ng/mL	0.830	>12.91	95%	65%
AGE PS	ng/mL	0.850	<11.00	81%	80%
AGE AF	ng/mL	0.960	<4.184	95%	90%
A1AT PS	mg/L	0.530	<2.341	81%	33%
A1AT AF	mg/L	0.870	<0.3180	76%	86%
DNA/RNA OSDP PS	pg/mL	0.510	<40.30	80%	40%
DNA/RNA OSDP AF	pg/mL	0.730	>31.76	84%	58%

A1AT, alpha-1-antitrypsin; AF, amniotic fluid; ApoE, apolipoprotein E; AGE, advanced glycation end product; AUC, area under receiver operating characteristic (ROC) curve; OSDP, oxidative stress damage product; PS, plasma.

8. Discussion

The current trend in prenatal testing represents a massive rearrangement from invasive to non-invasive or less-invasive sampling procedures [102]. The introduction of noninvasive prenatal testing using cffDNA was a breakthrough moment for prenatal aneuploidy screening [103]. It is reasonable to anticipate that further advances in prenatal screening development could lead to improvements in biochemical screening accuracy, following the promising result obtained by the potential introduction of omics methods into the prenatal screening [104]. Our review reveals that, while the successful use of omics techniques in prenatal screening has been reported, many challenges still exist. Moreover, the omics methods can be used for rapid and precise screening of large amounts of samples. Unfortunately, processing of biological material often requires complex preparations, a large amount of various reagents, and the work of a specialized group of scientists [105]. Moreover, the testing remains expensive, which limits its introduction into routine diagnostics, especially in less-developed and medium-developed countries [106]. From the other hand, the complexity of omics data analysis requires data integration and pipeline validation supported by bioinformatics and biostatistics.

Therefore, constantly updated databases are needed to standardize the proposed T21 biomarker reference values and improve data management. Further integration of these approaches exploits the advantages of these techniques, providing a rapid and accurate on-site method for T21 detection and quality control of potential screening biomarker validation [107]. In this case, omics methods could be effectively incorporated from research laboratories to everyday routine diagnostics as costs and processing time for sample analyses continue to decrease, which has been noticed. This translates to an increased contribution of omics methods in clinical trials, which may result in including them in the standards and recommendations of diagnostic procedures [103,108]. Especially in prenatal screening—the results obtained with the used of omics could be particularly useful in the early and precise fetal defects screening. Up to date, ELISA was a useful tool which meet the challenge of introducing results obtained with omics-based methods into daily routine diagnostics with subsequent validation of different procedures. Accordingly, due to the recent advance in genomics, current prenatal testing has evolved mainly tocell-based assays and cffDNA [103].

However, there is still a need for novel, omics-based studies in order to improve T21 prenatal screening and, more importantly, to discover potential medical targets. Proposed T21 therapy has focused on pharmacological treatment to improve cognition. A number of compounds have been shown to exhibit potential beneficial properties, reported to improve learning and congenital anomalies [109]. Chronic treatment with picrotoxin or

pentylenetetrazol improved deficits in hippocampus-based learning and long-term potentiation. Nevertheless, these trials are still carried out on a mouse model, Ts65Dn (which displays various DS phenotypes), which extends the time before the proposed solutions are implemented in routine clinical management [20–22]. The integrated use of omics in T21 evaluation should be thoroughly investigated in the nearly future [110]. Personalized medicine and omics technologies together provide global understanding of the mechanisms responsible for T21 occurrence. Advances in omics results should be correlated with the congenital disabilities and others comorbidities occurring during T21 development, moving this trend toward a personalized medicine and management course to clarify the molecular mechanisms underlying T21 pathogenesis. Simultaneously, the discovery of potential prenatal biomarkers and therapeutic targets could provide more detailed patient stratification and personalized treatment improving clinical management [111].

9. Conclusions

The introduction of integrated omics methods into routine non-invasive prenatal screening could increase the detection rate of fetal aneuploidy including T21. Based on our literature search, it can be concluded that cbDNA and cffDNA analysis demonstrate the vast potential in NIPT. However, there is still a need to provide useful data in order to validate their usefulness. Moreover, the development of fully automated systems remains essential to introduce modern technologies in prenatal screening. Accordingly, novel approaches have provided new insights into the complex pathophysiology of T21, which could be further used in novel therapeutic strategy evaluation.

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The Significance of Apolipoprotein E Measurement in the Screening of Fetal Down Syndrome

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Abstract: Prenatal screening for Down syndrome (DS) is based on both noninvasive and invasive methods. Noninvasive, cell-free fetal DNA genetic tests are expensive, whereas biochemical methods remain imprecise. Amniocentesis is the most frequently used invasive diagnosis procedure, characterized by 99.8% diagnostic efficiency and less than 1% risk of miscarriage. The aim of this study was to evaluate the screening value of apolipoprotein E (ApoE) as a potential noninvasive biomarker for prenatal DS assessment. This study was conducted on a group of female patients who decided to undergo routine amniocentesis between the 15th and 18th week of pregnancy at the Department of Reproduction and Gynecological Endocrinology of the Medical University of Bialystok, Poland. For the purpose of this study, 20 women with DS fetuses were selected as the study group, and 20 healthy pregnant women with euploid fetus karyotypes as the control group. The plasma levels of ApoE were significantly higher in the study group compared to healthy subjects ($p < 0.05$). The area under the receiver operating characteristic (ROC) curve was 0.978 ($p < 0.001$), with the cut-off set to 1.37 mg/mL, which was characterized by 80% of sensitivity and 100% of specificity. The high sensitivity and specificity demonstrate the screening utility of maternal ApoE concentration in prenatal fetal DS screening.

Keywords: Down syndrome; prenatal diagnosis; apolipoprotein E

1. Introduction

Trisomy 21, also known as Down syndrome (DS), is one of the most frequently occurring chromosomal disorders worldwide [1,2]. The disease affects 1 in every 787 live births and is characterized by the abnormal division of genetic material resulting in an additional chromosome 21 or its part [3]. Trisomy 21 is the major cause of DS, accounting for about 95% of cases [4]. This disease results in unequal distribution of DNA material and metabolic pathway dysfunction, including lipid metabolism disturbances, increased oxidative stress, mitochondrial dysfunction, and tau phosphorylation [5]. Maternal age above 35 years and the occurrence of balanced translocation in one of the parents are the main risk factors.

Apolipoprotein E (ApoE), a glycoprotein with a linear polypeptide chain rich in arginine, is a hydrophilic component of high-density lipoprotein (HDL), very low-density lipoprotein (VLDL),

lipoproteins, and chylomicrons [6]. It is mainly synthesized in the cell periphery of hepatocytes, but also in macrophages, astrocytes, lungs, kidneys, spleens, and muscle cells, with the highest expression in the liver and brain. In the brain, the main supply of ApoE is in the blood–brain barrier, and it is observed in the cerebrospinal fluid at concentrations of ~5 mg/L; it is mainly produced by astrocytes, neurons, and damaged microglia [6,7]. The maturation of the fetus is dependent on the undisrupted development of the nervous system during its growth. Approximately 23% of lipids are accumulated in the brain, particularly in neurons and astrocytes. During the first few weeks of gestation, the developing fetus mainly uses maternal cholesterol. Fetal cholesterol and apolipoproteins (ApoA1, ApoE, and ApoB), together with HDL, LDL, or VLDL are crucial for moderating embryonic signaling pathways [8]. Therefore, ApoE is considered to be a promoter of myelination, synaptogenesis, and other processes related to neurodevelopment that involve lipids.

Nowadays, prenatal screening of DS is based on both noninvasive methods, which estimate the risk of DS-affected pregnancy, and invasive techniques, which verify the presence of chromosomal aberrations. Serum screening and ultrasounds are intended to identify women with pregnancies at high risk of chromosomal abnormalities. However, diagnostic testing is indicated in international guidelines, mainly focusing on individual risk assessment based on historical, biochemical, and biophysical variables [9]. Amniocentesis is the most frequently used invasive procedure, which has a diagnostic efficacy of 99.8% and poses less than 1% risk of miscarriage [10–12]. The discovery of genetic testing using free fetal DNA (ffDNA), whose concentration can be measured in maternal peripheral blood, was a significant breakthrough in noninvasive screening. In addition to the 0.5% false-positive rate, this technique is still comparatively expensive [13–17]. Therefore, it is important to find a cost-effective and noninvasive screening biomarker with high sensitivity and specificity that would provide indisputable benefits, subsequently reducing the number of incorrect indications for amniocentesis diagnosis. Moreover, more comprehensive knowledge of DS pathogenesis, including the understanding of metabolic pathway alterations, may introduce new treatment targets and improve patients' quality of life.

The aim of the study was to evaluate the screening usefulness of maternal ApoE measurement as a potential noninvasive marker in prenatal diagnostics of DS.

2. Experimental Section

The study and control groups consisted of women who underwent routine amniocentesis between the 15th and 18th weeks of gestation at the Department of Reproduction and Gynecological Endocrinology of the Medical University of Bialystok, Poland. The indication for amniocentesis was an increased risk of chromosomal aberrations in noninvasive prenatal screening or patient age above 35 years. Exclusion criteria were as follows: chronic or acute diseases, hormonal treatment, anti-inflammatory treatment, high-risk pregnancy, or preterm delivery in the patient's medical history. All participants were informed about potential risks prior to the procedure and received relevant information regarding the study. Study participants were matched according to age, ethnicity, socioeconomic status, the course of pregnancy, body mass index (BMI), and the number of pregnancies with marked episodes of pregnancy pathology. The patient recruitment period started in 2017 and lasted 2 years. Following karyotype test result analysis, 20 women carrying fetuses with DS and 20 women with euploid (non-DS) fetuses were enrolled in the study. All patients were submitted to amniocentesis within a determined period, then randomized. An adequate sample size to detect a difference was demonstrated using power analysis [18]. Venous blood (5.5 mL) was obtained from participants at the day of amniocentesis, centrifuged, and plasma was subsequently separated and frozen at −80 °C.

Plasma ApoE concentration was determined using an enzyme-linked immunosorbent assay (ELISA) (ELISA Kit for apolipoprotein E (ApoE); Cloud-Clone Corp., Wuhan, Hubei 430056, China, SEA704Hu) according to the manufacturer's protocol and observing the principles of internal laboratory control for the performed determinations. Samples and controls were measured in the same run using

the blind analysis method. Duplicate samples were assessed and the average of the two results was calculated [19]. Statistical analyses were performed using Statistica 13.3 (StatSoft, Tibco Software Inc., Palo Alto, CA, USA). During the analysis, the normality of data distribution was demonstrated ($p > 0.05$). Thus, the groups were compared using a parametric two-way ANOVA test; $p < 0.05$ was considered statistically significant. In addition, the receiver operating characteristic (ROC) curves were determined using a medical package included in the Statistica program. Diagnostic sensitivity and specificity were calculated using a cut-off value that was calculated by the Youden's index (as a criterion for selecting the optimum cut-off point) [20].

The procedures were approved by the Local Ethics Committee of the Medical University of Bialystok, Poland, and written informed consent was obtained from each participant (R-I-002/36/2014).

3. Results

In this research, the first participant was entered on 1 January 2015. The last participant was randomized on 12 April 2019, at the end of the trial. A total value of 100 pregnant women were screened, and 40 were randomized and enrolled for the subsequent analysis.

Statistical Analyses

Basic statistics that were measured in the study, control, and total group, such as average ApoE concentration values, minimum and maximum values, and standard error, are presented in Table 1. ApoE concentrations were significantly higher in the study group compared to healthy subjects ($p < 0.001$). The comparison of ApoE concentrations measured in the study vs. control group is presented in Figure 1.

Table 1. Basic statistics of plasma ApoE measurement (data presented in mg/L).

Parameter	Study Group (n = 20)	Control Group (n = 20)	Total Group (n = 40)
Minimum value	1.27	0.66	0.66
Maximum value	2.18	1.31	2.18
Mean	1.57	1.02	1.3
SD	0.25	0.18	0.35

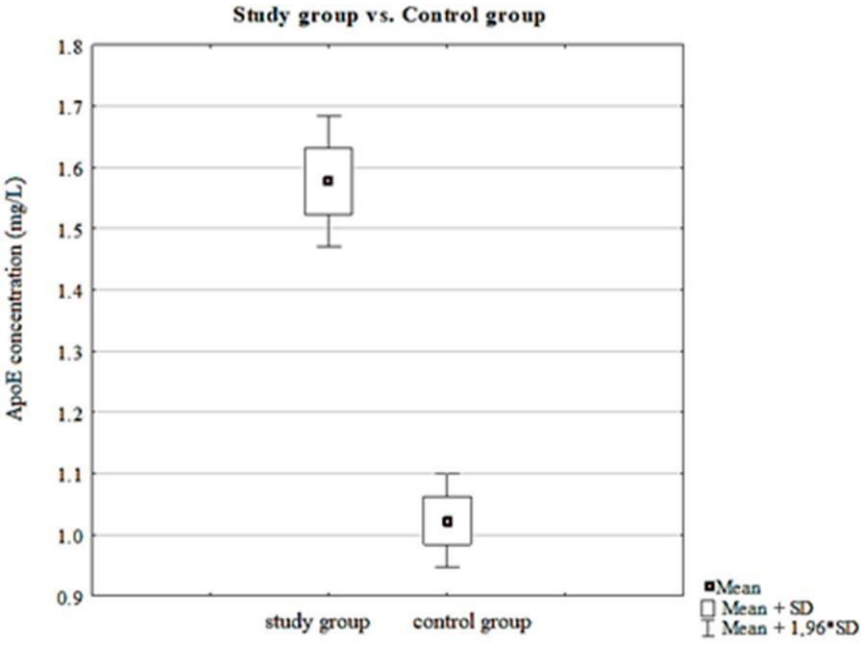

Figure 1. Concentration of apolipoprotein E (ApoE) measurements in study vs. control group. SD, standard deviation.

To determine the diagnostic utility of the ApoE test, the ROC curve was calculated as an illustration of the relationship between sensitivity and specificity (Figure 2). The cut-off point was set at 0.85 using Youden's index, simultaneously establishing the diagnostic norm of ApoE as 1.37 mg/L. The sensitivity, accuracy, specificity, and positive and negative predictive values (PPV and NPV, respectively) are presented in Table 2.

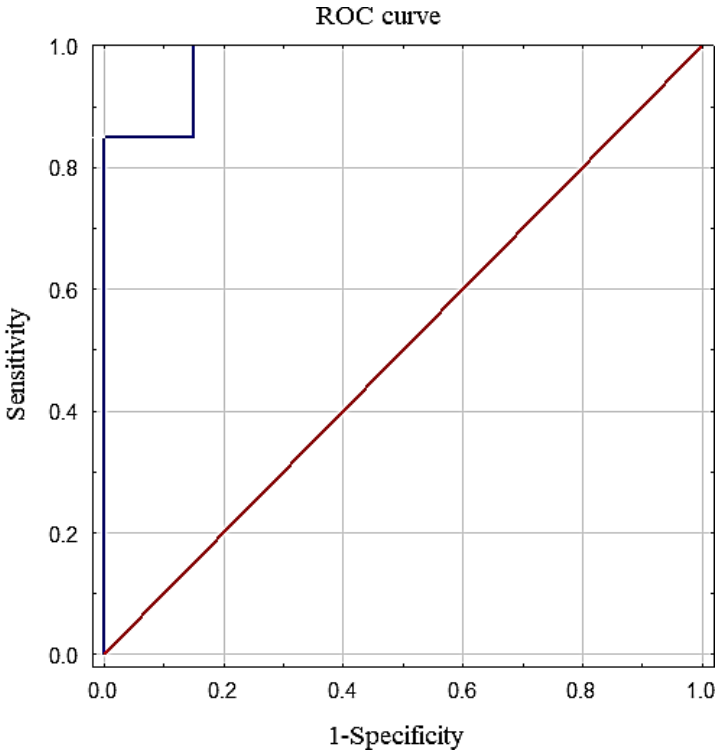

Figure 2. Receiver operating characteristic (ROC) curve for ApoE in Down syndrome (DS) screening.

Table 2. Statistical parameters of ApoE measurement.

Parameter	Sensitivity	Accuracy	PPV	NPV	Specificity
ApoE (cut-off point = 1.37 mg/L)	80%	82%	100%	83%	100%

NPV, negative predictive value; PPV, positive predictive value.

To evaluate the clinical applicability of ApoE as a prenatal screening tool, the area under the ROC curve (AUC) was evaluated. The AUC value was 0.978, which was significantly higher in comparison to AUC 0.5, which is the threshold of the diagnostic usefulness of a test ($p < 0.001$).

4. Discussion

Despite DS being the most common chromosomal aberration occurring in all races, the pathological process, as well as the incorrect division of DNA material, are not yet fully understood [21–23]. It has been demonstrated that pregnant women with a DS fetus suffer from lipid disorders related to disturbed cholesterol metabolism and lipid transport; incorrect distribution of VLDL, LDL, and lipid peroxidation [4]; and altered concentration of sphingolipids, which leads to improper myelination of fetal neurons [24]. These patients also show insufficient endothelial function, which is related to the inflammation process, oxidative stress, and dysregulated lipid metabolism, which may result in insufficient cell division [25].

Screening tests are used to survey a population by measuring a specific marker to define screening cut-off levels, with subsequent identification of a high-risk group for a particular disorder [26]. Multiple screening tests are used, involving the combination of a few biochemical tests, usually combined

with maternal age or an ultrasound examination, to estimate the risk of DS occurrence [27]. To compare the diagnostic efficiency, the combination of ultrasound examination with pregnancy-associated plasma protein A (PAPP-A) and serum-free human chorionic gonadotropin (B-HCG) measurement, using a 5% screen-positive rate, allows for the detection of 82–87% DS pregnancies [28]. Increased maternal serum ApoE concentration in DS-affected pregnancies has been previously explored, but the diagnostic utility of this marker has not been comprehensively illustrated [27].

The first attempt to evaluate the influence of ApoE on adolescent neurodevelopment was made by Tratnik et al., who conducted research on the association between prenatal exposure to mercury (Hg) and child neurodevelopment, while considering genetic ApoE polymorphism. The study revealed that the presence of the APOE ε4 allele combined with Hg exposure resulted in a decline in cognitive performance in the studied children [29]. Pinto et al. demonstrated that women carrying a fetus with DS may display impaired lipid metabolism [30]. Pranami et al. demonstrated that women carrying the APOE ε4 allele and having increased cholesterol levels have impaired microcirculation in capillaries, which may cause atherosclerosis of the microcirculation vessels surrounding ovarian follicles that may result in incorrect meiotic division 2, indirectly leading to DS [31].

In our study, plasma ApoE levels were considered low, but comparable to those found in the study by Kaneva et al., where the shift in plasma ApoE toward lower levels in European residents as a result of specific features of lipid metabolism was demonstrated [32]. We also demonstrated the screening utility of maternal serum ApoE measurement as a potential noninvasive marker of DS. Comparing this result with the commonly used biochemical markers, the screening usefulness of the test was much higher (AUC = 0.978) compared to PAPP-A (AUC = 0.7771) and B-HCG (AUC = 0.6682) or combined PAPP-A + B-HCG (AUC = 0.8533) measurements [33]. Our results indicate that ApoE concentration could be added in a combined test screening approach or proposed as an alternative examination. However, further evaluation with subsequent data validation with ultrasound tests and combined screening tests using a larger cohort is required.

The study performed by Rindler et al. identified the placental ApoE synthesis as the major maternal lipid profile modifier during pregnancy. ApoE has been suggested to play a supportive role in regulating maternal and fetal homeostasis [34–36]. ApoE may also balance the oxidative and antioxidative processes through LDL oxidation inhibition and methylation reduction [37]. The elevated levels of oxidative stress markers were observed in DS fetuses, as well as in DS pregnancies. Referring to the result above and that obtained by Melhem et al. where the secretion pattern demonstrated a predominant maternal orientation [37], it can be concluded that maternal ApoE synthesis emphasizes its pleiotropic role in preventing fetal abnormalities [38,39]. Thus, preconception ApoE screening in women may be of clinical importance in the prediction of fetus health complications.

In our study, women with a confirmed DS pregnancy had significantly higher plasma ApoE concentrations compared with healthy subjects. The traceability of commonly used biochemical noninvasive tests can be characterized by PAPP-A tests, with sensitivity estimated at 90% with 5% false-positive results, and the triple test (combination of three markers: free β chorionic gonadotropin, α-fetoproteins, and unconjugated estriol), whose sensitivity is estimated at 60–70% [9–14]. Screening based on Caucasian reference ranges has a detection rate of 86.8% for contingent first trimester screening, 76.2% for second trimester screening, and 83.8% for their combination. However, first trimester screening had a higher false-positive rate compared to second trimester screening (13.7% vs. 7.7%) [40]. Regarding ApoE, the sensitivity, accuracy, specificity, and PPV and NPV were 80%, 82%, 100%, 83%, and 100%, respectively. The diagnostic power of the test was proven by the determination of an AUC of 0.978. Introducing maternal ApoE measurement to the methods commonly used in DS risk assessment may increase the sensitivity and specificity of noninvasive prenatal screening. However, the present investigation is a preliminary study, and further research on a larger cohort is required.

5. Conclusions

Our study demonstrated the relationship between maternal ApoE and fetal DS occurrence, and showed that ApoE can be used as a predictive marker of this disease, but further studies are required. The discovery of dysregulated metabolic pathways could lead to the establishment of new diagnostic targets, which may enable optimal early diagnosis, resulting in improved therapy application and enhanced quality of life.

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Prenatal Screening of Trisomy 21: Could Oxidative Stress Markers Play a Role?

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Abstract: Despite significant progress in trisomy 21 (T21) diagnostic tools, amniocentesis is stillused for the confirmation of an abnormal fetal karyotype. Invasive tests carry the potential risk of miscarriage; thus, screening biomarkers are commonly used before undergoing invasive procedures. In our study, we investigated the possible application of oxidative stress markers in the prenatal screening of trisomy 21. The DNA/RNA oxidative stress damage products (OSDPs), advanced glycation end (AGE) products, ischemia-modified albumin (IMA), alfa-1-antitrypsin (A1AT), asprosin, and vitamin D concentrations were measured in both maternal plasma and amniotic fluid in trisomy 21 (T21) and euploid pregnancies. The obtained results indicated increased levels of DNA/RNA OSDPs and asprosin with simultaneous decreased levels of vitamin D and A1AT in the study group.The diagnostic utility of the plasma measurement based on the area under the received operative characteristic (ROC) curve (AUC) calculation of asprosin (AUC = 0.965), IMA (AUC = 0.880), AGE (AUC = 0.846) and DNA/RNA OSDPs (AUC = 0.506) in T21 screening was demonstrated. The obtained results indicate a potential role for the application of oxidative stress markers in the prenatalscreening of T21 with the highest screening utility of plasma asprosin.

Keywords: trisomy 21; Down syndrome; oxidative stress; antioxidant protein; prenatal screening

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1. Introduction

Trisomy 21 (T21), also known as Down syndrome, is an autosomal aneuploidy, appearing in 1/700 live births. An additional copy of chromosome 21 is a result of the incorrect separation during gametogenesis (95% of patients) [1–3]. Trisomy 21 is a complex condition associated with congenital anomalies, which include intellectual developmental disorder, congenital heart defects, gastrointestinal anomalies, immune system defects, thyroid disease, bone defects, genitourinary system defects, strabismus, and many other diseases. Additionally, an increased risk of many chronic diseases typically associated with older age such as Alzheimer's disease, dementia, and obesity is observed [4]. In T21 prenatal screening, serum biomarkers combined with ultrasound examination and cell-free fetal DNA are used to calculate the risk of T21 occurrence [5–9]. Despite the fact that cell-free fetal DNA evaluation is characterized by high accuracy (almost 99%), it is still combined with high costs which has not yet allowed for very wide diffusion to the general population, or acceptance by various national healthcare systems into their protocols [10]. Furthermore, mothers with a high calculated risk of trisomy 21 (either by combined test

and/or cell-free fetal DNA) should be counselled, and invasive testing, such as chorionic villus sampling (CVS) and/or amniocentesis, should be offered. The application of novel biochemical screening markers may result in the elevation of the sensitivity and specificity of noninvasive prenatal tests and may reduce the unjustified use of invasive procedures while simultaneously decreasing the risk of miscarriage, combined with the use of invasive tests [11]. Data in the literature underline the connection between fetal chromosomal aberrations and disturbances in oxidative stress with antioxidant processes [12–15]. It was previously hypothesized that the upregulated oxidative stress level is related to T21 pathogenesis; this was later proved by Žitňanová et al., who demonstrated upregulated levels of oxidative stress markers measured in T21 individuals [16]. Thus, it seems necessary to evaluate the hypothesis about oxidative stress biomarkers in T21 prenatal screening. Considering the fact that crucial genes of the oxidative stress pathway are mapped on chromosome 21 [17], the hypothesis of the significance of oxidative stress, not only in T21 postnatal pathology but also in prenatal diagnosis, needs to be evaluated. Accordingly, the biomarkers of oxidative stress measurements could be relevant in the screening of T21 [18]. The aim of this study was to assess the utility of selected parameters of oxidative stress markers in maternal plasma and amniotic fluid for T21 screening. DNA/RNA oxidative stress damage products (OSDPs), as well as other commonly used oxidative stress markers (ischemia-modified albumin (IMA) and advanced glycation ends products (AGE)), were evaluated in this study. Furthermore, novel antioxidant proteins—asprosin and alfa-1-antitrypsin (A1AT)—and vitamin D were also assessed and compared between T21 and euploid pregnancies.

2. Materials and Methods

2.1. Experimental Overview—Patient Recruitment

This was a prospective case–control study. The study and control groups consisted of women who underwent routine amniocentesis between the 15th and 18th weeks of gestation at the Department of Reproduction and Gynecological Endocrinology of the Medical University of Bialystok, Poland. A total amount of 100 pregnant women underwent screening procedures between 2017 and 2020, and 40 were included and recruited for the subsequent evaluation. The increased risk of chromosomal aberrations in noninvasive prenatal screening and an age greater than 35 years were indications for amniocentesis. Chronic or acute diseases, hormonal treatment, anti-inflammatory treatment, high-risk pregnancy, and preterm delivery in the patient's medical history were the exclusion criteria [19]. All participants were aware of the potential risks prior to the amniocentesis procedure and received relevant and necessary information about the study. The study group did not differ with respect to the course of pregnancy and body mass index (BMI). A necessary sample size to detect the significant differences in all studied parameters between groups was confirmed using power analysis [20]. Considering a 5% margin of error and 95% confidence level, the recommended sample size of our preliminary study was 16. Following karyotype test analysis, 20 women carrying T21 fetuses and 20 women with euploid fetuses qualified for the study. All participants had 5.5 ml of venous blood drawn on the day of amniocentesis. The biological material was centrifuged, with subsequent plasma separation, and frozen at −80 °C. Amniotic fluid samples with possible blood contamination were excluded from the study.

2.2. Ethics Statement

The experimental protocol was approved by the Bioethics Committee of the Medical University of Bialystok, Poland (APK/002/351/2020), and confirmation consent was received from each participant.

2.3. Laboratory Examinations

The IMA, AGE, A1AT, and asprosin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) (Enzyme-linked Immunosorbent Assay Kit; Cloud-

Clone Corp., Wuhan, China; CEA825Hu, CEB353Ge, SEB697Hu, and SEA332Hu, respectively) according to the manufacturer's instructions. The DNA/RNA OSDP concentrations were assayed using an immunoassay kit (DNA/RNA Oxidative Damage (High Sensitivity) ELISA Kit, Cayman Chemicals, Ann Arbor, Michigan, MI, USA, 589320). This kit enabled the simultaneous detection of DNA/RNA OSDPs, such as 8-hydroxyguanosine (8-OHG), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and 8-hydroxyguanine. The vitamin D concentration was evaluated using a commercial kit for 25-OH Vitamin D Total ELISA (Gentaur, Sopot, Poland, KAP1971). The total vitamin D measurement was evaluated through the chemiluminescence method using Cobas E411, from Roche company (07464215). The samples and controls were randomized, then measured in the same run, using the blind analysis method.

2.4. Data Management and Statistical Analysis

Statistical analyses were performed using Statistica 13.3 (StatSoft, Tibco Software Inc., Palo Alto, CA, USA) and GraphPad Prism v. 9.0 (GraphPad Software, Inc., San Diego, CA, USA). During the analysis, the lack of data distribution normality was demonstrated using the Shapiro–Wilk test. Thus, the groups were compared using the nonparametric Mann–Whitney test, and $p < 0.05$ was considered statistically significant. The Spearman test for multiple comparisons was used to perform correlation analyses between the concentrations of all the studied parameters in plasma and amniotic fluid samples. In addition, the receiver operating characteristic (ROC) curves were determined with simultaneous sensitivity and specificity calculations. Screening cutoff points were determined using Youden's index [21]. Odds ratios (ORs) were calculated using commercially available MedCalc software [22].

3. Results

3.1. The Comparison of Oxidative Stress-Related Parameters between the Study and Control Groups

Following the oxidative stress marker analyses, the concentrations of the DNA/RNA OSDPs were found to be significantly higher in amniotic fluid samples in T21 individuals compared to those from the control group ($p < 0.05$). No significant difference was observed in the plasma concentrations of the DNA/RNA OSDPs between the study and control groups. In the T21 group, the AGE concentrations were found to be significantly lower in both plasma and amniotic fluid samples compared to those from healthy subjects ($p < 0.001$). Additionally, the maternal plasma IMA concentrations were also lower in the T21 group in comparison to control group ($p < 0.0001$).

Considering the antioxidant parameters assessed in the study group, the total vitamin D plasma concentrations were significantly lower when compared to the control group ($p < 0.05$). To verify vitamin D deficiency, the 25-OH vitamin D concentrations were measured in plasma and amniotic fluid samples. Significant differences between the study and control groups were not proven, but 25-OH vitamin D levels lower than recommended were observed in both groups.

Novel antioxidant protein concentrations were also determined. The study group asprosin concentrations were significantly higher in both plasma and amniotic fluid samples compared to euploid pregnancies ($p < 0.001$). Interestingly, the A1AT concentrations were found to be significantly lower in amniotic fluid samples in the T21 group than in euploid pregnancies ($p < 0.001$). We did not notice any significant difference in plasma A1AT between the study and control groups (Figure 1).

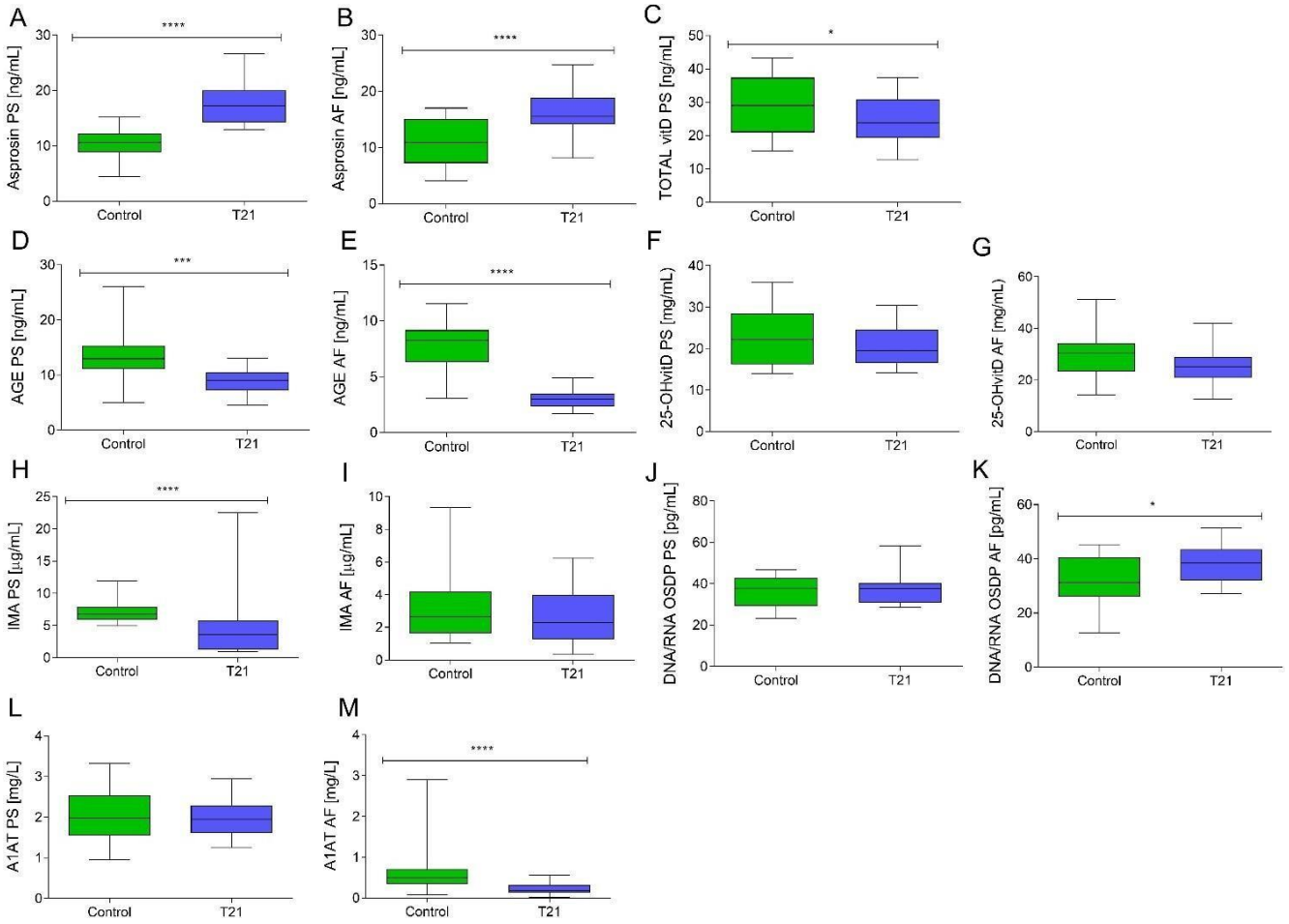

Figure 1. The studied protein concentrations measured in the plasma and amniotic fluid samples. Different asterisks above the bars indicate significant differences compared to the control (* $p \leq 0.05$; *** $p \leq 0.001$; **** $p \leq 0.0001$). (**A**) Plasma asprosin; (**B**) amniotic fluid asprosin; (**C**) plasma total vitamin D; (**D**) plasma advanced glycation end products; (**E**) amniotic fluid advanced glycation end products; (**F**) plasma 25-OH vitamin D; (**G**) amniotic fluid 25-OH vitamin D; (**H**) plasma ischemia-modified albumin; (**I**) amniotic fluid ischemia-modified albumin; (**J**) plasma DNA/RNA oxidative stress damage product; (**K**) amniotic fluid DNA/RNA oxidative stress damage products; (**L**) plasma alfa-1-antitrypsin; (**M**) amniotic fluid alfa-1-antitrypsin. A1AT, alfa-1-antitrypsin; AF, amniotic fluid; AGE, advanced glycation end products; Control, control group; IMA, ischemia-modified albumin; OSDP, oxidative stress damage product; PS, plasma; T21, trisomy 21.

Table 1 substantiates all the parameters analyzed with the concentrations found in T21 and control samples and the statistical comparison results (Table 1). No differences were observed between T21 plasma and amniotic fluid 25-OH vitamin D concentrations ($p > 0.05$). However, 25-OH vitamin D in the control group was higher in amniotic fluid than in plasma samples ($p < 0.01$). In the case of asprosin and DNA/RNA OSDP, no differences were noted between plasma samples and amniotic fluid in either euploid pregnancy or T21 groups ($p < 0.05$). AGE and A1AT concentrations in the control and T21 groups were higher in plasma than in amniotic fluid (AGE: $p < 0.01$; $p < 0.0001$; A1AT: $p < 0.001$; $p < 0.0001$, respectively). Control group IMA concentrations were lower in amniotic fluid than in plasma samples ($p < 0.0001$).

Table 1. Basic statistics and comparison of studied protein concentrations measured in the plasma and amniotic fluid samples.

Marker	Material	Study Group	Unit	Median Value	Min	Max	*p* Value (Control vs. T21)	*p* Value (between Study Material)	
								Control PS vs. Control AF	T21 PS vs. T21 AF
25-OH vitamin D	PS	Control	mg/mL	22.22	14.00	35.92	NS	$p < 0.01$	NS
		T21		19.51	14.24	30.44			
	AF	Control		30.60	14.24	51.34	NS		
		T21		25.20	12.59	42.07			
Asprosin	PS	Control	ng/mL	10.57	4.45	15.17	$p < 0.0001$	NS	NS
		T21		17.28	12.94	26.59			
	AF	Control		10.87	4.01	17.03	$p < 0.0001$		
		T21		15.53	8.09	24.77			
AGE	PS	Control	ng/mL	12.96	4.96	26.03	$p < 0.001$	$p < 0.01$	$p < 0.0001$
		T21		9.16	4.52	13.01			
	AF	Control		8.27	3.06	11.55	$p < 0.0001$		
		T21		3.00	1.67	4.89			
IMA	PS	Control	µg/mL	6.79	5.00	12.00	$p < 0.0001$	$p < 0.0001$	NS
		T21		3.61	0.90	22.52			
	AF	Control		2.64	1.05	9.34	NS		
		T21		2.28	0.33	6.23			
A1AT	PS	Control	mg/L	1.98	0.95	3.38	NS	$p < 0.001$	$p < 0.0001$
		T21		1.95	1.26	1.69			
	AF	Control		0.49	0.08	2.90	$p < 0.0001$		
		T21		0.18	0.01	0.56			
DNA/RNA OSDP	PS	Control	pg/mL	37.81	23.21	46.83	NS	NS	NS
		T21		37.57	28.53	58.40			
	AF	Control		31.16	12.64	45.22	$p < 0.05$		
		T21		38.48	27.06	51.46			

A1AT, alfa-1-antitrypsin; AF, amniotic fluid; AGE, advanced glycation end products; IMA, ischemia-modified albumin; NS, not significant; OSDP, oxidative stress damage product; PS, plasma; T21, trisomy 21.

3.2. Correlations between Examined Parameters

Spearman coefficients to describe the relationships between the studied parameters were calculated; the obtained results are presented on correlation matrices (Figure 2). Among the biochemical parameters measured in the control group, positive correlations were observed between total plasma vitamin D and plasma 25-OH vitamin D (r = 0.85; p < 0.001), and between plasma total vitamin D and amniotic fluid A1AT (r = 0.59; p < 0.05). Additionally, positive correlations were noticed between the control group's amniotic fluid 25-OH vitamin D and A1AT (r = 0.47; p < 0.05), as well as between amniotic fluid asprosin and A1AT (r = 0.45; p < 0.05). Accordingly, a positive correlation was demonstrated between A1AT and DNA/RNA OSDPs measured in plasma (r = 0.44; p < 0.05). A negative correlation was also demonstrated between amniotic fluid A1AT and plasma DNA OSDPs in the control group (r = −0.45; p < 0.05) (Figure 2A).

Considering the study group, strong positive correlations were observed between plasma total vitamin D and 25-OH vitamin D (r = 0.80; p < 0.001), as well as between plasma total vitamin D and plasma IMA (r = 0.45; p < 0.05). A positive correlation between amniotic fluid IMA and amniotic fluid 25-OH vitamin D was observed in the study group (r = 0.52, p < 0.05). Negative correlations between amniotic fluid 25-OH vitamin D and plasma asprosin measurements (r=−0.54 p < 0.05), along with plasma AGE and plasma A1AT, were also demonstrated (r = −0.60; p < 0.05). Additionally, a negative correlation between the T21 group's amniotic fluid DNA/RNA OSDPs and amniotic fluid A1AT was observed (r = −0.54; p < 0.05) (Figure 2B). No significant correlation was observed between the plasma and the amniotic fluid for the corresponding parameters, either in the control or the study group.

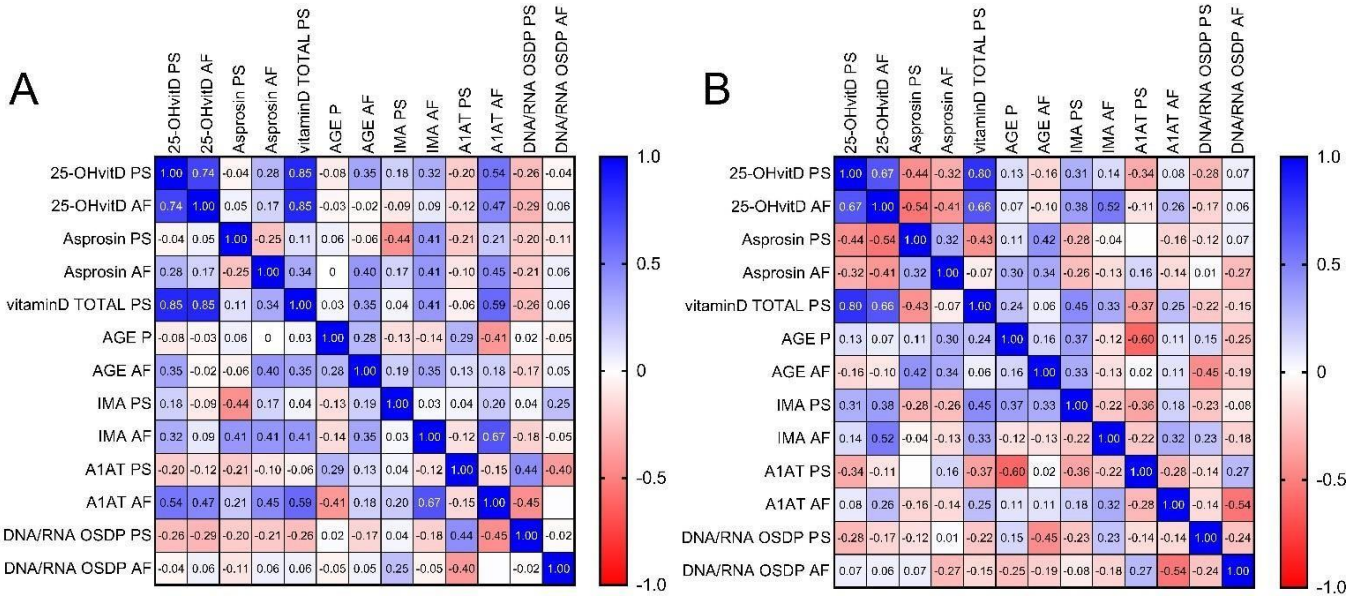

Figure 2. Graphical Spearman correlation matrix of the biochemical parameters in (**A**) the control group and (**B**) the study group. A1AT, alfa-1-antitrypsin; AF, amniotic fluid; AGE, advanced glycation end products; IMA, ischemia-modified albumin; OSDP, oxidative stress damage product; PS, plasma; Vit D, vitamin D.

3.3. Screening Utility of the Tested Parameters

To determine the diagnostic utility of the tested parameters, the ROC curve was calculated (Table 2), and an illustration of the relationship between sensitivity and specificity is presented in the ROC graphs (Figure 3). The cutoff values were set using Youden's index. The highest sensitivity was observed for plasma and amniotic fluid asprosin, as well as amniotic fluid AGE (1.00; 0.95; and 0.95, respectively). Plasma IMA and amniotic fluid AGE demonstrated the highest specificity in the T21 screening (1.00 and 0.90, respectively).

Differences regarding the chance of detection (OR) for T21 patients based on the studied parameter concentrations are also shown. Relationships between T21 occurrence and amniotic fluid asprosin (OR 22.78), AGE (OR 2.11), IMA (OR 0.18) and plasma asprosin (OR 8.20) and A1AT (OR 5.75) concentrations were noted ($p < 0.05$).

Table 2. Diagnostic criteria of the receiver operating characteristic (ROC) curve for the tested parameters.

Marker	Unit	AUC	*p* (AUC = 0.50)	Cut Off Value	Sensitivity	Specificity	OR	*p*
25-OH vitamin D PS	mg/mL	0.59	NS	<26.18	0.85	0.40	1.62	NS
25-OH vitamin D AF	mg/mL	0.66	NS	<31.21	0.85	0.50	3.27	NS
Asprosin PS	ng/mL	0.97	<0.0001	>12.70	1.00	0.85	8.20	$p < 0.05$
Asprosin AF	ng/mL	0.83	<0.001	>12.91	0.95	0.65	22.78	$p < 0.05$
AGE PS	ng/mL	0.85	<0.001	<11.00	0.81	0.80	1.00	NS
AGE AF	ng/mL	0.96	<0.0001	<4.184	0.95	0.90	2.11	$p < 0.05$
IMA PS	µg/mL	0.84	<0.001	<4.798	0.67	1.00	1.05	NS
IMA AF	µg/mL	0.54	NS	<1.798	0.38	0.76	0.18	$p < 0.05$
A1AT PS	mg/L	0.53	NS	<2.341	0.81	0.33	5.75	$p < 0.05$
A1AT AF	mg/L	0.87	<0.0001	<0.3180	0.76	0.86	0.71	NS
DNA/RNA OSDP PS	pg/mL	0.51	NS	<40.30	0.80	0.40	3.27	NS
DNA/RNA OSDP AF	pg/mL	0.73	<0.05	>31.76	0.84	0.58	3.78	NS

A1AT, alfa-1-antitrypsin; AF, amniotic fluid; AGE, advanced glycation end products; AUC, area under the received operative characteristic (ROC) curve; IMA, ischemia-modified albumin; NS, not significant; OR, odds ratio; OSDP, oxidative stress damage product; PS, plasma.

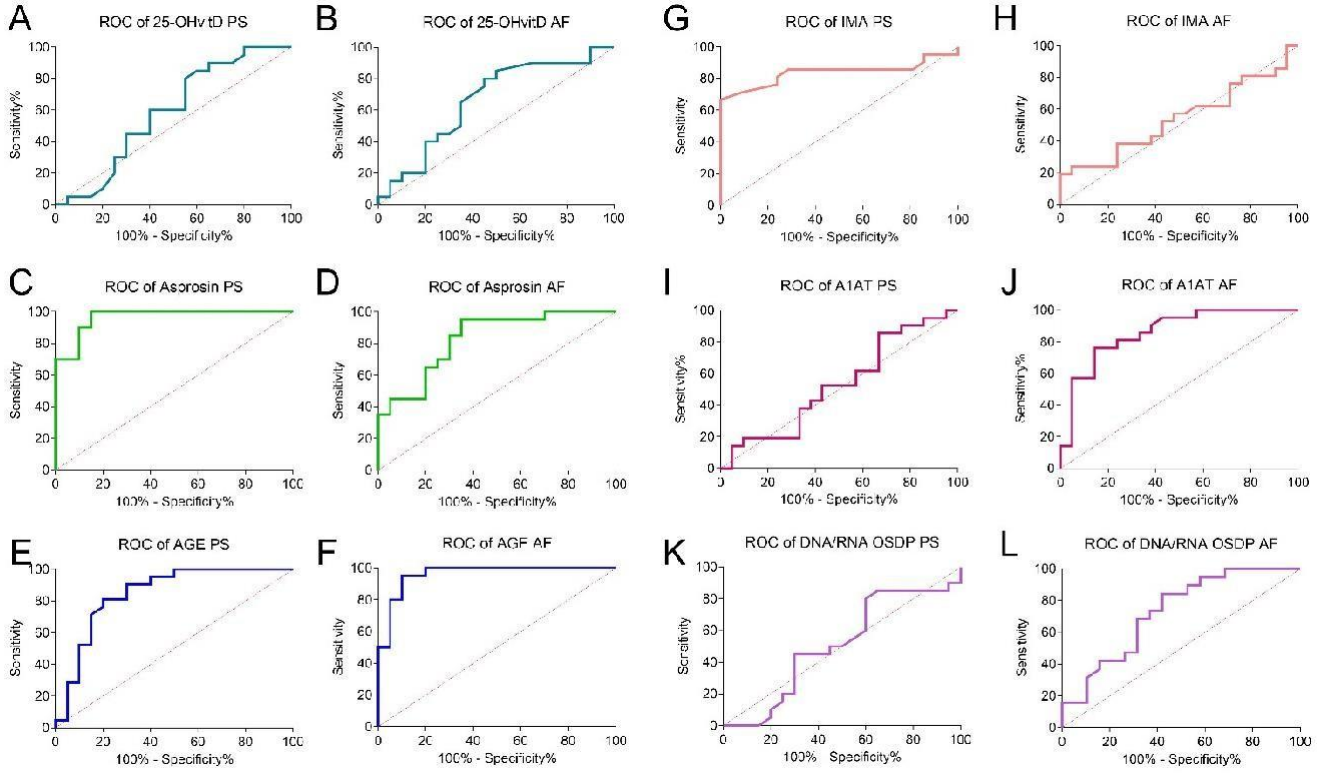

Figure 3. ROC curves of the studied parameters. (**A**) Plasma 25-OH vitamin D; (**B**) amniotic fluid 25-OH vitamin D; (**C**) plasma asprosin; (**D**) amniotic fluid asprosin; (**E**) plasma advanced glycation end products; (**F**) amniotic fluid advanced glycation end products; (**G**) plasma ischemia-modified albumin; (**H**) amniotic fluid ischemia-modified albumin; (**I**) plasma alfa-1-anitrypsin; (**J**) DNA/RNA oxidative stress damage product; (**K**) plasma DNA/RNA oxidative stress damage products; (**L**) amniotic fluid DNA/RNA oxidative stress damage products. AF, amniotic fluid; PS, plasma; OS, oxidative stress.

To evaluate the diagnostic usefulness of asprosin, A1AT, IMA, AGE, and DNA/RNA OSDPs as prenatal screening tools, the areas under the ROC curves (AUCs) were calculated and compared to AUC = 0.50 (borderline of the diagnostic usefulness of a test). Asprosin and A1AT demonstrated the highest screening value. The highest AUC value was demonstrated for plasma asprosin (0.97; $p < 0.0001$) and amniotic fluid AGE (0.96; $p < 0.001$). The amniotic fluid A1AT assay was characterized by AUC = 0.87 ($p < 0.001$). Furthermore, the AUC value of the DNA/RNA OSDP concentration in amniotic fluid samples was calculated to be AUC = 0.73 ($p < 0.05$). The 25-OH vitamin D (both in plasma and amniotic fluid), amniotic fluid IMA, plasma A1AT, and DNA/RNA OSDPs concentrations demonstrated no diagnostic usefulness in T21 screening ($p > 0.05$) (Figure 3).

4. Discussion

4.1. Main Findings

In our study, we determined the T21 screening utility of DNA/RNA OSDPs, aswell as other commonly used oxidative stress markers: IMA and AGE. Furthermore, novel antioxidant proteins—asprosin and A1AT—and vitamin D were also assessed and compared between T21 and euploid pregnancies. To the best of our knowledge, this is the first comparative analysis of oxidative stress biomarkers in prenatal T21 in both amniotic fluid and maternal plasma. Significant differences in plasma asprosin, AGE, and IMA, as well as amniotic fluid asprosin, AGE, DNA/RNA OSDPs, and A1AT, were observed between T21 and euploid pregnancies, suggesting the substantial role of oxidative stress in T21 pathology. Referring to the fact that the maternal compartment is constantly connected to the fetus [23–25], these parameters were analyzed in maternal plasma to determine the potential screening utility. Moreover, a concentration comparison between maternal plasma and amniotic fluid was evaluated to determine the insufficient metabolic pathway origins during T21 prenatal development.

It has been noticed that upregulated oxidative stress levels in T21 pathogenesis may result in the oxidation of polyunsaturated fatty acids, and therefore induce cell membrane-destructive effects. This oxidation process has been suggested as one of the major causes of cognitive disabilities observed in this disease [13]. Studies have also indicated that an increased level of oxidative stress results in DNA injury, cytoskeletal and chromatin reorganization, defects in apoptotic cell pathway, and aberrant cell cycle checkpoint function [15,26–29]. To evaluate the degree of DNA damage and the effectiveness of DNA repair processes in T21 pregnancy, concentrations of the DNA/RNA OSDPs, such as 8-OHG, 8-OHdG, and 8-hydroxyguanine, were determined in both plasma and amniotic fluid samples [30]. Our results showed an increased level of amniotic fluid DNA/RNA OSDPs measured in the study group. The lack of a strong correlation observed between DNA/RNA OSDPs and other oxidative stress markers suggests that oxidative stress in T21 pregnancy is a multifactorial and complex process [31]. Referring to the fact that we did not observe any significant difference in the maternal plasma DNA/RNA OSDPs between the study and control groups, it could be hypothesized that the processes associated with increased oxidative stress are more likely related to disturbed metabolic pathways in the fetal compartment. Interestingly, deregulated measurements were detected mainly in the amniotic fluid and did not transfer through maternal circulation. Additionally, the upregulated oxidative stress levels are a potentially important link of the pathological mechanism of abnormal fetal development [26,32].

Following the increased oxidative stress status in T21 pregnancy, we also evaluated the antioxidant state in which the key modulator is considered to be vitamin D. Despite bone mineralization, vitamin D is also involved in many biological processes, such as immune system modulation and antioxidation [33,34]. Vitamin D components can be divided into five types (D1–D5); their biological functions are triggered by 1.25 OH vitamin D, which is activated in the mitochondria from the 25-OH form [35]. Vitamin D supplementation is associated with a decrease in oxidative stress, improvement in anti-inflammatory defense, and activation of DNA repair processes [36,37]. In 2017, Zubillaga et al. proved that adults

with T21 are at greater risk of vitamin D deficiency, and the additional supplementation brings beneficial results [38]. Palacios et al. showed that vitamin D supplementation is necessary for decreasing the risk of pregnancy-related abnormalities, including pre-eclampsia, preterm birth, decreased birth weight, and other related diseases [35,36,38–42]. In our study, decreased levels of vitamin D were found among women carrying T21 fetuses, similar to the results received in T21 individuals [41]. The data obtained in the study showed decreased 25-OH vitamin D concentrations below the recommended level (<30 ng/mL) [32]. Furthermore, decreased vitamin D concentrations suggest insufficient antioxidant potential in the maternal compartment, which may result in a more severe subsequent course of fetal comorbidities [43]. Accordingly, in our study, the decreased 25-OH vitamin D concentration observed in T21 pregnancies was found to be positively correlated with another antioxidant and anti-inflammatory protein—A1AT [44]. A1AT, also known as serpin 1, protects neurons and glial cells from oxidative stress and glucose deprivation [45,46]. It is known that A1AT deficiency is a rare disease that significantly increases the risk of serious lung and/or liver diseases [47]. In our research, the concentration of amniotic fluid A1AT was significantly lower in the study group compared to the control group. The results suggest that a decrease in the A1AT concentration combined with aggravated inflammation processes and oxidative stress observed in T21 pregnancy may negatively impact plural comorbidities and the occurrence of fetal malformations [48–50]. A1AT deficiency combined with the decreased vitamin D levels observed in our study could have a multitude of effects of deregulated paths in T21 pregnancy development [44]. Furthermore, the negative correlation demonstrated between DNA/RNA OSDPs and A1AT showed that an increased degree of oxidative stress is combined with A1AT deficiency observed in amniotic fluid. Nevertheless, we did not observe any significant difference in the A1AT plasma concentration between T21 and euploid pregnancies.

In our study, elevated levels of the novel antioxidant protein asprosin were found in T21 amniotic fluid and plasma compared to those in the euploid control group. Asprosin is a hormone secreted by white adipose tissue activated by fasting as a response to low plasma glucose concentrations [51]. Interestingly, Zhang et al. proved that asprosin upregulates the activity of the antioxidant enzyme superoxide dismutase 2, which is associated with a decrease in the concentration of reactive oxygen species (ROS) and apoptosis processes [52]. An increased asprosin concentration may result from antioxidant maternal protection related to the developing T21 fetus [51–54]. Vitamin D deficiency is inversely associated with asprosin concentrations, which, combined with increased DNA/RNA OSDP levels, confirms that the antioxidant deficiency caused by the developing T21 fetus is insufficiently counteracted by the maternal organism.

The results of the present study are convergent with those obtained by other authors. Perrone et al. suggested that an increased oxidative stress level is detectable in amniotic fluid samples in early T21 pregnancy. In their study, upregulated isoprostane concentrations, a novel marker of free radical-catalyzed lipid peroxidation related to increased oxidative stress, were noticed. Their hypothesis, based on these outcomes, referred to the fact that T21 fetal development is interrupted by an environment with increased oxidative stress, which may injure many tissues [32]. These results were updated by Perlugi et al.,where decreased levels of glutathione (GSH) were observed and significantly increased levels of several markers of oxidative stress were found in T21 amniotic fluid. The sources of oxidative stress in pregnancy can be various, from the placenta to maternal and fetal tissues, and the induction of oxidative stress reactions could also come from external factors [55].

No significant correlations between corresponding parameters in plasma and amniotic fluid were demonstrated. We found it confusing that studied parameter concentrations in the amniotic fluid were not directly related (proportional) to the concentrations in the maternal plasma—in a number of cases, higher maternal content was not readily translated into higher concentrations in the amniotic fluid. This would also indicate that the relationship between maternal and fetal oxidative stress is complex beyond a

simple diffusion. The source of oxidative stress in pregnancy manifests in the placenta, in particular, but also originates from maternal and/or fetal cells and external factors [55]. It can be hypothesized that the correlation is described by some monotonic, but not linear function. Studies on the transfer mechanisms between the maternal and fetal compartment are needed to determine the association between the parameters studied in the amniotic fluid and maternal plasma.

4.2. Strength and Limitations

It was previously hypothesized that the upregulated oxidative stress level is related to T21 pathogenesis; this was later proved by Žitňanová et al., who measured and reported upregulated levels of oxidative stress markers in T21 individuals [22]. Thus, it seemed necessary to evaluate the hypothesis about oxidative stress biomarkers in T21 prenatal screening. The results indicate a potential role of the application of oxidative stress markers in the pre-natal screening of T21 with the highest screening utility of plasma asprosin. Moreover, the origins of the disturbed metabolic pathways were analyzed. Our study indicates that oxidative stress-related parameters in the maternal plasma were not directly related to concentrations in the amniotic fluid. It seems that disturbed metabolic processes in the fetal compartment are not particularly counteracted by additional syntheses of antioxidant substances in maternal circulation [38]. Furthermore, the analyzed protein's direct functions as antioxidants were not thoroughly examined. In this case, our study has indicated the novel possibilities in basic research, especially referring to the fact that insufficient antioxidants properties were established during T21 fetus development. These analyses are of great importance in understanding the role of oxidative stress in the pathophysiology of T21. Furthermore, the number of studies performed on T21 individuals to establish the negative impact of increased oxidative stress status is still insufficient. Preclinical studies concerning the impact of oxidative/antioxidative state on the development of T21 are still needed [56]. However, in our study, the low diagnostic utility of measurements of the oxidative stress marker IMA in T21 pregnancy were demonstrated. Although IMA and AGE have never been measured in T21 pregnancy before, extensive data in the literature suggest their promising diagnostic utility in pre-eclampsia and pregnancy hypertension [57,58]. Despite confirmation of a higher level of oxidative stress, the IMA and AGE levels have been shown to not be remarkably increased in various complications related to T21 gestation [34,42,59–61]. Moreover, referring to the limited size of the experimental group, further evaluation and data validation using a larger cohort are required to confirm the diagnostic usefulness of the studied oxidative stress parameters.

4.3. Implications and Future Perspectives

Considering that oxidative stress markers are still investigated for their possible screening utility, the oxidative stress markers in T21 pregnancy screening were evaluated. The commonly used noninvasive prenatal test for calculating the risk of T21, which combines ultrasound markers with biochemical markers of pregnancy-associated plasma protein A (PAPP-A) and serum-free human chorionic gonadotropin (B-HCG), is characterized by 93% accuracy. The separate diagnostic utility in maternal plasma has been proven (AUC for PAPP-A = 0.777; AUC for B-HCG = 0.668; AUC for combined PAPP-A + B-HCG = 0.8533) [62]. Comparing these data to the plasma asprosin measurement, characterized by AUC = 0.965, the diagnostic utility of maternal plasma asprosin as a potential noninvasive marker in T21 prenatal screening was demonstrated. Moreover, these results are also comparable with the free fetal DNA measurement, characterized by 99% accuracy [9]. Additionally, the possible association of the occurrence of T21 comorbidities and prenatal determination of asprosin in follow-up studies should be evaluated. The OR calculation has shown that deregulated concentration of plasma and amniotic fluid asprosin, A1AT, and amniotic fluid IMA during the second trimester increased the risk of Down syndrome among pregnant women ($p < 0.05$).

The preventive effects of antioxidants counteracting the harmful impact of ROS or acting as treatment for oxidative stress-related diseases are still constantly being examined. Accordingly, the potential beneficial effect of antioxidant administration during T21 development could reduce the cognitive and neuronal dysfunctions associated with T21 [63]. The evidence from studies performed in vitro and in vivo to evaluate the positive effects of dietary antioxidants seems compelling [56,64,65]. However, nonconclusive results in this area clearly demonstrate that more attention should be paid to the performance of high-quality randomized controlled trials [64–67]. Despite this, Nachvak et al. proved that alpha-tocopherol supplementation decreases the levels of oxidative stress markers in T21 [68]. Furthermore, antioxidant supplementation in adult T21 individuals could slow the development of dementia and Alzheimer's disease, which are the most strongly related to T21 diseases. Our results confirm the antioxidant deficiencies of pregnant women with fetal T21 and the potential of antioxidant treatment of pregnant women. In this case, this study has uncovered novel targets for evaluations in future preclinical trials [56,69]. Despite the relevant value of our research, this study should be considered as preliminary. In future research, long-term follow-up studies performed on large cohort study groups are of utmost importance.

5. Conclusions

The diagnostic utility in the prenatal screening of T21 of plasma measurements of asprosin, IMA, AGE, and DNA/RNA OSDPs was demonstrated. The obtained results indicate a potential role of the application of oxidative stress markers in the prenatal screening of T21, with the highest screening utility of asprosin measurement. Decreased A1AT with vitamin D and increased asprosin and DNA/RNA OSDP concentrations are related to T21 development. However, based on the present study, it is reasonable to speculate that oxidative stress occurs in the T21 fetal compartment rather than in the maternal compartment, and the maternal organism is inefficient in overcoming the antioxidant deficiencies caused by the developing T21 fetus. Thus, antioxidant applications in T21 pregnancy should still be evaluated.

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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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7. STRESZCZENIE

7.1. Streszczenie w języku polskim

Pomimo znacznego postępu w diagnostyce trisomii 21 (T21), amniopunkcja jest nadal wykorzystywana do potwierdzenia wystąpienia nieprawidłowego kariotypu u płodu. Ze względu na to, że testy inwazyjne wiążą się z potencjalnym ryzykiem poronienia, biomarkery przesiewowe są powszechnie stosowane przed poddaniem się zabiegom inwazyjnym. Wprowadzenie nowych metod do diagnostyki przesiewowej może zmniejszyć ilość wykonywanych zabiegów inwazyjnych. W przyszłości wprowadzenie zintegrowanych metod omicznych do rutynowych badań prenatalnych może zwiększyć odsetek wykrywalności aneuploidii płodu, w tym T21 metodami nieinwazyjnymi. Na podstawie przeglądu literatury można stwierdzić, że analiza cbDNA i cffDNA wykazuje ogromny potencjał w badaniach przesiewowych T21. Jednak nadal istnieje potrzeba dostarczenia danych w celu potwierdzenia ich użyteczności. Ponadto rozwój w pełni zautomatyzowanych systemów pozostaje niezbędny do wprowadzenia nowoczesnych technologii w badaniach prenatalnych. W związku z tym przytoczone badania prenatalne prowadzone z wykorzystaniem metod omicznych dostarczyły nowych informacji na temat złożonej patofizjologii T21. Dodatkowo, zaproponowane wykorzystanie metod omicznych może umożliwić szybszą ocenę nowych strategii terapeutycznych. Przypuszcza się, iż dalsza walidacja wyników z wykorzystaniem metody ELISA umożliwi wydobycie zalet wyników uzyskanych metodami omicznymi, zapewniając szybką i dokładną detekcję T21. Dane literaturowe podkreślają związek aberracji chromosomowych płodu z zaburzeniami procesów zależnych od

potencjału procesów oksydacyjno-antyoksydacyjnych. Obecność dodatkowego chromosomu 21 powoduje zaburzenie szeregu szlaków metabolicznych, skutkujących wystąpieniem wad wrodzonych u płodu. Dodatkowo, biorąc pod uwagę fakt, że kluczowe geny szlaku stresu oksydacyjnego są zmapowane na chromosomie 21, należałoby ująć znaczenie stresu oksydacyjnego nie tylko w patogenezie T21, ale także w diagnostyce prenatalnej. Z tego względu, w przeprowadzonym badaniu, poddano ocenie możliwość zastosowania oznaczania Apolipoproteiny E oraz wybranych markerów stresu oksydacyjnego w prenatalnych badaniach przesiewowych stosowanych w celu określenia ryzyka wystąpienia T21.

Stężenie następujących białek: apolipoproteiny E (ApoE), produktów oksydacyjnego rozpadu DNA/RNA (OSDP), produktów końcowe glikacji (AGE), albuminy modyfikowanej niedotlenieniem (IMA), alfa-1-antytrypsyny (A1AT), asprosinu i witaminy D oznaczono zarówno w osoczu matki, jak i w płynie owodniowym, pozyskanym od kobiet w ciąży z potwierdzoną T21 u płodu, stanowiąca grupę badaną, i u kobiet w ciąży z potwierdzoną euploidalną ciążą, stanowiąca grupę kontrolną. Uzyskane wyniki wskazują na podwyższony poziom ApoE, OSDPs DNA/RNA i białka asprosin przy jednoczesnym obniżeniu stężeniu witaminy D i A1AT w grupie badanej. Użyteczność diagnostyczna oznaczeń powyższych białek w badaniu przesiewowym T21 w oparciu o powierzchnię pola pod krzywą ROC (AUC) były następujące: ApoE (AUC=0,975); asprosin (AUC = 0,965), IMA (AUC = 0,880), AGE (AUC= 0,846) i OSDP DNA/RNA (AUC = 0,506). Uzyskane wyniki wskazują na potencjalną użyteczność oznaczania stężenia ApoE oraz wybranych markerów stresu oksydacyjnego w prenatalnych badaniach przesiewowych T21, przy czym największą

użytecznością przesiewową charakteryzowałosię oznaczenie stężenia białka ApoE oraz asprosinu.

7.2. Streszczenie w języku angielskim

Despite the significant progress in the diagnostic tools applied for prenatal screening, amniocentesis is still used to confirm abnormal fetus karyotype. Invasive testing carries a potential risk of miscarriage; therefore, screening biomarkers are commonly used before undergoing invasive procedures. The introduction of novel screening methods may subsequently reducing the number of incorrect indications for amniocentesis diagnosis. In the future, the introduction of integrated omics methods into routine non-invasive prenatal screening could increase the detection rate of fetal aneuploidy including T21. Based on our literature search, it can be concluded that cbDNA and cffDNA analysis demonstrate the vast potential in NIPT. However, there is still a need to provide useful data in order to validate their usefulness. Moreover, the development of fully automated systems remains essential to introduce modern technologies in prenatal screening. Accordingly, novel approaches have provided new insights into the complex pathophysiology of T21, which could be further used in novel therapeutic strategy evaluation. Up to date, ELISA was a useful tool which meet the challenge of introducing results obtained with omics-based methods into daily routine diagnostics with subsequent validation of different procedures.

Furthermore, literature data emphasize the relationship of fetal chromosomal aberrations with disturbed processes dependent on the

potential of oxidative-antioxidant processes. The presence of an additional chromosome 21 disrupts a number of metabolic pathways, resulting in birth defects in the fetus. In addition, given that key genes in the oxidative stress pathway are mapped on chromosome 21, the importance of oxidative stress not only in the pathogenesis of T21, but also in prenatal diagnosis should be included. In this case, in this study, we examined the possibility of using the determination of Apolipoprotein E and selected markers of oxidative stress in prenatal screening of trisomy 21.

Concentrations of Apolipoprotein E (ApoE), concentration of DNA / RNA damage products after increased oxidative stress influence (OSDP), advanced glycation products (AGE), ischemia modified albumin (IMA), alpha 1- antitrypsin (A1AT), asprosin and vitamin D concentrations were determined in both maternal plasma and amniotic fluid in trisomy 21 (T21) and euploid pregnancies. The obtained results indicate an increased level of ApoE, OSDPs DNA / RNA and asprosin protein with a simultaneous decrease in the level of vitamin D and A1AT in the study group. Diagnostic utility in screening T21 of the above parameters based on the area under the obtained ROC curve (ROC curve) was as follows: ApoE (AUC = 0.975); asprosin (AUC = 0.965), IMA (AUC = 0.880), AGE (AUC = 0.846) and OSDP DNA / RNA (AUC = 0.506) at the T21 screening. The obtained results indicate the potential role of the use of ApoE determination and selected markers of oxidative stress in the prenatal screening tests T21, where the greatest screening utility was demonstrated by the determination of the concentration of ApoE and asprosin protein.

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20. Zbucka-Kretowska, M.; Niemira, M.; Paczkowska-Abdulsalam, M.; Bielska, A.; Szalkowska, A.; Parfieniuk, E.; Ciborowski, M.; Wolczynski, S.; Kretowski, A. Prenatal circulating microRNA signatures of foetal Down syndrome. *Sci. Rep.* **2019**, *9*, doi:10.1038/s41598-018-35876-5.

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9. SUPLEMENT

9.1. Informacje o charakterze udziału współautorów w publikacjach wraz z szacunkowym określeniem procentowego wkładu każdego z nich oraz oświadczenia o zgodzie na wykorzystanie publikacji w rozprawie doktorskiej

1. Angelika Buczyńska; Iwona Sidorkiewicz; Anna Trochimiuk; Sławomir Ławicki; Adam Jacek Krętowski; Monika Zbucka-Krętowska

Novel approaches to an integrated route for Trisomy 21 evaluation

Biomolecules, 2021, 11(9), 1328.
Doi: 10.3390/biom11091328

Imię i nazwisko współautora	***Charakter udziału***	***Procentowy wkład***
doktorant – mgr Angelika Buczyńska	Udział w planowaniu planu pracy, przeprowadzanie przeglądu literatury prac prezentowanych w pracy, opracowanie i analiza wyników, przygotowanie manuskryptu, przygotowanie tabel wchodzących w skład manuskryptów	60%
dr Iwona Sidorkiewicz	Pomoc przy przeglądzie literatury i współtworzenie manuskryptu, korekta językowa	8%

Mgr Anna Trochimiuk	Udział w przeglądzie literatury, tworzeniu bazy danych	2%
Prof. dr hab. Sławomir Ławicki	Konsultacja merytoryczna	2%
Prof. dr hab. Adam Jacek Krętowski	Konsultacja merytoryczna	10%
dr hab. Monika Zbucka-Krętowska	Stworzenie koncepcji pracy, pomoc przy przygotowaniu manuskryptu, nadzór merytoryczny	18%

Oświadczam, że wszyscy współautorzy wyrazili zgodę na wykorzystanie powyższej publikacji w pracy doktorskiej mgr Angeliki Buczyńskiej.

2. Angelika Buczyńska; Iwona Sidorkiewicz; Sławomir Ławicki; Adam Jacek Krętowski; Monika Zbucka-Krętowska

The Significance of Apolipoprotein E Measurement in the Screening of Fetal Down Syndrome

Journal of Clinical Medicine, 2020, 9(12), 3995.

Doi: 10.3390/jcm9123995

Imię i nazwisko współautora	***Charakter udziału***	***Procentowy wkład***
doktorant – mgr Angelika Buczyńska	Udział w planowaniu eksperymentów, przeprowadzanie eksperymentów prezentowanych w pracy, opracowanie i analiza wyników, przygotowanie manuskryptu, przygotowanie tabel wchodzących w skład manuskryptów	60%
dr Iwona Sidorkiewicz	Pomoc przy przeprowadzeniu oznaczeń i współtworzenie manuskryptu, korekta językowa	10%
Prof. dr hab. Sławomir Ławicki	Konsultacja merytoryczna	2%
Prof. dr hab. Adam Jacek Krętowski	Konsultacja merytoryczna	10%
dr hab. Monika Zbucka-Krętowska	Stworzenie koncepcji pracy, udział w planowaniu eksperymentów, pomoc	18%

	przy przygotowaniu manuskryptu, nadzór merytoryczny	

Oświadczam, że wszyscy współautorzy wyrazili zgodę na wykorzystanie powyższej publikacji w pracy doktorskiej mgr Angeliki Buczyńskiej.

3. Angelika Buczyńska; Iwona Sidorkiewicz; Sławomir Ławicki; Adam Jacek Krętowski; Monika Zbucka-Krętowska

Prenatal Screening of Trisomy 21: Could Oxidative Stress Markers Play a Role?
Journal of Clinical Medicine, 2021, 10(11), 2382.
Doi: 10.3390/jcm10112382

Imię i nazwisko współautora	***Charakter udziału***	***Procentowy wkład***
doktorant – mgr Angelika Buczyńska	Udział w planowaniu eksperymentów, przeprowadzanie eksperymentów prezentowanych w pracy, opracowanie i analiza wyników, przygotowanie manuskryptu, przygotowanie tabel wchodzących w skład manuskryptów	60%
dr Iwona Sidorkiewicz	Pomoc przy przeprowadzeniu oznaczeń i współtworzenie manuskryptu, korekta językowa	10%
Prof. dr hab. Sławomir Ławicki	Konsultacja merytoryczna	2%
Prof. dr hab. Adam Jacek Krętowski	Konsultacja merytoryczna	10%
dr hab. Monika Zbucka-Krętowska	Stworzenie koncepcji pracy, udział w planowaniu eksperymentów, pomoc	18%

	przy przygotowaniu manuskryptu, nadzór merytoryczny	

Oświadczam, że wszyscy współautorzy wyrazili zgodę na wykorzystanie powyższej publikacji w pracy doktorskiej mgr Angeliki Buczyńskiej.

mgr Angelika Buczyńska

Centrum Badań klinicznych

Uniwersytet Medyczny w Białymstoku

OŚWIADCZENIE

Oświadczam, iż mój udział w przygotowaniu publikacji:

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Jednocześnie stwierdzam, iż mój indywidualny udział w powstaniu niniejszej pracy wyniósł 60%.

2. Angelika Buczyńska; Iwona Sidorkiewicz; Sławomir Ławicki; Adam Jacek Krętowski; Monika Zbucka-Krętowska, *The Significance of Apolipoprotein E Measurement in the Screening of Fetal Down Syndrome;* Journal of Clinical Medicine, 2020, 9(12), 3995.Doi:10.3390/jcm9123995, wchodzącej w skład rozprawy doktorskiej polegał na: udziale w planowaniu eksperymentów, przeprowadzaniu eksperymentów prezentowanych w pracy, opracowaniu i analizie wyników, przygotowaniu manuskryptu, przygotowaniu tabel wchodzących w skład manuskryptów.

Jednocześnie stwierdzam, iż mój indywidualny udział w powstaniu niniejszej pracy wyniósł 60%.

3. Angelika Buczyńska; Iwona Sidorkiewicz; Sławomir Ławicki; Adam Jacek Krętowski; Monika Zbucka-Krętowska, *Prenatal Screening of Trisomy 21: Could Oxidative Stress Markers Play a Role?*; Journal of Clinical Medicine,2021, 10(11), 2382.Doi:10.3390/jcm10112382, wchodzącej w skład rozprawy doktorskiej polegał na: udziale w planowaniu eksperymentów, przeprowadzeniu eksperymentów, opracowaniu i analizie wyników, przygotowaniu manuskryptu, przygotowaniu tabel wchodzących w skład manuskryptu.

Jednocześnie stwierdzam, iż mój indywidualny udział w powstaniu niniejszej pracy wyniósł 60%.

Angelika Buczyńska

dr hab. Monika Zbucka-Krętowska

Kierownik Zakładu Endokrynologii Ginekologicznej

i Ginekologii Wieku Rozwojowego

Uniwersytet Medyczny w Białymstoku

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Jednocześnie potwierdzam, iż indywidualny udział Pani mgr Angeliki Buczyńskiej w powstaniu niniejszej pracy wyniósł 60%.

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dr Iwona Sidorkiewicz

Centrum Badań klinicznych

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Iwona Sidorkiewicz

Prof. dr hab. Adam Jacek Krętowski

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Prof. dr hab. Sławomir Ławicki

Zakład Medycyny Populacyjnej i Prewencji Chorób Cywilizacyjnych

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Ławicki S.

mgr Anna Trochimiuk

Klinika Endokrynologii, Diabetologii i Chorób Wewnętrznych

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Anna Trochimiuk