Lek. Katarzyna Zawadzka-Starczewska

Nowe czynniki ryzyka rozwoju choroby Gravesa i Basedowa oraz orbitopatii Gravesa, ze szczególnym uwzględnieniem czynników genetycznych

Rozprawa na stopień naukowy doktora nauk medycznych
i nauk o zdrowiu w dyscyplinie nauki medyczne

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SŁOWA KLUCZOWE

orbitopatia Gravesa, choroba Gravesa i Basedowa, autoimmunizacja, przeciwciała przeciw receptorowi dla TSH, czynniki ryzyka, ludzkie antygeny leukocytarne, HLA, allele podatności, cholesterol LDL

Graves' orbitopathy, Graves' disease, autoimmunity, TSH receptor antibodies, risk factors, human leukocyte antigens, HLA, susceptibility alleles, LDL cholesterol

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za możliwość rozwoju, ogrom wiedzy, zaufanie i nieocenione wsparcie.
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niezwykle doceniam poświęcony czas, zaangażowanie podczas współpracy
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Jestem wdzięczna moim przyjaciołom i współpracownikom
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Spis treści

WYKAZ PUBLIKACJI STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

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Lp.	Autorzy	Tytuł	Źródło	IF	Punktacja MEiN
1.	*Zawadzka-Starczewska Katarzyna*, Tymoniuk Bogusław, Stasiak Bartłomiej, Lewiński Andrzej, Stasiak Magdalena.	Actual Associations between HLA Haplotype and Graves' Disease Development	J Clin Med. 2022 : 11, 9, 2492	4.964	140
2.	*Zawadzka-Starczewska Katarzyna,* Stasiak B, Wojciechowska-Durczyńska Katarzyna, Lewiński Andrzeja, Stasiak Magdalena.	Novel Insight into Non-Genetic Risk Factors of Graves' Orbitopathy.	Int J Environ Res Public Health. 2022 Dec 16;19(24):16941.	4.614	140
3.	Stasiak Magdalena, *Zawadzka-Starczewska Katarzyna,* Tymoniuk B, Stasiak B, Lewiński Andrzej.	Significance of HLA in the development of Graves' orbitopathy. (praca przyjęta do druku)	Genes Immun. 2023 Jan 13. doi: 10.1038/s41435-023-00193-z	4.248	100
			Łącznie	**13.826**	**380**

Punktacja zgodna z rokiem wydania publikacji.

WYKAZ STOSOWANYCH SKRÓTÓW

- AITD: *autoimmune thyroid disease* – choroba autoimmunizacyjna tarczycy
- aTg – przeciwciała przeciw tyreoglobulinie
- aTPO – przeciwciała przeciw peroksydazie tarczycowej
- EUGOGO: *European Group of Graves' Ophthalmopathy* - Europejska Grupa ds. Orbitopatii Gravesa
- FT4: wolna tyroksyna
- GD: *Graves' disease* – choroba Graves'a i Basedowa
- GO: *Graves' orbitopathy* – orbitopatia Gravesa
- HDL: *high-density lipoproteins* - lipoproteiny o wysokiej gęstości
- HLA: *human leukocyte antigen* – ludzki antygen leukocytarny
- IQR: interquartile range - rozstęp kwartylny
- LD: linkage disequilibrium - nierównowaga sprzężeń
- LDL: *low-density lipoprotein*s – lipoproteiny o niskiej gęstości
- MCV: *mean corpuscular volume* – średnia objętość erytrocytu
- MHC: *major histocompatibility complex* – główny kompleks zgodności tkankowej
- NGS: *next-generation sequencing* – sekwencjonowanie nowej generacji
- OR: *odds ratio* - iloraz szans
- PLT: *platelet count* – liczba płytek krwi
- QoL: *quality of life* – wskaźnik jakości życia
- RAI: *radioactive iodine* – jod promieniotwórczy
- ROS: *reactive oxygen species* – reaktywne formy tlenu
- SD: *Standard Deviation* – odchylenie standardowe
- SLEs - *stressful life events* – stresujące wydarzenia życiowe
- SSP-PCR - *Single Specific Primer-Polymerase Chain Reaction* – łańcuchowa reakcja polimerazy z zastosowaniem sekwencji specyficznych primerów
- TC: *total cholesterol* – cholesterol całkowity
- TG: *triglycerides* – triglicerydy
- TRAb*: TSH-receptor antibodies* – przeciwciała przeciw receptorowi dla TSH
- TSH: *thyroid stimulating hormone (thyrotropin*) – tyreotropina
- USG - badanie ultrasonograficzne
- 25(OH)D: *25-hydroxycholecalciferol* – 25-hydroksycholekalcyferol

OMÓWIENIE CYKLU PUBLIKACJI

Założenia i cele pracy wraz z uzasadnieniem połączenia publikacji w cykl.

Choroba Gravesa i Basedowa (GD) jest chorobą autoimmunizacyjną tarczycy (AITD) związaną z wytwarzaniem swoistych przeciwciał przeciwko receptorowi dla tyreotropiny (TRAb), które zazwyczaj stymulują receptor tyreotropiny (TSH), powodując nadmierną produkcję hormonów tarczycy. Choroba Gravesa jest najczęstszą przyczyną nadczynności tarczycy [1] w państwach z prawidłową podażą jodu, do których należy Polska. Chorobowość wynosi ok. 1-1,5 %, z zapadalnością w przedziale 20-30 nowych przypadków/100 tys./rok [2].

Przeciwciała przeciwko receptorowi TSH mogą wykazywać powinowactwo nie tylko do tarczycy, ale również do tkanek oczodołów, skóry, kości czy mięśni. Orbitopatia Gravesa (GO) jest najważniejszą pozatarczycową manifestacją GD. Występuje ona stosunkowo rzadko, szacowana częstość występowania wynosi 0,54–0,9 przypadków/100 000/rok u mężczyzn, 2,67–3,3 przypadków/100 000/rok u kobiet [3]. GO stanowi zespół objawów wynikających z zapalenia tkanek miękkich oczodołu i powodujących niejednokrotnie znacznie obniżoną jakość życia (QoL), do których należą m.in. wytrzeszcz, obrzęk, zaczerwienienie, łzawienie czy bóle gałek ocznych. GO może prowadzić do ciężkich powikłań, w tym do neuropatii nerwu wzrokowego czy uszkodzenia rogówki [4]. Do rozwoju GO dochodzi zwykle w trakcie nadczynności tarczycy, ale może także wystąpić w okresie eutyreozy czy niedoczynności tarczycy.

Postępowanie w GO zależy od aktywności zapalenia (faza GO aktywna lub nieaktywna zapalnie) oraz stopnia zaawansowania klinicznego (ciężkości) – GO łagodna, umiarkowana do ciężkiej lub zagrażająca utratą wzroku – biorąc pod uwagę również QoL pacjenta [5]. Najczęściej występują przypadki łagodnej GO, postać umiarkowana do ciężkiej rozwija się w 5-6 % GO [3].

Objawy oczne związane z nadczynnością tarczycy opisano już wiele wieków temu. Według dostępnych doniesień, Ismail ibn al-Husayn al-Jurjani już w 1110 roku opisał przypadek pacjenta z wolem, wytrzeszczem i kołataniem serca w swojej perskiej encyklopedii medycznej [6]. W XVI wieku, przy frontowej bramie Kolegium Świętego Jana Uniwersytetu w Cambridge, gdzie odbywa się sympozjum okulistyczne tegoż uniwersytetu, wyryto w kamieniu wizerunek twarzy mężczyzny z wytrzeszczem. Kolejne opisy dotyczące orbitopatii tarczycowej pochodzą z XIX wieku. Caleb Parry w 1825 r.

zaprezentował przypadek pacjentki po porodzie z wolem, wytrzeszczem i kołataniem serca oraz odnotował wystąpienie podobnych objawów po narażeniu na stres [7]. On właśnie stał się w ten sposób jednym z pierwszych autorów, którzy opisali potencjalne czynniki ryzyka rozwoju GO. Robert James Graves w 1835 roku powiązał występowanie nadczynności tarczycy z wytrzeszczem, a w 1840 roku Karl Adolph von Basedow z Merseburga opisał dokładnie chorobę charakteryzującą się wolem, wytrzeszczem i tachykardią – tzw. trójca merseburska [7].

Mimo obecności od wielu wieków w historii medycyny opisów współistnienia nadczynności tarczycy i wytrzeszczu, wciąż trwają badania nad patogenezą zarówno GD, jak i orbitopatii tarczycowej. Podobnie jak większość chorób autoimmunizacyjnych, GD jest wywoływana przez czynniki środowiskowe u osób predysponowanych genetycznie. Podłoże genetyczne może mieć istotny wpływ na rozwój chorób tarczycy oraz na ich przebieg. W ostatnich latach udowodniono, że ryzyko wystąpienia podostrego zapalenia tarczycy oraz przebieg tej choroby i ryzyko jej nawrotu są zależne od haplotypów HLA (ludzkie antygeny leukocytarne) [8,9]. W aspekcie GD, postulowano wcześniej związek pewnych antygenów i alleli HLA (m.in. –B*46, *-DRB1*08:03, -DRB1*09:01, -DRB1*14:03,* -DRw8, -DQw4, -B5, -Dw12, -A11) z występowaniem tej choroby w populacji azjatyckiej [10-12], choć wyniki różnych autorów były sprzeczne, prawdopodobnie wskutek małej liczebności grup badanych i stosowanych metod o niskiej rozdzielczości, w tym głównie metod serologicznych [10-11]. Ponadto, wyników dla populacji azjatyckiej nie można odnosić do populacji kaukaskiej, gdyż grupy etniczne różnią się pod kątem obecności poszczególnych alleli HLA.

W odniesieniu do populacji kaukaskiej postulowano znaczenie m.in. HLA-DRB1, -DQA1, -DQB1, -A*68 i -B*08 jako możliwych czynników ryzyka rozwoju i nawrotu GD [13], jednakże wyniki te również nie były powtarzalne. Również w tym przypadku najbardziej prawdopodobną przyczyną rozbieżności wyników były niedostatecznie liczne grupy pacjentów oraz zastosowanie metod o niskiej rozdzielczości. Dotychczas nie stwierdzono, czy którykolwiek z alleli HLA wpływa na przebieg GD i potencjalnie może być związany z większym ryzykiem rozwoju GO.

W chwili rozpoczęcia badań w ramach prezentowanego projektu, dane w zakresie zależności między HLA a GO były skąpe, nawet dla populacji azjatyckiej [11,14-18], a w odniesieniu do populacji kaukaskiej w dostępnej literaturze nie było takich doniesień. Wśród badań dotyczących populacji azjatyckiej dominowały prace

przeprowadzone z zastosowaniem mało dokładnych metod serologicznych, które obecnie nie są rekomendowane z uwagi na wysokie ryzyko błędu [19]. Wnioski z tych badań serologicznych były sprzeczne [11, 14-15]. Przeprowadzone badania w większości cechowały się niską rozdzielczością metody bądź małą liczebnością grupy. Pojedyncze badania zostały wykonane metodą wysokiej rozdzielczości w populacji azjatyckiej [17-18], ale tylko jedno z nich obejmowało adekwatnie dużą grupę pacjentów [18]. Podsumowanie wyników uzyskanych we wcześniejszych badaniach oceniających potencjalne allele ryzyka GO w populacji azjatyckiej przedstawiono w Tabeli 1.

Tabela 1. Podsumowanie wyników uzyskanych we wcześniejszych badaniach oceniających potencjalne allele ryzyka GO w populacji azjatyckiej

Autorzy	**Ref.**	**Populacja**	**Metoda**	**Liczba osób w grupie GO**	**Liczba osób w grupie GD bez GO**	**Liczba osób w grupie kontrolnej**	**Wynik – związek HLA z ryzykiem GO**
Inoue i wsp.	[11]	Azjatycka (japońska)	serologiczna	42	88	186	-DQw4 (+) i -A31 (-) -B5 (+) i -Dw12 (+) -A11 (+) i -DPw2 (+)
Inoue i wsp.	[14]	Azjatycka (japońska)	serologiczna	23	88	186	-DQw3 (+), -DPQ2(+)
Ohtsuka i Nakamura	[15]	Azjatycka (japońska)	serologiczna	48	94	767	-DR14 (+), -DQ1 (+) -B35 (-), -B54 (-), -DR4 (-), -DQ4 (-)
Mehraji i wsp.	[16]	Azjatycka (japońska)	SSP-PCR	45	80	180	Brak związku
Shin i wsp.	[17]	Azjatycka (koreańska)	NGS	35	71	142	*-C*03:03 (+)* *-B*54:01 (-)*
Huang i wsp.	[18]	Azjatycka (chińska)	NGS	82	272	411	*-B*38:02* (+) *-DQA1*01:02* + *-DRB1*16:02 (+)* *-DQA1*01:02* + *-DQB1*05:02* (+)

NGS, sekwencjonowanie nowej generacji; SSP-PCR, łańcuchowa reakcja polimerazy z zastosowaniem specyficznych primerów (metoda niskiej rozdzielczości); GD, choroba Gravesa i Basedowa; GO, orbitopatia Gravesa

Wobec braku miarodajnych danych, pochodzących z badań w populacji kaukaskiej, przeprowadzonych metodą wysokiej rozdzielczości, głównym celem bieżącego cyklu badawczego stało się wykazanie z zastosowaniem metod ultraczułych (ang. *next generation sequencing*, NGS), czy obecność konkretnych alleli HLA wiąże się ze zwiększonym lub zmniejszonym ryzykiem rozwoju GD oraz GO w tej populacji.

Jak wspomniano powyżej, na podstawie dotychczas opublikowanych badań wiadomo, że GO rozwija się częściej u osób, u których istnieją pewne kliniczne czynniki ryzyka, które wyzwalają chorobę u osób z podatnością genetyczną. Dotychczas

potwierdzone czynniki ryzyka GO to palenie tytoniu, wysokie stężenia TRAb w surowicy, leczenie radiojodem, niekontrolowana nadczynność lub niedoczynność tarczycy, a również podwyższone stężenie cholesterolu całkowitego (TC) i lipoprotein o niskiej gęstości (LDL) w surowicy [4, 20]. Ponadto inne parametry kliniczne, takie jak wiek, stres czy niedobór witaminy D [21-24], były postulowane jako czynniki ryzyka GO. Wielu czynników, w tym parametrów laboratoryjnych, nigdy nie porównywano u pacjentów z GO i pacjentów z GD bez GO w grupach o odpowiedniej liczebności. Drugim celem bieżącego cyklu badawczego stała się zatem ocena również innych niż genetyczne czynników ryzyka wystąpienia GO.

Hipoteza badawcza zakładała zatem, że podłoże genetyczne wynikające z obecności konkretnych alleli HLA oraz występowanie niegenetycznych czynników ryzyka wiążą się ze zwiększoną częstością rozwoju GD i GO, a niektóre allele HLA mogą potencjalnie mieć działanie protekcyjne, chroniąc przed wystąpieniem GD i/lub GO, niezależnie od innych czynników ryzyka.

Celem całości projektu było zatem wykazanie znaczenia nowych czynników ryzyka rozwoju GD oraz GO oraz zweryfikowanie znaczenia czynników opisanych dotychczas. Wyodrębnienie konkretnych genetycznych i niegenetycznych czynników ryzyka stworzy możliwość indywidualnej oceny ryzyka oraz personalizacji profilaktyki i leczenia, co pozwoli zapobiec obniżeniu jakości życia oraz wystąpieniu ciężkich, zagrażających utratą wzroku, powikłań.

Do grupy badanej we wszystkich pracach cyklu włączeni byli pacjenci z rozpoznaną GD, przebiegającą z GO lub bez GO. Chorobę Gravesa i Basedowa rozpoznawano w oparciu o następujące kryteria [25]: biochemiczne cechy nadczynności tarczycy (rozumiane jako obniżone stężenie TSH i podwyższone stężenie wolnych hormonów tarczycy), podwyższone stężenie TRAb i charakterystyczny obraz badania ultrasonograficznego (USG). Orbitopatię Gravesa rozpoznawano na podstawie obecności retrakcji powiek i wytrzeszczu oraz zajęcia tkanek miękkich oka (zaczerwienienie, obrzęk), zgodnie z wytycznymi European Group on Graves' Orbitopathy (EUGOGO) [4, 26].

Pacjenci z GD i bez GO zostali włączeni do prospektywnego ramienia tego badania, w którym zastosowano obserwację długoterminową (3 lata) w celu potwierdzenia przynależności pacjentów do grupy. Wszyscy pacjenci, u których wystąpiły objawy GO podczas obserwacji, zostali przeklasyfikowani do grupy GO.

Od wszystkich pacjentów uzyskano świadomą zgodę na udział w badaniu, po pełnym wyjaśnieniu celu i założeń projektu. Badanie zostało zatwierdzone przez Komisję Bioetyczną ICZMP w Łodzi.

Wyniki wszystkich analiz zebrano i opublikowano w postaci cyklu publikacji – trzech prac oryginalnych, które wzajemnie się uzupełniają i tworzą logiczny ciąg nowych, oryginalnych wniosków.

Za połączeniem prac w cykl publikacji przemawiał fakt, że ich tematyka jest ze sobą ściśle powiązana, ponieważ dotyczą one czynników ryzyka GD oraz GO. Wszystkie trzy badania przeprowadzone zostały w grupach chorych z GD, przy czym w pierwszej publikacji cyklu grupę z GD analizowano jako jedną kohortę, natomiast w następnych badaniach, chorych z GD podzielono na pacjentów z GO i bez GO. Oba badania analizujące znaczenie HLA miały tę samą grupę kontrolną, obejmującą 2217 zdrowych dawców komórek hematopoetycznych. Tak duża grupa była konieczna, by uzyskać reprezentatywną dla populacji częstość występowania poszczególnych alleli. Poza związkiem merytorycznym, publikacje łączy podobny zespół współautorów zaangażowanych w ich tworzenie oraz zbliżona metodologia, zarówno laboratoryjna, jak i statystyczna.

Cykl obejmuje:

1) publikację identyfikującą zależności między obecnością alleli HLA a ryzykiem rozwoju GD
2) artykuł opisujący znaczenie alleli HLA w rozwoju GO
3) publikację obejmującą ocenę niegenetycznych czynników ryzyka GO.

Omówienie osiągnięć badawczych kandydata opisanych w cyklu publikacji, na tle aktualnego stanu wiedzy.

Cykl publikacji otwiera praca, której głównym założeniem było wyodrębnienie alleli HLA związanych z ryzykiem wystąpienia GD u osób rasy kaukaskiej oraz alleli HLA, które można uznać za protekcyjne. Jak już wspomniano powyżej, wcześniejsze prace dotyczące alleli HLA potencjalnie związanych z GD, odnosiły się głównie do rasy azjatyckiej, a ponadto były często przeprowadzane metodami serologicznymi, które cechują się znacznie niższą dokładnością niż metoda NGS, która charakteryzuje się wysoką rozdzielczością, pozwalającą uzyskać specyficzność alleliczną. Ponadto symbole poszczególnych alleli oceniane według wcześniej dostępnych metod różnią się od obecnie stosowanych i niektóre antygeny, poprzednio oznaczane jednym symbolem, podczas oceny metodą NGS rozdzielane są jako kilka odrębnych alleli, co wywiera istotny wpływ na dokładność i spójność wyników. Starsze metody zapewniają wyniki dla całej grupy allelicznej, a nie dla konkretnego allelu HLA, co może powodować błędne wnioski i rozbieżności w wynikach badań w zależności od metody.

W trakcie projektu przeprowadzono typowanie HLA przy użyciu metody NGS (Illumina platform, Illumina, USA) u łącznie 159 pacjentów z GD. Uzyskane wyniki drogą analizy statystycznej porównano z wynikami dla zdrowej populacji ogólnej, obejmującej – jak wcześniej wspomniano – 2217 zdrowych dawców komórek hematopoetycznych (już wcześniej przeprowadzone typowanie HLA grupy kontrolnej metodą NGS). Typowaniu zostały poddane geny *HLA-A, -B, -C* (MHC klasy I) oraz *-DQB1* i *-DRB1* (MHC klasy II).

Niniejsze badanie obejmowało największą z dostępnych w momencie publikacji pracy grupę pacjentów z GD rasy kaukaskiej, u której zastosowano metodę NGS do analizy alleli HLA obu klas MHC.

Na podstawie przeprowadzonej analizy wykazano istotny związek między ryzykiem GD i allelami: *HLA-B*08:01, -B*39:06, -B*37:01, -C*07:01, -C*14:02, -C*03:02, -C *17:01, -DRB1*03:01, -DRB1*11:01, -DRB1*13:03, -DRB1*01:03, -DRB1*14:01, -DQB1*03:01, -DQB1*02:01*.

Allele *HLA-B*39:06, -B*37:01, -C*14:02, -C*03:02, -C*17:01, -DRB1*14:01* stanowią całkowicie nowe, wcześniej nieopisywane czynniki ryzyka rozwoju GD. Każdy z nich stanowi niezależny czynnik ryzyka, gdyż nie istnieje między nimi, ani między nimi a wcześniej raportowanymi allelami, zjawisko nierównowagi sprzężeń (ang. *linkage*

disequilibrium, LD), związane z większą częstością występowania konkretnych alleli z powodu ich bliskiego sąsiedztwa na chromosomie i częstszego łącznego dziedziczenia. Dzięki temu, ich znaczenie dla patogenezy GD jest szczególnie istotne.

Omawiane badanie wykazało silny związek GD z obecnością kilku alleli należących do obu klas MHC. Fakt, iż każdy z nich może być traktowany jako silny niezależny czynnik ryzyka potwierdzają moje obserwacje, iż obecność nawet tylko jednego z nich jest wystarczająca do rozwoju GD. Do alleli wysokiego ryzyka, które w analizowanej grupie pacjentów z GD były obecne jako pojedynczy allel należą: *HLA-B*39:06, -C*03:02, -C*07:01, -C*14:02, -DRB1*14:01* i *-DQB1*03:01*.

Należy podkreślić, iż w oparciu o uzyskane wyniki zaobserwowałam silną korelację między GD a kombinacją trzech alleli: *HLA-B*08:01, -DRB1*03:01* i *-DQB1*02:01* (współistnienie tych 3 alleli odnotowano u 22 % pacjentów z GD, a więc 4 razy częściej niż współobecność tych trzech alleli w grupie kontrolnej – 5,87%). Tak bliski związek między *HLA-B*08:01* (allel MHC klasy I) i allelami MHC klasy II był wcześniej postulowany w populacji kaukaskiej [27]. Obecne badanie potwierdziło ten fenomen i po raz pierwszy wykazało jego znaczenie u pacjentów z GD. To odkrycie rzuca nowe światło na możliwą nierównowagę sprzężeń między allelami z różnych klas MHC i wymaga dalszych badań populacyjnych.

W mojej grupie pacjentów tylko u 4 osób wystąpienie GD było poprzedzone przez przewlekłe autoimmunizacyjne zapalenie tarczycy (typu Hashimoto) i u wszystkich był obecny allel *HLA-DQB1*02:01* (obecnie opisany przez nas jako jeden z alleli wysokiego ryzyka GD), który pozostaje w nierównowadze sprzężeń z *-DRB1:03:01* - allelem typowym dla autoimmunizacji tarczycy. Współwystępowanie tych dwóch alleli stwierdzono u wszystkich pacjentów z współistnieniem GD i autoimmunizacji pozatarczycowej (tj. u dwóch pacjentów z chorobą Addisona i dwóch pacjentów z cukrzycą typu 1). To zjawisko z pewnością wymaga dalszych badań, ponieważ opisywane w badaniu podgrupy były zbyt małe, aby uzyskać wyniki istotne statystycznie.

W obecnym badaniu ujawniono również allele HLA o charakterze ochronnym w odniesieniu do GD, których częstość występowania była istotnie niższa w grupie z GD w porównaniu z osobami zdrowymi. Należą do nich: *HLA-B*07:02, -C*07:02, -C*03:04, DRB1*07:01, -DQB1*02:02* oraz *-DQB1*03:03*. Spośród wymienionych alleli, potencjalnie ochronne znaczenie pierwszych trzech zostało po raz pierwszy opisane w wynikach bieżącego badania.

W zaprezentowanej publikacji przedstawiono zatem, iż istnieje ścisły związek pomiędzy ryzykiem rozwoju GD a obecnością konkretnych alleli HLA, które zwiększają to ryzyko, bądź je redukują. Dane te pozwalają na opracowanie wiarygodnego narzędzia do spersonalizowanej oceny ryzyka GD, opartego na profilu HLA danego pacjenta.

Celem drugiej publikacji z omawianego cyklu prac było porównanie częstości występowania alleli HLA u pacjentów z GD w dwóch grupach: bez orbitopatii i z towarzyszącą GO. Grupy porównywano do siebie nawzajem oraz do grupy kontrolnej, obejmującej zdrowych dawców komórek hematopoetycznych (jak opisano powyżej). Jak już wspomniano w założeniach rozprawy, dotychczas nie uzyskano wiarygodnych wyników dotyczących alleli HLA potencjalnie zwiększających lub obniżających ryzyko GO dla populacji kaukaskiej.

W ramach projektu przeprowadzono typowanie HLA przy użyciu metody NGS (Illumina platform, Illumina, USA) u 91 pacjentów z GD bez orbitopatii oraz 70 pacjentów z towarzyszącą orbitopatią (łącznie 161 pacjentów z GD). Wcześniej przeprowadzono również typowanie HLA metodą NGS dla zdrowej populacji ogólnej, obejmującej 2217 zdrowych dawców komórek hematopoetycznych. Jak już opisano w przypadku pierwszego badania, tak i w tym badaniu typowaniu zostały poddane allele *HLA-A, -B, -C* (MHC klasy I) oraz allele *-DQB1* oraz *-DRB1* (MHC klasy II).

W oparciu o porównanie grup z GO i bez GO stwierdzono, że z istotnie wyższym ryzykiem rozwoju GO wiąże się obecność alleli *HLA-A*32:01, -B*39:01* i *-C*08:02,* należących wyłącznie do MHC klasy I. Z drugiej strony, obecność alleli *HLA-C*04:01* i *-DRB1*15:02* wiązała się z istotnie niższym ryzykiem rozwoju GO u chorych z GD. Zestawienie wyników w zakresie alleli zwiększających bądź obniżających ryzyko GO wraz z przedstawieniem siły oddziaływania (iloraz szans) podsumowano w Tabeli 2.

Tabela 2. Podsumowanie wyników dotyczących korelacji alleli HLA z ryzykiem GO

Allele zwiększające ryzyko GO	***OR***	**Allele obniżające ryzyko GO**	***OR***
*A*32:01*[a]	-	*C*04:01*[a,b]	*0.4*
*B*39:01*[a,b]	*2.8*	*DRB1*15:02*[a]	-
*C*08:02*[a]	*6.9*	*C*03:04*[b]	*0.1*
*A*01:01*[b]	*1.8*	*C*07:02*[b]	*0.2*
*B*37:01*[b]	*4.5*		
*B*42:01*[b]	*2.8*		
*C*03:02*[b]	8.3		
*DRB1*14:01*[b]	6.2		
*DRB1*03:01*[b]	1.9		
*DQB1*02:01*[b]	1.9		

[a] – w odniesieniu do grupy z GD bez GO; [b] – w odniesieniu do zdrowej grupy kontrolnej;
OR - iloraz szans

Porównując grupę pacjentów z GO z grupą kontrolną, allele o wyższej częstotliwości w GO stwierdzono zarówno w MHC klasy I, jak i klasy II. Różnice były statystycznie istotne dla alleli *HLA-A*01:01, -B*37:01, , -B*39:01, -B*42:01, -C*03:02, -DRB1*14:01, -DRB1*03:01, -DQB1*02:01*. Z kolei częstość występowania *HLA-C*04:01,-C*03:04* i *-C*07:02* była istotnie niższa w grupie z GO, w porównaniu z grupą kontrolną (Tabela 2).

Jak wynika z przeprowadzonych analiz, największe ryzyko wystąpienia GO było związane z obecnością *HLA-B*37:01, -C*03:02, -C*08:02,* i *-DRB1*14:01* (iloraz szans odpowiednio 4.5; 8.3; 6.9 oraz 6.2).

Allelami o istotnie wyższej częstości w grupie pacjentów z GD bez orbitopatii w porównaniu do grupy kontrolnej były z kolei: *HLA-B*08:01, -B*39:06, -B*51:01, HLA-C*03:02, -C*07:01, -C*14:02, C*16:02, -C*17:01, -DRB1*01:03, -DRB1*03:01, -DRB1*15:02, -DQB1*03:01, -DQB1*02:01*. Korelację między obecnością większości z wymienionych alleli, a ogólnym ryzykiem GD wykazano w poprzedniej publikacji, jednak znaczenie *HLA-B*51:01* i *-C*16:02* nigdy wcześniej nie zostało odkryte. W obecnym badaniu potwierdzono zaobserwowane już w pierwszej – omówionej

powyżej – publikacji zwiększone ryzyko GD u nosicieli alleli *HLA-B*08:01, -C*07:01* i *-DQB1*03:01*. Również w aspekcie alleli o potencjalnie ochronnym działaniu w stosunku do GD bez orbitopatii, w obecnej pracy potwierdzono pionierskie obserwacje odnotowane w publikacji poprzedniej, dotyczące takiego działania *HLA-B*07:02* i *-C*07:02*. Spójność wyników uzyskanych po wyodrębnieniu grup z GO i bez GO z wynikami pierwszego badania świadczy dodatkowo o wysokiej wiarygodności stosowanej metody i adekwatnej wielkości grup.

Kolejna, ostatnia już z omawianego cyklu publikacji, praca dotyczy niegenetycznych czynników ryzyka GO. Do badania włączono 161 pacjentów, obejmujących grupę z GO (70 osób) i bez GO (91 osób), analogicznie jak opisano powyżej. Z badania wykluczono pacjentów z innymi niż GD chorobami, które mogły mieć wpływ na wyniki badań laboratoryjnych. W ramach tego etapu projektu przeanalizowano wyniki badań laboratoryjnych uzyskanych w badanej grupie pacjentów, oraz dane demograficzne i kliniczne, pochodzące z wywiadu dostarczonej dokumentacji pacjenta (m.in. dotyczące leczenia jodem promieniotwórczym lub wykonania zabiegu tyreoidektomii). Próbki krwi od wszystkich pacjentów pobrano w momencie postawienia diagnozy. Jako osoby palące kwalifikowano palaczy aktywnych w momencie rozpoznania GD. Wydarzenia stresujące oceniano na podstawie kwestionariusza skali stresu Holmesa-Rahe'a (z wynikiem >150 uznanym za pozytywny dla wydarzenia stresującego) [28].

Palenie tytoniu postulowano jako czynnik ryzyka GO w wielu pracach, w tym w aktualnych wytycznych EUGOGO [4,20]. Moje analizy potwierdziły silny związek między GO a paleniem papierosów ($p<0.001$). Palenie silnie indukuje stres oksydacyjny, a – jak wykazano – reaktywne formy tlenu (ROS), których podwyższone stężenia stwierdzono we krwi i moczu pacjentów z GO, stymulują w oczodołach proliferację fibroblastów, syntezę glikozoaminoglikanów i mediatorów stanu zapalnego [29]. Stąd korzystne jest antyoksydacyjne działanie selenu u pacjentów z GO [29].

W wielu badaniach wykazano istotnie wyższe stężenie TRAb u pacjentów z GO niż u pacjentów z GD bez GO, co również potwierdziłam w obecnym badaniu. Lantz i współpracownicy wykazali zwiększone ryzyko GO u pacjentów z TRAb > 6,3 IU/l w momencie rozpoznania GD [30]. W omawianym badaniu różnica stężeń TRAb była istotna, ale średnie stężenia TRAb były znacznie wyższe niż 6,3 IU/L w obu grupach (tj. 17,59 vs. 13,65 IU/L odpowiednio dla grupy z GO i bez GO). Dlatego rzeczywisty

próg stężeń TRAb dla ryzyka rozwoju GO należy poddać dalszej ocenie, ponieważ może być znacznie wyższy niż poprzednio postulowano [30].

W bieżącym badaniu, potwierdziłam również obserwację, iż starszy wiek jest czynnikiem ryzyka rozwoju GO, a osoby w grupie bez GO są istotnie statystycznie młodsze. Niestabilna nadczynność tarczycy jest uważana za czynnik ryzyka GO [4]. W obecnym projekcie nie udało się zaobserwować tej korelacji, ponieważ zdecydowana większość pacjentów zarówno w grupie GO, jak i bez GO w momencie rozpoznania miała nasiloną nadczynność tarczycy. Wynikało to z doboru grupy, gdyż byli to przede wszystkim pacjenci wymagający opieki ośrodka referencyjnego. W mojej grupie chorych z GO odnotowałam istotnie mniej nasiloną tyreotoksykozę niż u chorych bez GO. Już poprzednio były obserwowane niższe stężenia wolnej tyroksyny (FT4) u pacjentów z GO [31]. Powszechnie wiadomo, że tyreotoksykoza związana z GD jest zwykle znacznie cięższa u młodszych pacjentów. Jak wspomniano, opisywane badanie potwierdziło, że starszy wiek początku choroby jest czynnikiem ryzyka GO. Biorąc pod uwagę powyższe ustalenia, założyłam, że mniejsze nasilenie tyreotoksykozy w grupie GO może potencjalnie wynikać ze starszego wieku pacjentów, wymaga to jednak dalszych badań.

W omawianym badaniu zaobserwowano również kilka nowych, wcześniej nie opisywanych, a w obecnych analizach istotnych statystycznie, różnic w wynikach badań laboratoryjnych u chorych z GO i bez GO. Pierwszym z nich jest stężenie glukozy w surowicy, które było wyższe w momencie rozpoznania w grupie bez GO, co może mieć związek z większym nasileniem tyreotoksykozy w tej grupie.

Profil przeciwciał przeciwtarczycowych w GO jest przedmiotem wielu badań. W obecnym badaniu stwierdzono, że w grupie z GO stężenie przeciwciał przeciw tyreoglobulinie (aTg) było istotnie niższe, niż w grupie bez GO, co potwierdza poprzednie doniesienia [31], w których odnotowano również obniżone stężenie przeciwciał przeciw tyreoperoksydazie (aTPO) [31]. W aktualnym projekcie również uzyskano niższe stężenia aTPO w surowicy w grupie GO, jednak nie osiągnięto istotności statystycznej.

Ponadto w obecnym badaniu wykazano istotnie wyższe stężenia kreatyniny w surowicy w grupie GO w porównaniu z chorymi bez GO. Przypuszczałam, że ta obserwacja wynika głównie ze starszego wieku pacjentów, jednak stężenia mocznika w surowicy nie różniły się znacząco między grupami, a wręcz średnie stężenie mocznika było niższe w grupie GO (chociaż różnice nieistotne statystycznie). Dlatego opisana

obserwacja może wskazywać, że wyższe stężenia kreatyniny (choć nadal w zakresie wartości referencyjnych) mogą stanowić nowy czynnik ryzyka GO. Jednak konieczne są dalsze badania, aby potwierdzić tę zależność i ewentualnie zaproponować wartość odcięcia stężenia kreatyniny, którą można byłoby potencjalnie uznać za zwiększającą ryzyko rozwoju GO. Jeśli dalsze badania potwierdzą taki związek, poszukiwanie jego przyczyny będzie z pewnością procesem złożonym, a nie można wykluczyć, że pośrednie oddziaływanie np. stresu oksydacyjnego związanego z częstszym zjawiskiem palenia papierosów w tej grupie, przyniesie dalsze dowody konieczności podejmowania profilaktyki GO.

Ponadto, w obecnym badaniu, u pacjentów z GO stwierdzono istotnie wyższą wartość wskaźnika MCV (średnia objętość erytrocytu) w porównaniu do grupy bez GO. W opublikowanych w ostatnich latach badaniach wykazano, że u pacjentów z tyreotoksykozą stwierdzano niższą wartość MCV (wzrastającą podczas leczenia nadczynności tarczycy), ponadto postulowano odwrotną korelację poziomu MCV ze stężeniem hepcydyny [32, 33]. Podobnie, również zaobserwowałam niższą wartość MCV u pacjentów bez GO, u których tyreotoksykoza była cięższa. Jednakże, wyższa wartość wskaźnika MCV u pacjentów z GO może wynikać ze stresu oksydacyjnego, który wg dotychczasowych doniesień jest związany z wyższymi wartościami MCV [34].

Dotychczas nie analizowano stężenia kreatyniny oraz wartości MCV jako potencjalnych czynników ryzyka GO, dlatego potwierdzenie tej zależności wymaga dalszych badań.

Całkowicie nową obserwacją pochodzącą z obecnego badania jest również fakt, iż w grupie chorych z GO liczba płytek krwi (PLT) była istotnie niższa niż u pacjentów z GD bez GO. Istnieją doniesienia o autoimmunizacyjnej etiologii małopłytkowości związanej z chorobą Gravesa i Basedowa i nakładaniu się autoimmunizacji tarczycy i płytek krwi [35], co potwierdza znaczna poprawa liczby płytek w małopłytkowości opornej na dożylne leczenie immunoglobulinami i steroidami w trakcie leczenia choroby Gravesa i Basedowa [36]. Zatem, jednym z możliwych wyjaśnień patomechanizmu istotnie niższej liczby płytek krwi u pacjentów GO jest znacznie wyższe stężenie TRAb w surowicy w tej grupie chorych oraz większe nasilenie procesu autoimmunizacyjnego.

W ostatnich latach stwierdzono, że niezależnymi czynnikami ryzyka GO są wysokie stężenia cholesterolu całkowitego (TC) i cholesterolu LDL w surowicy [4,37]. Nie obserwowano korelacji między GO a stężeniami lipoprotein o wysokiej gęstości (HDL) i triglicerydów [38]. W obecnym badaniu potwierdzono te doniesienia

tj. wykazano wyższe stężenia cholesterolu całkowitego i LDL w surowicy w grupie pacjentów z GO w porównaniu do pacjentów z GD bez GO, bez istotnych różnic w stężeniach cholesterolu HDL i triglicerydów. Opisane dane potwierdza fakt, że leczenie obniżające stężenia cholesterolu poprawiło skuteczność dożylnej steroidoterapii (ivGC) u pacjentów z GO [38]. Dlatego aktualne wytyczne EUGOGO zalecają leczenie obniżające poziom cholesterolu u chorych z GD [4]. Cenną obserwacją pochodzącą z obecnego badania jest fakt, że nawet nieznaczne podwyższenie stężenia LDL, a być może nawet wysoki prawidłowy poziom LDL, są już czynnikami ryzyka rozwoju GO. Dlatego wyniki bieżącego projektu dostarczają dalszych dowodów potwierdzających istnienie wskazań do terapii obniżającej poziom cholesterolu u chorych z GD.

W ostatnim czasie postawiono hipotezę, że niedobór 25(OH)D (25-hydroksycholekalcyferolu) w surowicy jest niezależnym czynnikiem ryzyka GO [21]. W moim badaniu nie zaobserwowałam takiej korelacji, mimo podobnej liczebności analizowanych grup. Co ciekawe, zaobserwowałam wyższe stężenia 25(OH)D w grupie z GO, ale różnica nie była istotna statystycznie. Jednak w żadnej z analizowanych grup nie odnotowano ciężkiego niedoboru 25(OH)D (<10 ng/ml). Wydaje się zatem, że w przypadku występowania jedynie nieoptymalnego zaopatrzenia w witaminę D, t.j. stężenia 25(OH)D >20 ng/ml, parametr ten nie wpływa na ryzyko rozwoju GO. Możliwy wpływ ciężkiego niedoboru witaminy D na ryzyko rozwoju GO wymaga dalszych badań.

Dotychczas stwierdzono istotnie większą liczbę stresujących wydarzeń życiowych (SLEs) u pacjentów z GD w porównaniu z grupą kontrolną [23]. Ponadto częstość lęku i depresji w grupie GO była istotnie wyższa niż u osób zdrowych [24], jednak nie ma pewności, czy lęk i depresja poprzedzały GO, czy też były jej skutkiem. W obecnym badaniu SLEs występowały u 70 % pacjentów z grupy z GO i u 63 % pacjentów bez GO i różnica ta nie była statystycznie istotna. Ta kwestia wymaga z pewnością dalszych badań.

Jak powszechnie wiadomo, GD występuje częściej u kobiet (co potwierdzają również analizy w ramach opisywanego projektu), jednak dotychczasowe badania dotyczące związku między GO i płcią dostarczyły niespójnych wyników. W wielu pracach postulowano wyższą częstość występowania GO u kobiet [3], jednak różnice związane z płcią zmieniają się wraz z ciężkością GO: postać umiarkowaną do ciężkiej udokumentowano w podobnych proporcjach u kobiet i mężczyzn, natomiast wśród pacjentów z ciężką GO większy odsetek stanowili mężczyźni [24]. Obecne badanie

przeprowadzono w ośrodku referencyjnym, zatem większość pacjentów z GO stanowią chorzy z GO stopnia umiarkowanego do ciężkiego i – rzeczywiście – nie zaobserwowałam wśród chorych istotnej korelacji między płcią a ryzykiem GO.

Porównanie wyników badań laboratoryjnych, dla których uzyskano istotną statystycznie różnicę pomiędzy grupą pacjentów z GO i bez GO, a więc tych, które mogą być potencjalnie uważane za czynniki ryzyka GO, podsumowano w Tabeli 3. W Tabeli uwzględniono również omówione powyżej parametry, dla których nie odnotowano istotności statystycznej (w pracy oryginalnej zawarta jest tabela prezentująca wyniki dla wszystkich badanych parametrów).

Tabela 3. Podsumowanie najważniejszych danych dotyczących porównania wyników badań laboratoryjnych u pacjentów z GD: z GO i bez GO.

Parametr (wartości referencyjne i jednostki)	**GO**		**GD bez GO**		**Wartość *p***
	średnia ± SD (N)	mediana/IQR	średnia ± SD (N)	mediana/IQR	
TSH (0,27-4,2 µIU/ml)	0.18±0.59 (59)	0.01/0.04	0.10±0.48 (83)	0.01/0.01	**0.049***
FT3 (2.0-4.4 pg/ml)	9.59±8.89 (56)	4.85/10.55	12.85±8.06 (79)	11.63/11.95	**0.003***
FT4 (0.93-1.7 ng/dl)	2.64±2.09 (57)	1.80/1.86	3.58±1.96 (82)	3.15/2.86	**<0.001***
aTPO (<34 IU/ml)	182.57±195.05 (58)	77.50/268.85	211.54±204.57 (71)	170.40/236.50	0.23
aTg (<115 IU/ml)	347.22±894.99 (58)	25.45/218.22	406.41±640.99 (67)	250.00/484.30	**<0.001***
TRAb (<1.75 IU/l)	17.59±13.79 (68)	13.72/26.95	13.65±13.52 (88)	9.20/13.25	**0.021***
Glukoza (60-99 mg/dl)	94.10±24.76 (59)	89.00/11.50	98.26±18.74 (52)	96.00/11.00	**0.006***
Mocznik (19.3-42.3 mg/dl)	31.56±10.25 (48)	31.00/11.00	32.40±7.49 (45)	32.00/10.00	0.65
Kreatynina (0.66-1.25 mg/dl)	0.68±0.20 (58)	0.66/0.23	0.53±0.16 (54)	0.50/0.21	**<0.001***
PLT (150-400 x 10^3/µL)	238.37±59.11 (62)	235.00/79.25	270.11±72.74 (65)	259.00/89.00	**0.02***
MCV (kobiety 78-93 fl, mężczyźni 82-94 fl)	86.21±6.35 (58)	86.95/7.25	82.93±5.27 (60)	82.55/7.67	**<0.001***

IQR, rozstęp kwartylny; SD, odchylenie standardowe

Podsumowanie i wnioski.

Choroba Gravesa i Basedowa jest najczęstszą przyczyną nadczynności tarczycy, a związana z nią orbitopatia w wielu przypadkach stanowi poważne wyzwanie terapeutyczne. W prezentowanym cyklu badawczym wykazano istnienie silnej predyspozycji genetycznej związanej z allelami HLA do rozwoju GD i GO. Poza wyodrębnieniem alleli wysokiego ryzyka, określono również te, które wykazują działanie ochronne przed wystąpieniem GO i GD. Mając świadomość, że na opisaną predyspozycję genetyczną, muszą zadziałać również niegenetyczne czynniki ryzyka rozwoju GD i GO, w ostatniej publikacji cyklu wskazano na istnienie potencjalnych nowych klinicznych czynników ryzyka oraz dokonano weryfikacji znaczenia czynników poprzednio opisanych. Uzyskane wyniki pozwalają na udoskonalenie dotychczasowych zasad profilaktyki i leczenia, a także stwarzają możliwość indywidualizacji tych działań w oparciu o profil czynników ryzyka i czynników ochronnych u danego chorego. Wydaje się zatem, że całość cyklu dostarcza cennego narzędzia dla rozwoju precyzyjnej medycyny spersonalizowanej u chorych z GD. Zdaję sobie sprawę, że wraz z dalszym rozwojem metod badawczych odkryte zostaną z pewnością również inne – w tym genetyczne – istotne czynniki ryzyka rozwoju GD i GO. Mam nadzieję, że przedstawiony cykl publikacji będzie już teraz pomocnym narzędziem w indywidualizacji profilaktyki i leczenia GD i GO, w tym m.in. wyboru metody leczenia radykalnego GD, czy też sposobu profilaktyki i terapii GO. Ponadto, lekarze, mając świadomość ryzyka rozwoju GO w oparciu o obecność czynników tego ryzyka, mogą podjąć działania diagnostyczno-terapeutyczne już w momencie pojawienia się pierwszych objawów, a pacjenci mają szansę uniknąć narażenia na rozwój i/lub progresję GO i uzyskać optymalnie dostosowane leczenie.

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STRESZCZENIE W JĘZYKU POLSKIM

Tytuł: „Nowe czynniki ryzyka rozwoju choroby Gravesa i Basedowa oraz orbitopatii Gravesa, ze szczególnym uwzględnieniem czynników genetycznych”

Wstęp: Choroba Gravesa i Basedowa (GD) jest najczęstszą przyczyną nadczynności tarczycy w Polsce. Najważniejszą pozatarczycową manifestacją GD jest orbitopatia Gravesa (GO), mogąca nie tylko istotnie pogarszać jakość życia pacjenta, ale także zagrażać utratą wzroku. Podobnie jak inne choroby autoimmunizacyjne, GD i GO są zwykle wywoływane przez czynniki środowiskowe u osób predysponowanych genetycznie. Postuluje się znaczenie alleli HLA w rozwoju GD/GO, jednak dla populacji kaukaskiej nie potwierdzono dotychczas jednoznacznie takiej zależności dla ryzyka GD przy pomocy wiarygodnych metod genotypowania i na dużych grupach pacjentów. Natomiast, w przypadku GO, w populacji kaukaskiej takich badań do tej pory praktycznie nie wykonywano. Istnieje również potrzeba poszerzenia wiedzy dotyczącej niegenetycznych czynników ryzyka, zarówno w zakresie odkrycia nowych, jak i potwierdzenia znaczenia już znanych, czy ostatnio postulowanych. Znajomość genetycznych i niegenetycznych czynników ryzyka jest kluczowym warunkiem skutecznej profilaktyki, jak również właściwego dostosowania leczenia GD, a szczególnie GO.

Cele: Celem cyklu prac było znalezienie nowych, genetycznych i niegenetycznych, czynników ryzyka GD i GO oraz weryfikacja znaczenia czynników już opisywanych. W związku z brakiem publikacji dotyczących znaczenia HLA w rozwoju GO, najważniejszym celem było udokumentowanie istnienia alleli HLA związanych z ryzykiem GO i potencjalnie chroniących przed rozwojem GO.

Materiały i metody: Do grupy badanej w każdej z prac projektu włączono pacjentów z GD, w tym chorych z GO oraz bez GO. Grupy kontrolne stanowili zdrowi potencjalni dawcy komórek hematopoetycznych. U wszystkich badanych przeprowadzono genotypowanie HLA (*HLA-A, -B, -C, -DQB1, -DRB1*) przy użyciu metody sekwencjonowania nowej generacji (NGS). Ponadto u wszystkich pacjentów z GD/GO przeprowadzono panel badań laboratoryjnych oraz przeanalizowano dane z wywiadu i dokumentacji medycznej.

Wyniki: W pierwszym badaniu zaobserwowałam istotny związek ryzyka GD z następującymi allelami: *HLA-B*08:01, -B*39:06, -B*37:01, -C*07:01, -C*14:02, -C*03:02, -C*17:01, -DRB1*03:01, -DRB1*11:01, -DRB1*13:03, -DRB1*01:03,*

*-DRB1*14:01, -DQB1*03:01, DQB1*02:01*. Odnotowano również, że ochronne znaczenie w odniesieniu do GD ma występowanie *HLA-B*07:02, -C*07:02, -C*03:04, -DRB1*07:01, -DQB1*02:02, -DQB1*03:03*.

W drugim badaniu udokumentowano, że genetycznymi markerami zwiększonego ryzyka GO są allele *HLA-A*01:01, -A*32:01, -B*37:01, -B*39:01, -B*42:01, -C*08:02, C*03:02, -DRB1*03:01, -DRB1*14:01* i *-DQB1*02:01*. Ponadto stwierdzono, że obecność *HLA-C*04:01, -C*03:04, -C*07:02* i *-DRB1*15:02* może działać protekcyjnie w odniesieniu do GO.

W trzeciej części obecnego projektu potwierdzono także związek GO z paleniem tytoniu, starszym wiekiem, wyższymi stężeniami TRAb w surowicy oraz z hipercholesterolemią, nawet w sytuacji nieznacznie tylko podwyższonego stężenia cholesterolu LDL. Wykazano ponadto, że wśród nowych, potencjalnie użytecznych markerów ryzyka GO, znaczenie mogą mieć: wyższe stężenia kreatyniny w surowicy, wyższy wskaźnik MCV i niższa liczba płytek krwi.

Wnioski: Identyfikacja grup alleli HLA związanych ze zwiększonym ryzykiem GD/GO i chroniących przed tymi chorobami, stanowi ważny krok w rozwoju medycyny spersonalizowanej opartej na ocenie podatności genetycznej. Całość badań zawartych w omawianym projekcie dostarcza szeroki panel czynników ryzyka, które mogą stanowić wiarygodne narzędzie do indywidualnej oceny ryzyka GO, co może istotnie poprawić profilaktykę i leczenie GD/GO, poprzez precyzyjne ich dostosowanie do zindywidualizowanego wyniku oceny ryzyka dla danego pacjenta.

STRESZCZENIE W JĘZYKU ANGIELSKIM

Title: "Novel risk factors of Graves' disease and Graves' orbitopathy with special emphasis on genetic susceptibility markers"

Introduction: Graves' disease (GD) is the most common cause of hyperthyroidism in Poland. The most important extrathyroidal manifestation of GD is Graves' orbitopathy (GO), which may not only significantly worsen the patient's quality of life, but can also be a sight-threatening condition. Similarly to other autoimmune diseases, GD and GO are usually triggered by environmental factors in genetically predisposed individuals. The importance of HLA alleles in the development of GD/GO was postulated before, however, in the Caucasian population, such a relationship was not unequivocally confirmed using reliable genotyping methods and on large groups of GD patients. In the case of GO, no such studies were performed before. There was also a need to broaden the knowledge on non-genetic risk factors, in terms of both discovering new ones and confirming the importance of the already known or recently postulated ones. Knowledge on genetic and non-genetic risk factors is a crucial condition for effective prevention, as well as proper adjustment of GD treatment, especially GO.

Aim: The purpose of the presented series of articles was to find new genetic and non-genetic risk factors of GD and GO and to verify the significance of the factors already described. Due to the lack of studies on significance of HLA in GO development, the main aim of the study was to evaluate correlation between HLA alleles and increased or decreased risk of GO.

Materials and methods: Study groups included GD patients with or without GO. The control group consisted of healthy potential donors of hematopoietic cells. HLA genotyping (*HLA-A, -B, -C, -DQB1, -DRB1*) was performed in all subjects using a next-generation sequencing (NGS) method. In addition, laboratory tests were performed in all GD/GO patients and data from a medical history were analyzed.

Results: In the first study, I observed that GD was significantly associated with the following alleles: *HLA-B*08:01, -B*39:06, -B*37:01, -C*07:01, -C*14:02, -C*03:02, -C* 17:01, -DRB1*03:01, -DRB1*11:01, -DRB1*13:03, -DRB1*01:03, -DRB1*14:01, -DQB1*03:01, -DQB1*02: 01*. In addition, I found that alleles *HLA-B*07:02, -C*07:02, -C*03:04, -DRB1*07:01, -DQB1*02:02, -DQB1*03:03* may play a protective role.

In the second study, I demonstrated that genetic markers of increased GO risk are the alleles *HLA-A*-01:01, -A*32:01, -B*37:01, -B*39:01, -B*42:01, -C*08:02, -C*03:02, -DRB1*03:01, -DRB1*14:01* and *-DQB1*02:01*. I also observed that the presence of *HLA-C*04:01, -C*03:04, -C*07:02* and *-DRB1*15:02* may be protective against GO development.

In the third part of the project, I confirmed the association of GO with smoking, older age, higher serum TRAb concentrations and hypercholesterolemia, even in the case of only mild LDL elevation. I also showed that higher serum creatinine concentrations, higher MCV and lower platelet count may constitute new potentially useful markers of GO risk.

Conclusions: Identification of HLA alleles associated with increased and decreased risk of GD/GO is an important step in the development of personalized medicine based on the assessment of genetic susceptibility. All studies included in the project provided a wide panel of risk factors that can constitute a reliable tool for individual GO risk assessment, which can significantly improve personalized management of patients with GD/GO.

OPUBLIKOWANE PRACE

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Actual Associations between HLA Haplotype and Graves' Disease Development

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Abstract: The association between HLA and the risk of Graves' disease (GD) has been analyzed for many years. However, the results were often inconsistent and mostly regarded Asian populations. The purpose of our study was to perform HLA genotyping using a next-generation sequencing (NGS) method in Caucasians, to find out which alleles are eventually correlated with GD morbidity as well as which of them can be considered protective. *HLA-A, -B, -C, -DQB1, -DRB1* were genotyped using a next-generation sequencing method in 2376 persons, including 159 GD patients and 2217 healthy controls. We have demonstrated a significant association between the risk of GD and the following alleles: *HLA-B*08:01, -B*39:06, -B*37:01, -C*07:01, -C*14:02, -C*03:02, -C*17:01, -DRB1*03:01, -DRB1*11:01, -DRB1*13:03, -DRB1*01:03, -DRB1*14:01, -DQB1*03:01, DQB1*02:01.* The alleles *HLA-B*39:06, -B*37:01, -C*14:02, -C*03:02, -C*17:01, -DRB1*14:01* are novel GD-associated, previously not-reported independent ones with no linkage disequilibrium with other high-risk alleles. On the other hand, the frequencies of *HLA-B*07:02, -C*07:02, -C*03:04, DRB1*07:01, -DQB1*02:02, -DQB1*03:03* were significantly lower in GD compared to controls. This study demonstrated the actual relationships between HLA and GD based on the NGS method and provided a novel set of alleles as a reliable tool for an individual personalized risk assessment.

Keywords: Graves' disease; human leukocyte antigen; HLA; susceptibility alleles

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1. Introduction

Graves' disease (GD) is an autoimmune thyroid disorder characterized by the production of specific antibodies against the thyrotropin (TSH) receptor. These TSH-receptor antibodies (TRAb) most frequently stimulate thyroid hormone production resulting in hyperthyroidism. However, TRAb may also block the TSH-receptor or have an ambivalent character [1]. The prevalence of GD in the Caucasian population is about 0.5–2.0% [1,2]. Similar to other autoimmune diseases, GD is usually triggered by environmental factors in genetically predisposed individuals [2,3]. Among genes associated with the immune response, human leukocyte antigen (HLA) genes have been found to be associated with autoimmune thyroid diseases (AITD), including GD [4]. Other genes such as cytotoxic T lymphocyte-associated factor 4 (*CTLA-4*), thyroglobulin (*Tg*) or *CD40* genes can also be associated with an increased risk of GD [5,6]. However, taking into account the relevance of the major histocompatibility complex (MHC) for immune responses and high polymorphism of HLA region, it seems to play a prominent role as a molecular background of GD [4].

Previous studies on HLA-related susceptibility to GD indicated the existence of significant ethnic differences [2,3,7–12]. Furthermore, the obtained results in either Asian or Caucasian populations are not consistent [3,7–12]. Most of the previously published studies applied much older methods, especially serological ones, which had significantly lower accuracy than the high-resolution next-generation sequencing (NGS) method used in our study. Moreover, the symbols of particular alleles assessed by the previously applied methods differ from those currently used, and some antigens previously denoted by one symbol are separated as several individual alleles when assessed by the high-resolution method. This fact undoubtedly has an important impact on the accuracy and consistency of the already published results.

Several studies conducted in the Asian population postulated the importance of the presence of *HLA-B*46* in the development of GD [7–9], while others indicated a possible relationship with HLA-DRw8, -DQw4, -B5, Dw12 and -A11 antigens [13]. On the other hand, a Chinese study showed that the relationship between GD and *HLA-B*46* concerned only men [14]. The same study found that the risk of GD was higher in patients with *HLA-A*2*, -Cw1, *-DRB1*16:02, DRB1*03:01, DRB1*14:05, -DRB5*02, -DQB1*05:02*, while the presence of *-DRB1*15:01* and *-DQB1*03:01* played a protective role [14]. Another study of Chinese patients observed the relationship between HLA-DR9 and *-DQB1*03:03* in males [15]. Protective effects of HLA-DR12, previously described as an increased risk antigen, and of *HLA-DQA1*04:01* were also postulated [15]. In turn, Japanese authors showed that the most important factor in the development of GD was the presence of *HLA-DPB1*05:01* and/or *HLA-A*2*, with the risk being the highest in carriers of both of them [16]. In a Taiwanese study, *HLA-A*02:07* was found to be a GD risk factor [17], while other Taiwanese authors showed that there was a correlation between GD and *HLA-B*46:01*, *-DPB1*05:01*, *-DQB1*03:02*, *-DRB1*15:01* and *-DRB1*16:02* [18]. Despite the apparent discrepancies, the results of a number of studies conducted on the Asian population are consistent in terms of the relationship between GD and the presence of alleles such as *HLA-A*02:07*, *-B*46*, *-DRB1*08* or *-DPB1*05:01* [6,17,18].

In Caucasians, studies are more scarce. Their results are consistent only with regard to the increased risk of GD in people with *HLA-DRB1*03* and alleles remaining in linkage disequilibrium with *HLA-DRB1*03* for this population, i.e., *-DQA1*05: 01*, *-DQB1*02:01* [3,11,19,20]. However, it is well known that *HLA-DRB1*03* is associated with an increased risk of all thyroid autoimmune diseases, not exclusively with GD. In regard to other alleles, Heward et al. postulated a possible role of *HLA-DQB1*03:01/04* and *-DQB1*02* in GD occurrence [3]. More recently, Vita et al. demonstrated significantly higher frequency of *HLA-C*07*, *-C*17* and *-DRB1*04* in patients with GD as compared to controls [2].

The small amount of data for the Caucasian population and the inconsistency between the results of individual studies, related mainly to different methods used and the size of the groups, left the HLA-related genetic basis of GD for the Caucasian population not satisfactorily explained. Therefore, there was a need to re-analyze and to compare HLA profiles in large groups of patients with GD and healthy controls using a modern high-resolution NGS method. By application of this method, our research group has recently demonstrated novel strong correlations between HLA and subacute thyroiditis (SAT), including not only the risk of SAT but also its clinical course [21–25]. It can be supposed that the relationship between GD and HLA is also much more complicated and includes more alleles than it was previously reported.

The purpose of the study was to re-evaluate class I and class II MHC alleles in 159 patients with GD and 2217 healthy controls, and to clarify which HLA alleles are eventually associated with GD in the Caucasian population. Identification of an actual set of GD-related and GD-protective alleles would provide a novel reliable tool for the individual risk assessment and would constitute a great step in a development of personalized medicine.

2. Materials and Methods

2.1. GD Group and Control Group

A total number of 2376 persons were included into the study, with 2217 healthy Polish hematopoietic stem cell donors with no medical history of thyroid disease, and 159 patients who were diagnosed with GD in the Department of Endocrinology and Metabolic Diseases, Polish Mother's Memorial Hospital—Research Institute, Lodz, Poland, as well as in the Department-associated outpatient clinic. The size of the control group should be large enough to avoid any bias related to potential diseases which may appear in some members of this group in future, as well as to avoid any bias associated with random increased or decreased frequency of some alleles in a smaller control group.

2.2. HLA Typing Procedures

HLA-A, -B, -C, -DQB1 and *-DRB1* were genotyped using a high-resolution NGS method. DNA was isolated from whole blood collected to the anticoagulant (EDTA)-containing tubes. Further sample preparation consisted of several steps, including long-range PCR, genomic library preparation, and sequencing. Amplicons were quantified by fluorescence detection method, balanced, pooled and enzymatically fragmented. Afterwards, end repair and A-tailing of DNA fragments was performed followed by index adapter ligation. Genomic library was cleaned up and denatured prior to loading on NGS Illumina Platform (NextSeq). We analyzed sequencing data with NGS HLA Genotyping software MIA FORA. The data were considered sufficient whenever the coverage reached 40.

2.3. Statistical Analysis

Allele frequencies were reported as absolute values and in percentages. The statistical significance of the differences between groups was evaluated by the chi-square test and by binomial logistic regression analysis, with p values ≤ 0.05 considered significant. The statistical analysis was carried out using Statistica v 13.1 software (Statsoft Polska, Kraków, Poland).

2.4. Inclusion Criteria

In all patients included into the study, GD was diagnosed on the basis of standard criteria [1], including hyperthyroidism, elevated TRAb level, as well as typical ultrasound (US) pattern.

2.5. Biochemical and US Procedures

Serum levels of TSH, free triiodothyronine (FT3), free thyroxine (FT4) and TRAb were measured by the electrochemiluminescence immunoassay (ECLIA) with Cobas e601 analyzer (Roche Diagnostics, Indianapolis, IN, USA). Ultrasound examinations (US) were performed in every patient, using a 7–14 MHz linear transducer (Toshiba Aplio XG; Toshiba, Japan).

2.6. Ethics Procedures

Informed consent for all performed procedures was obtained from all of the patients after a full explanation of the purpose and nature of all procedures used. The study was approved by the Ethics Committee of the Polish Mother's Memorial Hospital—Research Institute, Lodz, Poland (approval code—108/2018).

3. Results

The mean age of patients at diagnosis of GD was 43.88 ± 17.44 years, with a male to female ratio of 1:4.48. The diagnosis of GD was based on the laboratory results (Table 1).

Table 1. Laboratory characteristics of the Graves' disease (GD) group.

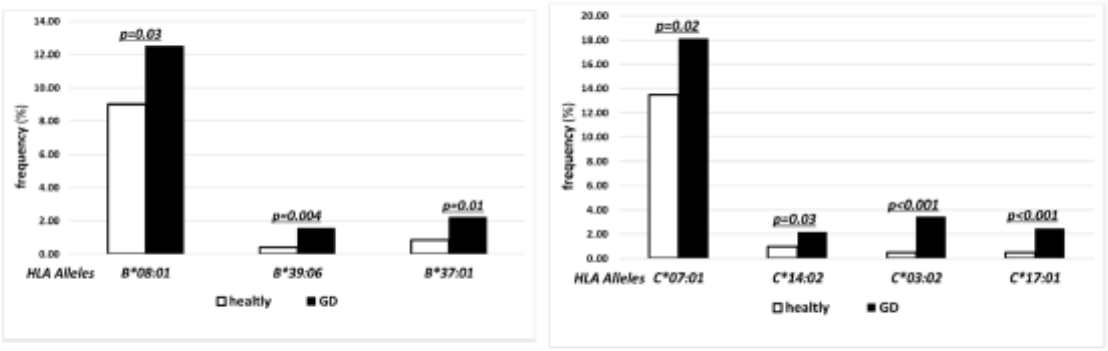
Parameter (Reference Range and Units)	Mean ± SD	Median
TSH (0.27–4.2 μIU/mL)	0.14 ± 0.43	0.05
FT4 (0.9–1.7 ng/dL)	3.35 ± 2.39	2.33
FT3 (2.0–4.4 pg/mL)	11.07 ± 8.38	7.86
TRAb (<1.7 IU/L)	15.04 ± 13.62	10.12

Abbreviations: FT3, free triiodothyronine; FT4, free thyroxine; SD, standard deviation; TRAb, TSH receptor antibodies; TSH, thyrotropin.

Statistically significant differences in the frequency of HLA alleles between patients with GD and control group were found with several alleles having higher frequency and others having lower frequency in GD as compared to controls.

3.1. Alleles with Higher Frequencies in GD

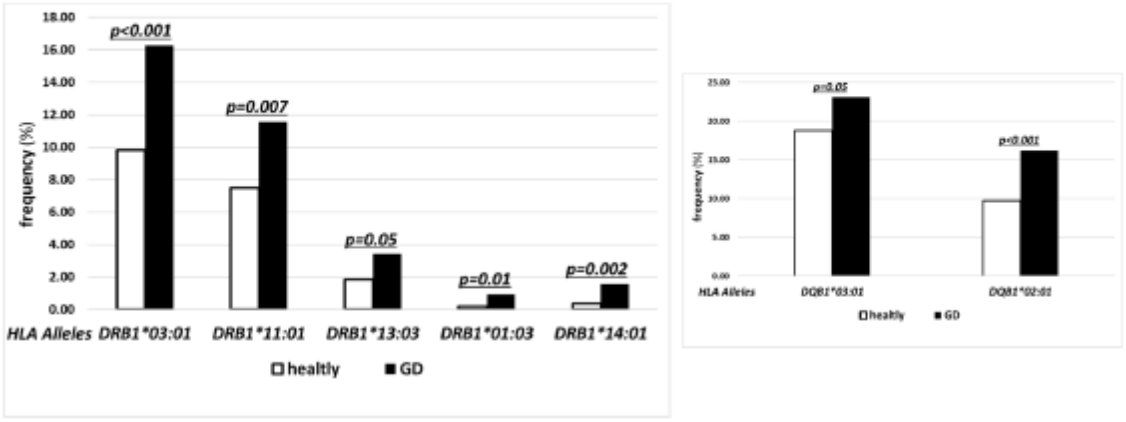
The alleles of higher frequency in GD as compared to controls were found in both MHC class I and class II. The differences were statistically significant for the following alleles of MHC class I: *HLA-B*08:01* (12.5% vs. 9.0%), *-B*39:06* (1.56% vs. 0.41%), *-B*37:01* (2.19% vs. 0.83%) (Figure 1a), *-C*07:01* (18.13% vs. 13.49%), *-C*14:02* (2.19% vs. 0.95%), *-C*03:02* (3.44% vs. 0.50%), *-C*17:01* (2.50% vs. 0.50%) (Figure 1b).



(a) (b)

Figure 1. Frequencies (%) of human leukocyte antigen (HLA) over-represented alleles with statistically significant difference between control (open bars) and Graves' disease (GD) patients (solid bars) for major histocompatibility complex (MHC) class I alleles: *HLA-B* (**a**) and *HLA-C* (**b**).

For the MHC class II, significant differences in the frequencies were found for the following alleles: *-DRB1*03:01* (16.25% vs. 9.83%), *-DRB1*11:01* (11.56% vs. 7.49%), *-DRB1*13:03* (3.44% vs. 1.87%), *-DRB1*01:03* (0.94% vs. 0.20%), *-DRB1*14:01* (1.56% vs. 0.36%) (Figure 2a), *-DQB1*03:01* (23.13% vs. 18.83%), *DQB1*02:01* (16.25% vs. 9.72%) (Figure 2b).



(a) (b)

Figure 2. Frequencies (%) of human leukocyte antigen (HLA_ over-represented alleles with statistically significant difference between control (open bars) and Graves' disease (GD) patients (solid bars) for major histocompatibility complex (MHC) class II alleles: *HLA-DRB1* (**a**) and *HLA-DQB1* (**b**).

3.2. Alleles with Lower Frequencies in GD

On the other hand, the frequencies of the following alleles were significantly lower in GD as compared to controls: *HLA-B*07:02* (5.94% vs. 10.53%), *-C*07:02* (4.38% vs. 11.41%), *-C*03:04* (1.56% vs. 5.19%), *-DRB1*07:01* (8.75% vs. 12.97%), *-DQB1*02:02* (5.63% vs. 9.47%), *-DQB1*03:03* (2.19% vs. 4.74%) (Figure 3).

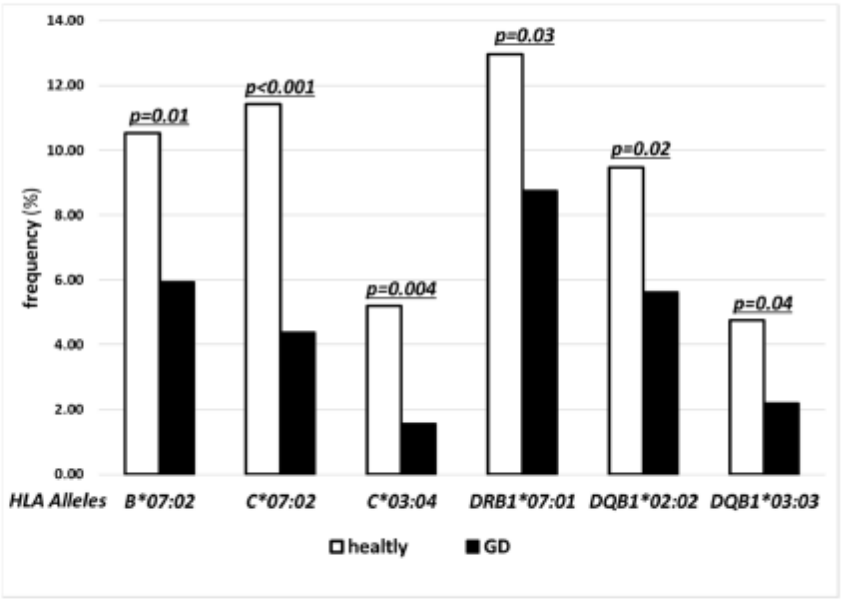


Figure 3. Frequencies (%) of human leukocyte antigen (HLA) under-represented alleles with statistical difference between controls (open bars) and Graves' disease (GD) patients (solid bars) for both major histocompatibility complex (MHC) class I and class II alleles.

3.3. Significance of a Single High Risk Allele and of Co-Presence of Alleles

In 26 patients (16.4%), only one of the alleles described above as correlated to a high risk of GD was found. Among this group, the following alleles were found: *HLA-B*39:06*, *-C*03:02*, *-C*07:01*, *-C*14:02*, *-DRB1*14:01* and *-DQB1*03:01* in two (7.7%), three (11.5%), six (23.1%), one (3.8%), one (3.8%) and thirteen (50%) patients, respectively.

In 33 patients (20.8%), two of the high-risk alleles were present. Among this group the co-presence of *HLA-DRB1*11:01* and *-DQB1*03:01* was observed the most frequently (45%). The co-presence of alleles which are not in linkage disequilibrium was observed in 9 patients (27.3%). Among this group, the most frequent was the combination of *HLA-C*14:02* with *-DQB1*03:01* (22.2%).

Interestingly, allele *HLA-B*08:01* was most frequently present with *-DRB1*03:01* and *-DQB1*02:01*. A combination of the three alleles, *-B*08:01, -DRB1*03:01-* and *DQB1*02:01*, occurred in 36 of 159 GD patients (22%), while the co-presence of these three alleles was found only in 5.87% of the control group. In only three patients (1.9%), the *HLA-B*08:01* allele occurred with other alleles (Table 2). In none of the patients did *-B*08:01* occur as the only high-risk allele.

Table 2. Frequencies and linkage disequilibrium of three-locus *HLA-B-DRB1-DQB1* haplotypes in Graves' disease (GD) patients depending on the presence of the *HLA-B*08:01* allele.

HLA Haplotype	Haplotype Frequency
*B*08:01- DRB1*03:01- DQB1*02:01*	22% [*n* = 36]
*B*XX:XX- DRB1*03:01- DQB1*02:01*	6.6% [*n* = 11]
*B*08:01- DRB1*XX:XX- DQB1*XX:XX*	1.9% [*n* = 3]

*B*XX:XX*—allele other than *-B*08:01; DRB1*XX:XX- DQB1*XX:XX*—alleles other than *-DRB1*03:01,- DQB1*02:01.*

4. Discussion

In the recent years, it has become more and more clear that GD is triggered by environmental factors such as infections, stress, smoking, etc., in genetically predisposed individuals [6,7]. This genetic susceptibility plays a critical role in the pathogenesis of GD and has been previously demonstrated to be HLA-dependent. Moreover, in both Asian and Caucasian populations, this genetic background was demonstrated to include MCH class I and class II. Therefore, the identification of alleles specifically related to GD seems to be a crucial step in the development of personalized medicine in regard to thyroid disorders. However, the results that have been reported for the last two decades in both populations are not consistent. Significant discrepancies in the results obtained by various authors can undoubtedly depend on the applied method. The use of high-resolution methods can change the results obtained with older methods. Genotyping methods of resolution that allow the achievement of allelic specificity is currently a gold standard of research that is expected to demonstrate high reliability, and to avoid method-dependent errors. Older, less accurate methods provide results for the entire allelic group, not for a particular allele. This may result in erroneous conclusions and discrepancies in the results of the studies depending on the method. According to the results obtained in a strictly controlled group of HLA typing performed for the purposes of bone marrow transplantation between 1996 and 2011, 29.1% discrepancies were found between older methods and NGS method [26] which was applied in the present study. Another important example of the significance of the genotyping method is *HLA-B*27* test commonly used to genetically confirm a diagnosis of ankylosing spondylitis. It has been recently demonstrated that HLA typing methods used so far gave insufficiently precise results, and two alleles, *HLA-B*27:06* and *HLA-B*27:09*, are probably not associated with the disease [27]. These examples clearly present the importance of the method and a possible role of a method-dependent factor in the inconsistency of the previous results.

To date, to the best of our knowledge, the present study included the largest Caucasian cohort to whom a modern high-resolution method was applied to analyze both

MCH classes. Thus, this study can summarize and clarify the actual HLA-related genetic background of GD.

The present study has confirmed a strong correlation between GD and *HLA-B*08:01*, *DRB1*03:01*, *-DQB1*02:01* (Figures 1a and 2a,b). Our observation is consistent with the previous reports [2,3,11,12,19,20]. Interestingly, in our study, *HLA-B*08:01* was accompanied by these two alleles in most cases of its presence although it belongs to a different MCH class (Table 2). The co-presence of these three alleles in GD group was 4 times more frequent than in controls. Additionally, *HLA-DRB1*03:01* and *-DQB1*02:01* were rarely present without *HLA-B*08:01* (Table 2). Such a close unexpected association between *HLA-B*08:01* and MCH class II alleles was previously postulated in the Caucasian population [28], but the present study has confirmed it and demonstrated its strength for GD patients for the first time. This finding sheds a new light on the possible linkage disequilibrium between alleles from different MCH classes. Moreover, it indicates the existence of specific mechanism of impact augmentation between these alleles in GD.

The currently discussed group of linkage disequilibrium includes also *HLA-DQA1*05:01* [27,29,30], previously reported as GD high risk allele [2,3,19]. We did not perform comparison of frequencies of *HLA-DQA1*05:01* between our groups because *HLA-DQA1* alleles are not reported for transplantation purposes and results performed using NGS method are unavailable either for our control group or for any other representatively large cohort in Poland. Comparison with any population with available lower resolution results could introduce unacceptable bias, as the main strength of the present study is the application of the most reliable method. However, a strong linkage disequilibrium between *HLA-DQA1*05:01* and *DRB1*03:01, DQB1*02:01*, as well as other alleles demonstrated as high-risk ones in the present study, i.e., *DRB1*01:03, DQB1*03:01* and *DRB1*13:03* (Figure 2) seems to confirm previous findings of *HLA-DQA1*05:01* being a GD risk allele [2,3,19]. The potential role of *HLA-DQB1*03:01* was previously postulated by Heward et al. [3]. Similar to our study, Martin et al. observed significantly higher frequency of *HLA-DRB1*11:01* and *-DRB1*13:03* [31]. Our study has confirmed the role of these alleles as well as has further supported the role of *HLA-DQA1*05:01* because of linkage disequilibrium between either *HLA-DRB1*11:01* or *-DRB1*13:03* and *HLA-DQA1*05:01* [29]. Both of these *HLA-DRB1* alleles are also in linkage disequilibrium with *HLA-DQB1*03:01* which has been confirmed in the present study as high-risk allele. To the best of our knowledge, this is the first report presenting strong susceptibility to GD related to *HLA-DRB1*01:03*—the allele that is also in linkage disequilibrium with *HLA-DQA1*05:01* and *-DQB1*03:01* [29].

Our study also demonstrated that the risk of GD is significantly increased in patients with a presence of *HLA-C*07:01*, being in linkage disequilibrium with previously discussed *-B*08:01* [32,33]. It is worth mentioning that the importance of *HLA-B*08:01* and *-C*07:01* was previously postulated in the literature, mostly as *-B*08* and *-C*07* with application of lower resolution methods and two-digit results [2,34]. Our study has confirmed this association for *HLA-B*08:01* and *-C*07:01* (Figure 1a,b). When analyzing the above-described associations, one should keep in mind that susceptibility associated with alleles being in linkage disequilibrium cannot be considered as fully independent. However, the single presence of any of them can be correlated with the risk of GD.

This study has confirmed the correlation of GD with *HLA-C*17:01* postulated before by Vita et al. [2]. Our study based on NGS method has demonstrated even stronger statistical significance than previously reported (Figure 1b). No linkage disequilibrium has been reported between *HLA-C*17:01* and other high-risk alleles [32,33], so *HLA-C*17:01* should be considered an independent one.

The most important finding of our present study is the significance of novel alleles which have been reported here as GD-related for the first time. This group includes *HLA-B*37:01, -B*39:06, -C03:02, -C14:02,* and *-DRB1*14:01* (Figure 1a,b and Figure 2a). These alleles belong to both I and II MCH classes and are not in linkage disequilibrium either with each other or with any previously discussed GD-related alleles [27,32,33]. Thus, their significance for the pathogenesis of GD is particularly relevant.

Similar to Vita et al. [2], we have not confirmed a higher frequency of *HLA-DRB1*08* previously postulated in North American and British Caucasians [34,35]. The specificity of the applied method may have played the most important role in these differences.

Our study has demonstrated strong association of GD with the presence of several alleles belonging to both MCH classes. Strong impact of any of them on the risk of GD can be additionally supported by our finding that the presence of a single allele from the high-risk group is sufficient to induce GD. In the present study, alleles *HLA-B*39:06, -C*03:02, -C*07:01, -C*14:02, -DRB1*14:01* and *-DQB1*03:01* were present as a single allele in GD patients. Most of them, i.e., *HLA-B*39:06, -C*03:02, -C*14:02, -DRB1*14:01*, have been reported for the first time as high-risk alleles in the present study. It is worth underlining that among patients with two high risk alleles, the co-presence of *HLA-DRB1*11:01* and *-DQB1*03:01*, being in linkage disequilibrium was the most common. However, in 27.3% of the patients, the co-present alleles were totally independent, with *HLA-C*14:02*—newly reported in the present paper—being the most frequent independent allele.

Predisposition for autoimmune disorders other than GD can also be HLA-related. Hashimoto's thyroiditis (HT) and GD share a variety of common etiological and pathophysiological factors, including HLA-based predisposition, a trend to aggregate in the same families or even to coexist in the same gland [36]. Moreover, several reports suggested the existence of a continuum between HT and GD [37–39]. In our GD group, only four patients had preceding Hashimoto's thyroiditis, and in all of them *HLA-DQB1*02:01* was present. This allele was demonstrated in our results as one of the alleles related to high risk of GD and it is in linkage disequilibrium with *-DRB1:03:01*—an allele typical for thyroid autoimmunity. Interestingly, the co-presence of these two alleles was found in all patients with coexistence of GD and non-thyroidal autoimmunity (i.e., in two patients with Addison's disease and two patients with diabetes type 1). These subgroups were too small to obtain any statistical results, but this phenomenon requires further analysis.

It was previously postulated that the presence of some HLA alleles may play a protective role against GD. Similar to the results on high-risk alleles, most of the papers published so far regard the Asian population. Yin et al. postulated that *HLA-B*33* can protect against GD [40], while Mehraji et al. demonstrated that *HLA-DQB1*02:01* and *-DQA1*02:01* play the protective role [41]. The results of studies in the Korean population did not confirm the previous findings and revealed that alleles *HLA-DRB1*01:01, DRB1-*07:01, -DRB1*12:02* and *-DRB1*13:02* were the protective ones [42] On the other hand, other Korean group confirmed the protective role of *HLA-DQA1*02:01* and *-DQB1*02:01* as well as *HLA-DRB1*12*, and additionally postulated the significance of *-DQA1*06:01* and *-DQB1*03:01* [43]. In the Chinese population, Cavan et al. postulated the significance of *-DQA1*04:01* and confirmed previously reported protective role of *HLA-DRB1*12* and *-DQB1*03:01* [15]. The results obtained by the different research groups were inconsistent and it is difficult to unambiguously distinguish the actually protective alleles. The main candidates in the Asian population seem to be *HLA-DRB1*12:02, -DQB1*02:01* and *DQB1*03:01*.

A similar situation of inconsistent results regards the Caucasian population, and in addition, the results are more scarce. Therefore, our present study aimed also to compare GD and control cohorts with regard to potentially protective alleles. We confirmed the previously reported significantly less frequent presence of *HLA-DRB1*07:01*, [12,34,35] and *-DQB1*02:02* [34] in patients with GD as compared to healthy controls (Figure 3). However, in the last study, the significance of *-DQB1*02* was postulated in two-digit presentation [34]. We have refined and clarified this finding using the NGS method. Our study revealed also lower frequency of *HLA-DQB1*03:03* in GD individuals (Figure 3). It has to be underlined that *HLA-DQB1*02:02* and *-DQB1*03:03* are in linkage disequilibrium with *-DRB1*07:01*, together with the previously postulated *-DQA1*02:01* [12,27].

The presence of these protective alleles can play a very important role in GD development. Proteins related to *HLA-DRB1*03:01* and its linkage disequilibrium alleles differ from those related to *-DRB1*07:01* and its linkage disequilibrium-related alleles at position β74, a crucial site in the binding pocket of the HLA allele, the residue being arginine and glu-

tamine, respectively [2,34]. It has been hypothesized that in patients with the co-presence of these two alleles, *HLA-DRB1*07:01* can cancel out the GD-susceptibility associated with *-DRB1*03:01* [2,34].

Similar to our findings on novel high-risk alleles, we have also demonstrated novel, previously not reported, protective associations. In the present study, the frequencies of *HLA-B*07:02* and *-C*07:02* were significantly lower in the GD cohort than in the control group (Figure 3). These alleles cannot be considered independent as there is also a linkage disequilibrium between them. However, as it was previously underlined, the presence of any of them can potentially be sufficient as a protective factor. Moreover, another novel independent allele was revealed as protective, i.e., *-HLA-C*03:04*, with a high statistical significance of $p = 0.004$ (Figure 3). This allele is not in linkage disequilibrium with any other potentially protective allele.

The differences in the results between Caucasian and Asian populations with regard to the MCH class II alleles, which are in linkage disequilibrium with *HLA-DQA1*05:01* can be considered unexpected. Among all of these alleles, only *-DRB1*03:01* was proved to be GD-related in the Asian population [14]. However, all of the above-described linkage disequilibrium-based correlations are common not exclusively for the Caucasian population but for all analyzed populations, including Asians [29]. Therefore, the question arises why the correlations present in Caucasians are absent in Asians, in whom completely different alleles are considered as high risk of GD. Furthermore, taking into account our present results and the literature data, we can observe a phenomenon of entirely opposite roles of *HLA-DQB1*02:01* and *-DQB1*03:01* in these populations. These alleles have been demonstrated as high risk in Caucasians in the present study and in some previous ones [3]. However, they are reported as protective in Asian studies [15,42,43]. Therefore, although *HLA-DRB1*03:01* has been demonstrated to be a high-risk allele in both populations, *-DQB1*03:01* being in linkage disequilibrium with *-DRB1*03:01* in both populations, is a high-risk allele in Caucasians and a protective allele in Asians. This fact demonstrates the impact of other factors influencing GD risk in the Asian population and points out the necessity for further analysis of this phenomenon.

5. Conclusions

The present study has demonstrated the actual associations between HLA haplotype and GD. The application of the NGS method to genotype both MCH I and II classes in large groups of patients and controls has clarified many discrepancies present in the previous results possibly due to a lack of allelic specificity and/or the size of the groups being too small. A significant association was found between the risk of GD and the following alleles: *HLA-B*08:01, -B*39:06, -B*37:01, -C*07:01, -C*14:02, -C*03:02, -C*17:01, -DRB1*03:01, -DRB1*11:01, -DRB1*13:03, -DRB1*01:03, -DRB1*14:01, -DQB1*03:01, DQB1*02:01*. Among these alleles, *-B*39:06, -B*37:01, -C*14:02, -C*03:02, -C*17:01, -DRB1*14:01* are novel, previously not-reported, independent alleles with no known linkage disequilibrium with other high-risk alleles. On the other hand, the frequencies of *HLA-B*07:02, -C*07:02, -C*03:04, -DRB1*07:01, -DQB1*02:02, -DQB1*03:03* were significantly lower in GD compared to controls, with the first three alleles being reported as protective for the first time. These alleles can be considered protective. This study has provided a novel set of alleles as a reliable tool for individual risk assessment. The identification of alleles which in a particular population are associated with GD and which play a protective role is an essential step in the development of personalized medicine.

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Abbreviations

AITD	autoimmune thyroid diseases
ATA	American Thyroid Association
CD40	cluster of differentiation 40
CTLA-4	cytotoxic T lymphocyte-associated factor 4
EDTA	ethylenediaminetetraacetic acid (anticoagulant)
FT3	free triiodothyronine
FT4	free thyroxine
GD	Graves' disease
HLA	human leukocyte antigens
MHC	major histocompatibility complex
NGS	next-generation sequencing
SAT	subacute thyroiditis
Tg	thyroglobulin
TRAb	TSH-receptor antibodies
TSH	thyroid stimulating hormone (thyrotropin)
US	ultrasound

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Significance of HLA in the development of Graves' orbitopathy

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Graves' disease (GD), similarly to most autoimmune disease, is triggered by environmental factors in genetically predisposed individuals. Particular HLA alleles increase or decrease GD risk. No such correlation was demonstrated for Graves' orbitopathy (GO) in Caucasian population. *HLA-A, -B, -C, -DQB1* and *-DRB1* genotyping was performed using a high-resolution method in a total number of 2378 persons including 70 patients with GO, 91 patients with non-GO GD and 2217 healthy controls to compare allele frequencies between GO, non-GO and controls. Significant associations between GO and HLA profile were demonstrated, with *HLA-A*01:01, -A*32:01, -B*37:01, -B*39:01, -B*42:01, -C*08:02, C*03:02, DRB1*03:01, DRB1*14:01* and *DQB1*02:01* being genetic markers of increased risk of GO, and *HLA-C*04:01, -C*03:04, -C*07:02* and *-DRB1*15:02* being protective alleles. Moreover, correlations between HLA alleles and increased or decreased risk of non-GO GD, but with no impact on risk of GO development, were revealed. Identification of these groups of GO-related and GO-protective alleles, as well as the alleles strongly related to non-GO GD, constitutes an important step in a development of personalized medicine, with individual risk assessment and patient-tailored treatment.

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INTRODUCTION

Graves' disease (GD) is an autoimmune thyroid disorder caused by production of specific antibodies against the thyrotropin (TSH) receptor (TRAb) [1] leading mainly to hyperthyroidism. The prevalence of GD in the Caucasian population is about 0.5–2.0% [1, 2]. Graves' orbitopathy (GO) is the major extrathyroidal manifestation of GD, occurring more frequently in women, with the estimated incidence of 0.54–0.9 cases/100 000/year in men and 2.67–3.3 cases/100 000/year in women [3]. GO has significant impact on quality of life (QoL) and may even constitute a sight-threatening condition [3]. The knowledge on risk factors which are associated with GO occurrence seems crucial for the prevention and management of GO. Smoking, severe/unstable hyperthyroidism as well as high serum TRAb levels are well-known risk factors of GO development and progression [3]. However, it seems clear that these factors act on some specific genetic background which is pivotal for the disease development. Autoimmune diseases, including GD, are typically triggered by environmental factors in genetically predisposed individuals [2, 4]. Among genes associated with the immune response, human leukocyte antigen (HLA) genes seem to play a prominent role as a molecular background of GD [5, 6]. Many different HLA alleles were postulated as GD risk factors. However until recently, the data regarding Caucasian population were not consistent, most probably due to small study groups as well as different methods applied by the authors, including low resolution or serological methods [5]. We have previously demonstrated that application of next generation sequencing (NGS) methods allowed to reveal actual HLA-related susceptibility for subacute thyroiditis, which appeared to include much more alleles than just *HLA-B*35*, influencing the disease course [7–11]. Recently, our research team applied the same NGS methods to demonstrate that GD is strongly HLA-dependent in Caucasian population. We have proved significant association between the risk of GD and the presence of the following alleles: *HLA-B*08:01, -B*39:06, -B*37:01, -C*07:01, -C*14:02, -C*03:02, -C*17:01, -DRB1*03:01, -DRB1*11:01, -DRB1*13:03, -DRB1*01:03, -DRB1*14:01, -DQB1*03:01, DQB1*02:01*. We have also demonstrated the protective role of *HLA-B*07:02, -C*07:02, -C*03:04, DRB1*07:01, -DQB1*02:02, -DQB1*03:03* [5].

There is currently no clear HLA-related susceptibility for GO confirmed in Caucasian population. Previously published studies on the relationship between HLA alleles and the occurrence of GO concerned almost exclusively Asian population and revealed highly inconsistent results. Using serological method in Japanese cohort, Inoue et al. observed that GO was associated with the following three HLA pairs: HLA-DQw4 without presence of -A31, HLA-A11 without presence of -DPw2, and a co-presence of HLA-B5 and -Dw12 [12]. However, in a study published a year earlier, Inoue et al. found only DQw3 as a GO risk factor [13]. Ohtsuka and Nakamura used the same methods, also in Japanese population, and obtained completely different results [14]. They postulated that HLA-DR14 and DQ1 antigens may be genetic markers of predisposition to severe GO, while HLA-B35, B54, DR4, and DQ4 may play protective role [14]. In 2017 Mehraji et al. reported lack of any differences in allelic distribution between GO and non-GO patients in Iranian cohort [15]. On the other hand, in Korean study, *HLA-C*03:03* was found more frequently in GO as compared to non-GO patients [6]. In a very recent Chinese study, the HLA alleles

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Table 1. Summary of the results of previous studies.

Authors	Ref.	Population	Method	No. of GO patients	No. of GD patients	No. of healthy controls	Result - HLA related to GO risk
Inoue et al.	[12]	Asian (Japanese)	serological	42	88	186	-DQw4 (+) and -A31 (-) -B5 (+) and -Dw12 (+) -A11 (+) and -DPw2 (+)
Inoue et al.	[13]	Asian (Japanese)	serological	23	88	186	DQw3 (+), DPQ2(+)
Ohtsuka and Nakamura	[14]	Asian (Japanese)	serological	48	94	767	-DR14 (+), DQ1 (+) -B35 (-), B54 (-), DR4 (-), DQ4 (-)
Mehraji et al.	[15]	Asian (Iranian)	SSP-PCR	45	80	180	none
Shin et al.	[16]	Asian (Korean)	NGS	35	71	142	*-C*03:03 (+) -B*54:01 (-)*
Huang et al.	[17]	Asian (Chinese)	NGS	82	272	411	*-B*38:02* (+) *-DQA1*01:02* + *-DRB1*16:02 (+) -DQA1*01:02* + *-DQB1*05:02* (+)

NGS – next generation sequencing.
SSP – single specific primer - polymerase chain reaction (low to intermediate resolution method).

including *HLA-B*38:02, -DRB1*16:02, -DQA1*01:02* and *-DQB1*05:02* were postulated as risk factors for GO [16]. The results of the previous studies were summarized in Table 1. Taking into account the inconsistency of the results, unambiguous conclusions are not possible to be drawn even in Asian population.

There are almost no consistent reports indicating the relationship between HLA and GO in the Caucasian population. Yin et al. did not demonstrate the existence of HLA-related susceptibility to GO in the group of patients with GD and postulated the importance of environmental or epigenetic factors only [17]. However, the authors of that study focused only on the frequency of *HLA-DR3*, without assessing the frequencies of other alleles.

The purpose of our study was to perform HLA genotyping using the NGS method in Caucasians, to find out which alleles are eventually correlated with high risk of GO, as well as which of them can be considered protective. Identification of a group of GO-related and GO-protective HLA alleles would constitute a great step in a development of personalized medicine as it would provide a new precise diagnostic tool for the individual risk assessment.

SUBJECTS AND METHODS

GD group and control group

A total number of 2378 persons were included into the study, with 2217 healthy Polish hematopoietic stem cell potential donors who did not have any medical history of thyroid disease or orbitopathy (control group), and 161 unrelated patients with GD diagnosed in the Department of Endocrinology and Metabolic Diseases, Polish Mother's Memorial Hospital-Research Institute, as well as in the Department-associated outpatient clinic. The GD group included 70 patients with GO (GO group) and 91 patients without GO (non-GO group). The large size of the control group was required to avoid any bias associated with potential diseases which might occur in currently healthy members of this group in future, and – additionally – to avoid any bias related to random changes (increase or decrease) in frequencies of some alleles in a smaller control group.

Inclusion criteria

In all patients included into the GD study group, the diagnosis of GD was made on the basis of standard criteria [1], including hyperthyroidism, elevated TRAb level, as well as typical ultrasound (US) pattern. The diagnosis of GO, as well as the assessment of GO activity and severity, was performed on the basis of the EUGOGO guidelines actual at the time of diagnosis, i.e. 2021 version [3] or 2016 version [18] the latter version used for patients diagnosed before the time when 2021 version was available. Patients with other diseases which may have influenced the obtained results were excluded from the study, except for two patients with latent autoimmune diabetes in adults (LADA), who were not excluded from the GO group as no potential error related to their HLA results was expected. LADA is associated with the presence of *HLA-DRB1*03* and *-DQB1*02:01* as well as *HLA-DRB1*04* and *-DQB1*0302* [19]. Alleles *HLA-DRB1*04* and *-DQB1*03:02* were not found as more frequent in our GO group, while *HLA-DRB1*03:01* is a well-known marker of many autoimmune diseases, and it is in linkage disequilibrium with DQB1*02:01. Therefore, taking into account the lack of any potential bias caused by LADA in these two patients, they were not excluded from the study.

Diagnostic procedures

Serum levels of TSH, free thyroxine (FT4), free triiodothyronine (FT3) and TRAb were measured by the electrochemiluminescence immunoassay (ECLIA) using Cobas e601 analyzer (Roche Diagnostics, Indianapolis, IN, USA). In all patients, ultrasound examinations (US) were performed using a 7–14 MHz linear transducer (Toshiba Aplio XG; Toshiba, Japan). In all GO patients, magnetic resonance imaging (MRI) was performed to unambiguously confirm GO diagnosis and to exclude any other orbital or intracranial pathological process.

HLA typing procedures

DNA was isolated from whole blood samples collected to the anticoagulant (EDTA)-containing tubes. *HLA-A, -B, -C, -DQB1* and *-DRB1* genotyping was performed using a standard high-resolution NGS method [20] with application of MIA FORA NGS FLEX 5 HT HLA Typing Kit [21] (Immucor Transplant Diagnostics, Inc. 35 Technology Drive South Warren, New Jersey 07059, USA) that supplies reagents for up to 1152 samples. However, during one run of high-resolution typing we performed genotyping of 576 samples. The MIA FORA NGS FLEX 5 HT HLA typing protocol uses long-range PCR to capture the clinically relevant Class I and II HLA genes. The core kit includes each of the Class I genes, *HLA-A, HLA-B,* and *HLA -C*, as well as the Class II genes, *HLA-DRB1* and *HLA-DQB1. HLA-A, HLA-B,* and *HLA-C* are sequenced in their entirety. We performed sample preparation divided into three distinct sections: long-range PCR, library preparation, and sequencing. During the first section we prepared six PCR mixes per sample. Each gene was amplified as one large piece in its entirety, except for DRB, which was amplified as two overlapping segments due to its large size. Within the MIA FORA system, these are referred to as DRB-5 and DRB-L. Following gene amplification amplicons were quantitated by fluorescence detection using PicoGreen™ reagent and a fluorescent plate reader. The PCR products per sample were balanced and pooled before proceeding with enzymatic fragmentation, end repair, A-tailing, and cleaned with magnetic beads. Index adaptor ligation: each

kit contains two sets of six individual index adaptor plates, with 96 adaptors per plate. These index adaptors contain index sequences (barcodes) and Illumina-compatible adaptor sequences that allow for sequencing in a multiplex format. Index Adaptors from identically-named Index Adaptor Plates cannot be combined into the same library. Each 96-well sample plate was consolidated into a single microcentrifuge tube and size-selected with the Pippin Prep before final PCR amplification. The library was quantitated by Qubit and concentration was adjusted according to the Illumina NextSeq library preparation protocol. This protocol describes semi-automated sample processing for high throughput sequencing, from long range PCR through library preparation, prior to sequencing on an Illumina instrument (Illumina 5200 Illumina Way San Diego, California 92122 U.S.A). Genomic library was cleaned with magnetic beads and denatured by 0.2 N NaOH before loading on NGS Illumina Platform. All automated sample processing was performed on the Biomek i7 Liquid Handler. Sequencing data were analyzed by MiaFora NGS software v. 4.5, IPD-IMGT/HLA database version 3.40. The data were considered sufficient whenever the coverage reached 40. We used advanced NGS HLA Genotyping Software MIA FOR A, a trademark owned by Sirona Genomics, Inc. Genotypes were computed from massive, paired-end sequencing reads derived from the Illumina Next Generation Sequencing (NGS) platform. The results of HLA-typing are available as Supplementary Materials.

Statistical analysis

Statistical calculations were performed for all alleles in all loci regardless of the frequency of their occurrence in the population. Regardless of whether patients or control group individuals were homozygous or heterozygous, each of them was counted once only. To make the results more readable, we presented results only for these alleles for which the statistically significant differences were achieved. Allele frequencies were reported in percentages. The statistical significance of the differences between groups was evaluated by the chi-square test and by binomial logistic regression analysis, with p values ≤0.05 considered significant. For small groups, the statistical significance of the differences between the groups was evaluated by Fisher exact test with p values ≤ 0.05 considered significant. Odds ratio (OR) was calculated for all comparisons in which a given allele was present in both of the compared groups. The statistical analysis was carried out using Statistica v 13 software (Statsoft Polska, Kraków, Poland).

Ethics procedures

All patients gave their informed consent for all procedures performed during the study. The consents were obtained after full explanation of the purpose and nature of all the procedures used in the study. The study was approved by the Ethics Committee of the Polish Mother's Memorial Hospital—Research Institute, Lodz, Poland (approval code—108/2018).

RESULTS

The mean age of the patients at the time of diagnosis of GD was 43.63 ± 17.59 years, with a male to female ratio of 1:4.75. Statistically significant differences in the frequency of HLA alleles between patients within GO and non-GO groups as well as between either of them and the control group were found, with several alleles of higher frequency and others of lower frequency either in GO or non-GO group. The deviation of genotypic frequencies from the Hardy-Weinberg Equilibrium at each HLA locus was analyzed for the control group. The p value results from GENEPOP vs. 4.7.5: Hardy-Weinberg test were as follows: *HLA-A* – 0.4428, *HLA-B* – 0.9006, *HLA-C* – 0.9482, *HLA-DRB1*–0.5317, *HLA-DQB1*–0.3989.

Comparison of GO and non-GO groups

The alleles of higher frequency in GO as compared to non-GO group were found in MHC class I only. The differences were statistically significant for *HLA-A*32:01* (7.14% vs 0.0%, $p = 0.01$), *-B*39:01* (8.57% vs. 0.0%, $p = 0.006$) and *-C*08:02* (7.14% vs. 1.0%, $p = 0.04$, OR 6.9) (Fig. 1). On the other hand, the frequency of *HLA-C*04:01* and *DRB1*15:02* was significantly lower in GO as compared to non-GO group (11.43% vs. 24.18%, $p = 0.04$, OR 0.4,and 0.0% vs. 6.59%, $p = 0.03$, respectively) (Fig. 2).

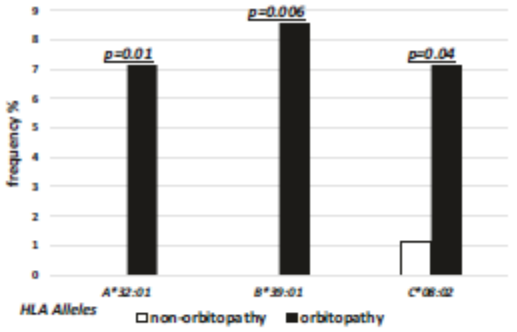

Fig. 1 Frequencies (%) of human leukocyte antigen (HLA) over-represented alleles with statistically significant difference between non-GO (open bars) and GO patients (solid bars).

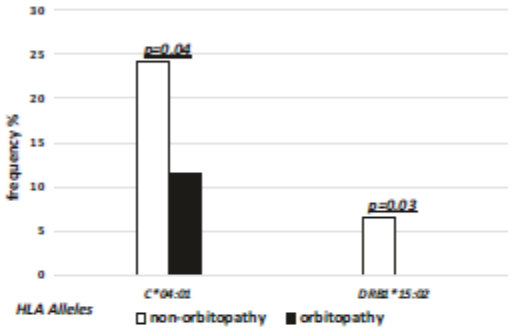

Fig. 2 Frequencies (%) of human leukocyte antigen (HLA) under-represented alleles with statistically significant difference between non-GO (open bars) and GO patients (solid bars).

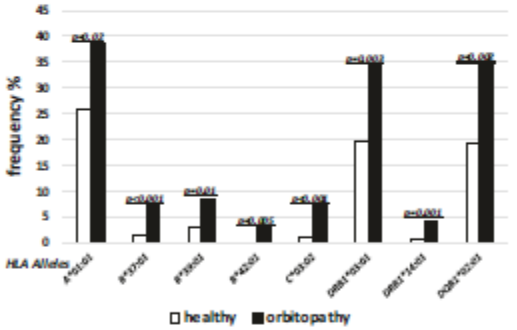

Fig. 3 Frequencies (%) of human leukocyte antigen (HLA) over-represented alleles with statistically significant difference between healthy (open bars) and GO patients (solid bars).

Comparison of GO and control group

The alleles of higher frequency in GO as compared to the controls were found in both MHC class I and class II. The differences were statistically significant for the following alleles of MHC class I: *HLA-A*01:01*(38.54% vs. 25.89%, $p = 0.02$, OR 1.8), *-B*37:01* (7.14% vs. 1.67%, $p < 0.001$, OR 4.5), *-B*39:01* (8.57% vs. 3.20%, $p = 0.01$, OR 2.8), *-B*42:01* (2.86% vs. 0.09%, $p = 0.005$, OR 2.8) and *-C*03:02* (7.14% vs. 0.99%, $p < 0.001$, OR 8.3) (Fig. 3). For the MHC class II,

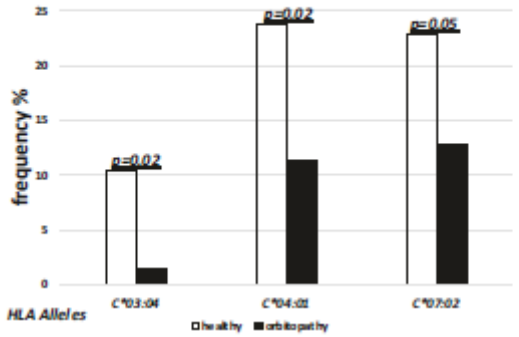

Fig. 4 Frequencies (%) of human leukocyte antigen (HLA) under-represented alleles with statistically significant difference between healthy (open bars) and GO patients (solid bars).

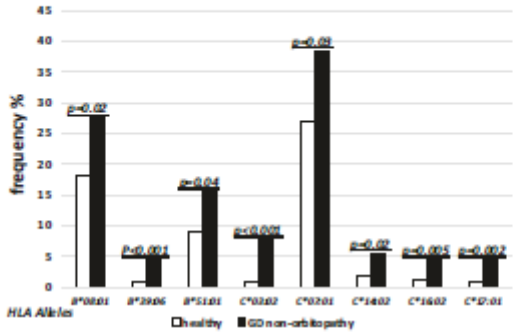

Fig. 5 Frequencies (%) of human leukocyte antigen (HLA) over-represented alleles with statistically significant difference between healthy (open bars) and non-GO patients (solid bars) for major histocompatibility complex (MHC) class I alleles.

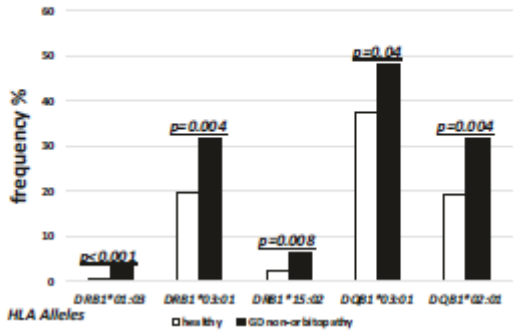

Fig. 6 Frequencies (%) of human leukocyte antigen (HLA) over-represented alleles with statistically significant difference between healthy (open bars) and non-GO patients (solid bars) for major histocompatibility complex (MHC) class II alleles.

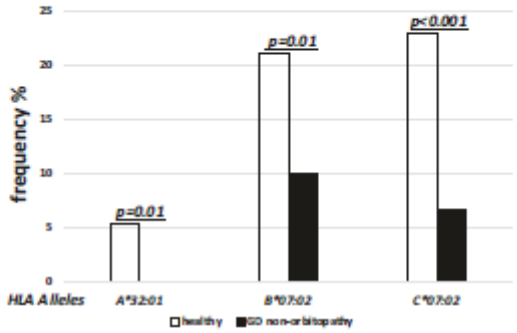

Fig. 7 Frequencies (%) of human leukocyte antigen (HLA) under-represented alleles with statistically significant difference between healthy (open bars) and non-GO patients (solid bars).

the frequencies of the following alleles were higher in GO as compared to the controls: *HLA-DRB1*03:01* (34.29% vs. 19.67%, $p = 0.003$, OR 1.9), *-DRB1*14:01* (4.29% vs.0.72%, $p = 0.001$, OR 6.2), *DQB1*02:01* (34.29% vs. 19.44%, $p = 0.002$, OR 1.9) (Fig. 3).

On the other hand, the frequencies of *HLA-C*04:01*, *-C*03:04* and *-C*07:02* were significantly lower in GO as compared to the controls (11.43% vs. 23.73%, $p = 0.02$, OR 0.4; 1.43% vs. 10.37%, $p = 0.02$, OR 0.1; and 12.86% vs. 22.92%, $p = 0.05$, OR 0.2, respectively) (Fig. 4).

Comparison of non-GO and control group

The alleles of higher frequency in non-GO as compared to the controls were found in both MHC class I and class II. The differences were statistically significant for the following alleles of MHC class I: *HLA-B*08:01* (27.47% vs. 18.0%, $p = 0.02$, OR 1.7), *-B*39:06* (4.40% vs. 0.81%, $p < 0.001$, OR 5.6), *-B*51:01* (15.38% vs. 9.02%, $p = 0.04$, OR 1.8) (Fig. 5), *HLA-C*03:02* (7.69% vs. 0.99%, $p < 0.001$, OR 7.7), *-C*07:01* (38.46% vs. 26.97%, $p = 0.03$, OR 1.6), *-C*14:02* (5.49% vs. 1.89%, $p = 0.02$, OR 3.0), *-C*16:02* (4.40% vs. 1,08%, $p = 0.005$, OR 4.2), *-C*17:01* (4.40% vs. 0.99%, $p = 0.002$, OR 4.6) (Fig. 5). For the MHC class II, the frequencies of the following alleles were higher in non-GO as compared to the controls: *HLA-DRB1*01:03* (3.30% vs. 0.41%, $p < 0.001$, OR 8.4), *-DRB1*03:01* (31.87% vs. 19.44 %, $p = 0.004$, OR2.1), *-DRB1*15:02* (6.59% vs. 2.26%, $p = 0.008$, OR 3.1),, *-DQB1*03:01* (48.35% vs. 37.66%, $p = 0.04$, OR 1.5), *-DQB1*02:01* (31.87% vs. 19.44%, $p = 0.004$, OR 2.2) (Fig. 6). No age-related difference in high risk allele frequency was found (data not presented). Comparison of high risk allele frequencies between males and females was not performed because of the incomparable sizes of males and females subgroups.

On the other hand, the frequencies of *HLA-A*32:01*, *-B*07:02* and *-C*07:02* were significantly lower in non-GO as compared to the controls (0.0% vs. 5.37%, $p = 0.01$; 9.89% vs. 21.06%, $p = 0.01$, OR 0.4; and 6.59% vs. 22.87%, $p < 0.001$, OR 0.5, respectively) (Fig. 7).

The summary of the relationships between HLA and GO development as well as between HLA and non-GO GD are presented in Table 2.

Frequencies of a single high risk allele and of co-presence of alleles

In 15 patients with GO (21.43%), only one of the alleles described above as correlated to a high risk of GO was found. These alleles were *HLA-A*01:01*, *-A*32:01*, *-B*39:01*, *-C*03:02*, *-C*08:02* with *HLA-A*01:01* and *-B*39:01* being the most commonly present. Each of them occurred as a single high risk allele in 4 patients with GO (5.7%).

In 11 patients (15.71%), two of the high-risk alleles were present. Among this group, the co-presence of *HLA-DRB1*03:01* and *-DQB1*02:01* was observed the most frequently (27.27%) and these two alleles are in linkage disequilibrium (LD) [22]. The co-presence of different alleles which are not in LD was observed in the rest of patients among this group. Interestingly, among the group of patients with three high risk alleles, *HLA-A*01:01* was most frequently present with *-DRB1*03:01* and *-DQB1*02:01*. A combination of these three alleles – *HLA-A*01:01*, *-DRB1*03:01-* and

Table 2. Summary of relationships between HLA and GO and non-GO GD development.

Increased risk of GO	OR	Increased risk of GO and non-GO[2]	OR, OR[2]	Increased risk of non-GO but not GO[2]	OR	Decreased risk of GO	OR	Decreased risk of non-GO[2]	OR
***A*32:01**[a]	-	*C*03:02*	*8.3; 7.7*[b]	*B*08:01*	*1.7*	***C*04:01***[a,b]	*0.4*[a,b]	*B*07:02*	*0.4*
B*39:01[a,b]	**2.8**[b]	*DRB1*03:01*	*1.9; 2.1*[b]	*B*39:06*	*5.6*	***DRB1*15:02***[a]	-	*C*07:02*	*0.5*
C*08:02[a]	**6.9**	*DQB1*02:01*	*1.9; 2.2*[b]	***B*51:01***	***1.8***	*C*03:04*[b]	*0.1*	***A*32:01***	-
A*01:01[b]	***1.8***			*C*07:01*	*1.6*	*C*07:02*[b]	*0.2*		
*B*37:01*[b]	*4.5*			*C*14:02*	*3.0*				
B*42:01[b]	**2.8**			***C*16:02***	**4.2**				
*DRB1*14:01*[b]	*6.2*			*C*17:01*	*4.6*				
				*DRB1*01:03*	*8.4*				
				DRB1*15:02	**3.1**				
				*DQB1*03:01*	*1.5*				

[a]vs non-GO; [b]vs healthy controls, alleles not previously reported as GD related unless GO and non-GO groups were distinguished are presented in bold.
GO Graves' orbitopathy, *OR* odds ratio.

*DQB1*02:01* – occurred in 11 out of 70 GO patients (15.7%), while the co-presence of other sets of three alleles was found only in 5.7% of GO patients. Among the group of patients with four risk alleles, only patients with the most common combination of three alleles (i.e. *HLA-A*01:01*, *-DRB1*03:01-* and *DQB1*02:01*) with additional presence of *HLA-A*32:01*, *-B*37:01* or *-DRB1*14:01* were found. None of the patients had more than four high risk alleles.

DISCUSSION

In the last decades, it has become more and more clear that autoimmune diseases are triggered by environmental factors such as infections, stress, smoking, etc. in genetically predisposed individuals [5, 23]. This genetic susceptibility seems to be crucial also in the pathogenesis of GD. Very recently, our research group has demonstrated the complex correlation between HLA alleles and GD development [5]. Those results clarified the previously existing discrepancies between different reports available for Caucasian population. Significant divergences in the results presented by various authors could undoubtedly depend on the applied method and the size of the study group. We previously confirmed in patients with GD as well as with SAT, that the use of high-resolution methods can significantly change the results obtained with less specific older methods. Application of modern methods of genotyping, which allow to achieve allelic specificity, is currently a gold standard of research because these methods demonstrate high reliability and allow to avoid method-dependent errors. Less specific methods obtain results for the entire allelic group, not for a particular allele and – therefore – may lead to erroneous conclusions. In a strictly controlled group of HLA typing performed for the purposes of bone marrow transplantation between 1996 and 2011, discrepancies between results obtained with older methods and the NGS method were found in as many as 29.1% of cases [24]. Therefore, the results of our recent study may be considered highly reliable, as it included the largest Caucasian cohort to whom a modern high-resolution method was applied up to date [5].

Having identified the alleles related to high risk of GD and the protective ones [5], we made an attempt to fill the knowledge gap regarding HLA background of GO in Caucasian population. As it was stated above, the data on this issue are available almost exclusively for Asians but even in that population the results are so divergent that no clear conclusion is possible to be drawn. In Caucasians, Yin et al. postulated lack of any genetic susceptibility to GO and concluded that environmental and epigenetic factors played crucial role in GO development [17]. However, taking into account the fact that some GO patients are practically free from environmental and biochemical risk factors (no smoking history, slightly elevated TRAb and thyroid hormone levels) the importance of genetic factors seems pivotal. Recently, several reports on significance of various gene polymorphisms in GO development in Caucasians have been published [25–28]. Additionally, the impact of *CD28/CTLA-4/ICOS* haplotypes on susceptibility to GD and GO was also postulated [29]. These results strongly support the significance of genetic background of GO.

Our present results further confirmed the role of genetic background in GO development by demonstrating the significance of HLA for GO risk in Caucasians, with the application of NGS method. As it was stated above, other results on HLA-related susceptibility for GO are lacking in Caucasians, so our results cannot be directly compared to other studies, especially those which used the same method. Contrary to Yin et al. [17], we have confirmed a strong correlation between GO and HLA, including identification of both high-risk and protective alleles.

In the present study, we have demonstrated that *HLA-A*32:01*, *-B*39:01*, *-C*08:02*, *-A*01:01*, *-B*37:01*, *B*42:01* and *DRB1*14:01* are associated with increased risk of GO while they are not associated with non-GO GD course. On the basis of OR obtained for our study the highest risk of GO was associated with the presence of *HLA-C*08:02* (OR 6.9) and *-B*37:01* (OR 4.5). This is a very important finding, especially considering the fact that *HLA-A*32:01*, *-B*39:01*, *-C*08:02* alleles were strongly GO-related as compared to non-GO group. *HLA-A*01:01* is a very common allele in Caucasian population, therefore the difference between GO and non-GO was not statistically significant, however the significance was clear when GO group was compared to the healthy controls. There is no LD between these three alleles [30, 31], so the presence of any of them constitutes independent high risk factor. It should be stressed that *HLA-DRB1*14:01* was previously postulated as GO-related in Japanese patients [14] (Table 1) and this is the only similarity between our results in Caucasians and currently published data for the Asian population. However, such a lack of consistency could be expected, as not only were the results in Asians highly divergent, but also HLA susceptibility for autoimmune diseases including GD often differs between the two populations [6, 12–16]. We have previously demonstrated that the only GD high risk allele confirmed for both Asians and Caucasians was *HLA-DRB1*03:01* [5] whose specificity for GD is quite low, because it is an allele typical for many autoimmune disorders.

In addition to the novel finding of GO-related HLA alleles, we have also identified alleles potentially protective against GO, but not against non-GO course of GD. Among the two of them, the protective effect of *HLA-C*04:01* was demonstrated when GO

group was compared either to non-GO or to control group. This allele was previously described as SAT high risk one [7]. The present finding of its protective role against GO can to some extent explain the phenomenon of extremely rare co-presence of SAT and GO. Previously, potential significance of HLA background on the course of SAT and GD in patients with co-presence of these two diseases was postulated [10]. The is no LD between *HLA-C*04:01* and the other GO protective alleles - *HLA-C*03:01*, *-C*07:02* or *DRB1*15:02* [22, 30, 31], thus each of them can be considered an independent protective factor. Interestingly, *HLA-B*15:02* was simultaneously found to be associated with an increased risk of non-GO GD as compared to control group. In our cohort, none of GO patients was *HLA-B*15:02* positive. This allele occurred exclusively in non-GO group. It is worth emphasizing that Chen et al. postulated a crucial role of this allele in GD development in Chinese cohort [32], however its frequency has never been analyzed separately in GO and non-GO groups.

All the three alleles related to the high risk of GO as compared to non-GO, i.e. *HLA-A*32:01*, *-B*39:01* and *-C*08:02*, were not found to be GD high risk alleles in our previous study [5]. Such correlation is clearly visible only if GO group is separated and compared to non-GO group. These three alleles are not associated with non-GO, thus when the GO group was not analyzed separately but together with non-GO, as the whole GD group [5], the difference could not be significant. Similar situation regards protective effect of *HLA-C*04:01* and *DRB1*15:01* against GO which has been observed in the present study but in our previous report no correlation between this allele and the overall risk of GD development was found [5].

On the contrary, all alleles found in the present study as associated with GD either with GO or without GO, were also demonstrated as related to the high risk of GD in our previous study [5], with *HLA-C*03:02* being an entirely novel finding there [5] and *HLA-DRB1*03:01* and *-DQB1*02:01* being earlier postulated by other authors [2, 4, 33–36]. Among this group of three alleles, *HLA-C*03:02* is an independent risk factor not being with LD with others. *HLA-DRB1*03:01* is in LD with *-DQB1*02:01* [22, 37] and it should be kept in mind that susceptibility associated with alleles being in LD cannot be considered fully independent if both of them are present. However, a single presence of any of them constitutes the risk factor of the disease. Being aware of this fact is especially important in regard to our results in patients with multi-allele susceptibility, as in most of the patients with three or four high risk alleles the co-presence of *HLA-DRB1*03:01* and *-DQB1*02:01* was found. In such cases, these alleles cannot be considered independent risk factors.

The present study has also identified the alleles which are associated with high probability of non-GO course of GD but not with GO. Correlation between the presence of most of these alleles and the overall GD risk was demonstrated in our previous study [5]. However, the significance of *HLA-B*51:01* and *C*16:01* has never been found before, and *HLA-DRB1*15:02* was only postulated as a high risk factor of GD in Asians, as it was stated above. The increased risk of GD in carriers of any of the rest three alleles, i.e. *HLA-B*08:01*, *-C*07:01*, and *DQB1*03:01*, had been postulated before [2, 4, 34] and confirmed in our previous [5] and present studies. It should be underlined that the entirely novel correlation of non-GO GD and *HLA-B*51:01*, reported here for the first time, should be considered potentially expected, because of LD between this allele and *HLA-C*14:02*, reported as a high risk factor of GD for the first time in our recent study [5, 30, 31]. Moreover, *HLA-C*16:02* – the second allele demonstrated as related to non-GO GD – is in LD with *-B*51:01* [30, 31]. Therefore, current demonstration of the significance of *HLA-B*51:01* and *-C*16:02* complements our previous findings. Among the rest of the alleles associated with increased risk of GD but not with GO, *HLA-B*08:01* is in LD with *C*07:01*, while *DRB1*01:03* is in LD with *-DQB1*03:01* [30, 31]. Therefore, in this group, only *HLA-B*39:06* can be considered fully independent, while the rest of them is not entirely independent if alleles being in LD are present together.

In our previous study [5], the protective effect of *HLA-B*07:02* and *-C*07:02* was described for the first time. The present study has confirmed the protective role of both of these alleles against non-GO GD but only *-C*07:02* against GO. The same role of both of them in regard to non-GO GD can be further proved by LD between them in Caucasian population [30, 31]. Therefore, they cannot be considered independent. Similarly to the previously discussed alleles, the significant differences between GO and non-GO patients with GD are clearly visible here. Both of these alleles were described as protective in regard to overall GD development in our previous study [5]. However, in the present study, when GO and non-GO groups were analyzed separately, the protective effect of both of these alleles appeared to concern non-GO group only. In GO group, the difference did not reach statistical significance for *HLA-B*07:02* allele. Interestingly, *HLA-A*32:01*, demonstrated here as associated with the high risk of GO, was found in none of non-GO patients. This fact can further confirm the role of this allele in GO development and one should not consider this allele as protective against non-GO course of GD, but rather as a highly potent GO risk factor. On the basis of our results, we can speculate that the presence of this allele constitutes such a strong susceptibility factor that all patients with GD and *HLA-A*32:01* will develop GO. Similarly, we did not find any patient without GO and with *HLA-B*39:01*. The strength of the correlation between GO and these two alleles may be – therefore – similar.

The present study has demonstrated for the first time strong associations between GO and HLA alleles, with *HLA-A*01:01, -A*32:01, -B*37:01, -B*39:01, -B*42:01, -C*08:02, C*03:02, DRB1*03:01, DRB1*14:01* and *DQB1*02:01* being genetic markers of increased risk of GO, and *HLA-C*04:01, -C*03:04, -C*07:02* and *DRB1*15:02* being the protective alleles. Moreover, we have found which alleles are associated with increased and decreased probability of non-GO GD but have no correlation with the risk of GO development. Identification of these groups of GO-related and GO-protective alleles, as well as the alleles strongly related to non-GO GD, fills the existing gap in the knowledge on genetic background of GO and constitutes a significant step in the development of personalized medicine. The present findings provide a precise diagnostic tool for the individual GO risk assessment, which can significantly facilitate tailoring the prevention strategy and treatment modality in an individual patient.

DATA AVAILABILITY

The source data are included as Supplementary Materials.

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AUTHOR CONTRIBUTIONS

MS and KZ-S were responsible for study design, data collection, data analysis, and writing of the manuscript. BT and BS contributed to data collection and data analysis. AL contributed to study design, and writing of the manuscript. All authors were involved in writing the paper and approved the submitted final version.

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COMPETING INTERESTS

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Article

Novel Insight into Non-Genetic Risk Factors of Graves' Orbitopathy

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Abstract: An assessment of the risk of Graves' orbitopathy (GO) is an important challenge in Graves' disease (GD) management. The purpose of this study was to compare non-genetic parameters in GD patients with and without GO in order to find novel risk factors and to verify the factors already reported. A total number of 161 people, 70 with GO and 91 non-GO patients were included in this study. GO was confirmed to be associated with smoking, older age, higher TSH receptor antibodies (TRAb) and lower thyroglobulin antibody (TgAb) levels and hypercholesterolemia. We demonstrated the latter correlation even for only a mild increase in LDL cholesterol. Importantly, our study provides novel potential GO risk factors, including higher serum creatinine levels, higher MCV and lower PLT. If further confirmed, these new, simple and easily accessible potential GO markers may constitute valuable auxiliary markers in GO risk assessments. We additionally proved that in moderate to severe GO, gender-related differences attenuate. No impact of vitamin D deficiency in GO development in patients with 25-hydroxyvitamin D [25(OH)D] > 20 ng/mL was found. The present report provides a set of GO risk factors, which can be used as a precise tool for an individual GO risk assessment.

Keywords: Graves' orbitopathy; Graves' disease; autoimmunity; TSH receptor antibodies; risk factors; LDL cholesterol; creatinine; oxidative stress

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1. Introduction

Graves' disease (GD) is an autoimmune thyroid disorder (AITD), resulting from an uncontrolled production of antibodies against thyroid stimulating hormone (TSH) receptors (TRAb). These antibodies usually stimulate TSH receptor, leading to excessive thyroid hormone production [1]. A prevalence of GD is approximately 1% to 1.5%, with an incidence ranging from 20 to 30 new cases/100,000/year [2]. TRAb may exhibit a high affinity not only to the thyroid gland but also to other tissues, including eyes (orbitopathy), skin (dermatopathy) and bone and muscles (acropachy) [2,3]. Graves' orbitopathy (GO), which is the most important extrathyroidal manifestation of GD, presents with lid retraction, exophthalmos, soft tissue involvement of the eye, spontaneous retrobulbar pain and pain on an attempted upward or downward gaze [4]. GO results in significantly reduced quality of life (QoL) and severe complications, including sight threatening conditions, such as optic neuropathy and/or corneal breakdown [4]. Our research team previously demonstrated that both genetic and non-genetic factors influence the clinical course of thyroid disorders [5]. Genetic susceptibility to GD was proved to be human leukocyte antigens (HLA)-related, with *HLA-B*08:01*, *-B*39:06*, *-B*37:01*, *-C*07:01*, *-C*14:02*, *-C*03:02*, *-C*17:01*, *-DRB1*03:01*, *-DRB1*11:01*, *-DRB1*13:03*, *-DRB1*01:03*, *-DRB1*14:01*, *-DQB1*03:01*, *DQB1*02:01* being GD high risk alleles [6]. Recently, we have demonstrated that the risk of GO development is also HLA-dependent [7].Knowledge on genetic the background provides new tools

for personalized patient management in GO prevention and treatment. However, it is a non-genetic factor that triggers GO in patients with a genetic predisposition. Therefore, the necessity to broaden the knowledge on factors, which induce GO or worsen its course is still growing, so as to further personalize the management. Several non-genetic factors are already known as GO-related, including smoking, higher TRAb levels, radioiodine therapy, uncontrolled hyper- or hypothyroidism and recently demonstrated higher total cholesterol (TC) and low density lipoprotein (LDL) cholesterol levels [4,8]. However, many other parameters, such as age, stress or vitamin D deficiency were postulated to be GO risk factors [9] but the results of different studies are inconsistent. Some laboratory parameters have never been compared between GO and non-GO GD patients in size-matched groups.

Knowledge on the risk factors associated with the onset or severity of GO can be fundamental in the prevention and treatment of GO. Thus, the aim of the present study was to compare clinical and laboratory features of patients belonging to GO and non-GO GD groups in order to find novel non-genetic GO risk factors and to verify the factors already reported. This analysis aims to provide novel tools for GO prevention and for the further development of a personalized GO management.

2. Materials and Methods

2.1. GD Rroup and Control Group

A total number of 161 people, 70 with GO and 91 non-GO patients who were diagnosed with GD in the Department of Endocrinology and Metabolic Diseases Polish Mother's Memorial Hospital Research Institute, Lodz, Poland and the Department-associated outpatient clinic were included in this study.

2.2. Inclusion Criteria

In all the patients included in this study, GD was diagnosed using the standard criteria [1], including hyperthyroidism, elevated TRAb level and typical ultrasound (US) patterns. GO was diagnosed on the basis of the presence of lid retraction, soft tissue involvement of the eye (redness, swelling) and exophthalmos, according to the guidelines [4]. Patients with GD and without GO were included in the prospective arm of this study in which long term follow up (>3 years) were applied to confirm patients' group affiliation. All patients in whom GO symptoms occurred during the follow up were reclassified and included in the GO group. Patients with other acute or chronic diseases, which may have influenced laboratory results were excluded from the study.

2.3. Laboratory Procedures

Blood samples from all the patients were collected at the time of diagnosis. The procedure was performed by a single venipuncture, using the vacutainer system technique. Samples were collected in the morning (6.00–8.00 A.M.) after overnight fasting. Serum levels of TSH, free triiodothyronine (FT3), free thyroxine (FT4), thyroglobulin antibodies (TgAb), thyroperoxidase antibodies (TPOAb), TRAb, morning cortisol and 25-hydroxycholecalciferol were measured by the electrochemiluminescence immunoassay (ECLIA) with a Cobas e601 analyzer (Roche Diagnostics, Indianapolis, IN, USA). The erythrocyte sedimentation rate (ESR) was determined by Ves-Matic Cube 30 (Diesse, Monteriggioni, Italy). C-reactive protein (CRP), total cholesterol, LDL and HDL cholesterol, triglycerides, urea, creatinine, glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin levels were determined by the VITROS® 4600 Chemistry System (Ortho Clinical Diagnostics, Raritan, NJ, USA).

2.4. Medical History Analysis

Medical history data regarding radioiodine treatment or thyroidectomy were recorded on the basis of patients' medical documentation. Active smokers at the time of GD diagnosis were recorded as smokers. Stressful events were evaluated on the basis of the Holmes–Rahe stress scale questionnaire with a result >150 considered positive for stressful event [10,11].

2.5. Statistical Analysis

Descriptive statistics of the collected data included median, mean, standard deviation and interquartile ranges. For comparisons between the groups, we used Student's *t*-test for normally distributed variables (i.e., age, urea and total cholesterol), the Mann–Whitney U test for the other real-valued data and the chi-square test for categorical variables. The normality of data distributions was assessed by the Shapiro–Wilk test. Additionally, a correlation analysis, including Pearson's coefficient and Spearman's rank correlation coefficient calculations between the CAS score and TRAb (both in linear and logarithmic scale) was performed. For all the tests a p value < 0.05 was considered significant. The sample size was calculated on the basis of the region population size and GD prevalence in the Polish population with 95% confidence interval. Python statistical libraries (SciPy stats) were used for all the computations.

2.6. Ethics Procedures

Informed consent for all the performed procedures was obtained from all of the patients after a full explanation of the purpose and nature of all procedures used. The study was approved by the Ethics Committee of the Polish Mother's Memorial Hospital-Research Institute, Lodz, Poland (approval code—108/2018).

3. Results

The mean age of the patients at the time of GD diagnosis was 41.3 ± 17.39 years, with a male to female ratio of 1: 4.57. The diagnosis of GD was based on laboratory results and the US pattern. For the whole GD group, median/IQR of TSH, FT4, FT3 and TRAb were 0.005/0.015 µIU/mL, 2.48/2.68 ng/dL, 7.95/13.43 pg/mL and 10.29/16.16 IU/L, respectively.

3.1. Comparison of GO and Non-GO Groups with Respect to Patients' Age and Laboratory Results

A statistically significant difference was found for the mean age at GD diagnosis with patients in the GO group being older than those in the non-GO group (44.25 ± 14.61 vs. 39.00 ± 19.04 years) (Table 1). The mean time lag between a GD diagnosis and the onset of GO in the GO group was 32 months.

Table 1. Comparison of GO and non-GO groups in regard to patients' age and laboratory results.

Parameter (Reference Range and Units)	GO		Non-GO		*p*-Value
	Mean ± SD (N)	Median/IQR	Mean ± SD (N)	Median/IQR	
Age at GD diagnosis (years)	44.25 ± 14.61 (71)	47.00/21.50	39.00 ± 19.04 (91)	40.00/28.00	0.049 *
TSH (0.27–4.2 µIU/mL)	0.18 ± 0.59 (59)	0.01/0.04	0.10 ± 0.48 (83)	0.01/0.01	0.049 *
FT3 (2.0–4.4 pg/mL)	9.59 ± 8.89 (56)	4.85/10.55	12.85 ± 8.06 (79)	11.63/11.95	0.003 *
FT4 (0.93–1.7 ng/dL)	2.64 ± 2.09 (57)	1.80/1.86	3.58 ± 1.96 (82)	3.15/2.86	<0.001 *
TPOAb (<34 IU/mL)	182.57 ± 195.05 (58)	77.50/268.85	211.54 ± 204.57 (71)	170.40/236.50	0.23
TgAb (<115 IU/mL)	347.22 ± 894.99 (58)	25.45/218.22	406.41 ± 640.99 (67)	250.00/484.30	<0.001 *
TRAb (<1.75 IU/L)	17.59 ± 13.79 (68)	13.72/26.95	13.65 ± 13.52 (88)	9.20/13.25	0.021 *
Morning cortisol (>10 µg/dL)	12.87 ± 5.08 (15)	11.00/5.27	14.12 ± 6.80 (18)	14.47/8.38	0.56
ESR (<12 mm/h)	21.40 ± 20.18 (40)	17.00/23.50	19.00 ± 20.38 (30)	15.50/20.00	0.34
CRP (<1.00 mg/dL)	0.71 ± 0.56 (54)	0.50/0.10	0.89 ± 1.10 (48)	0.50/0.13	0.86
Glucose (60–99 mg/dL)	94.10 ± 24.76 (59)	89.00/11.50	98.26 ± 18.74 (52)	96.00/11.00	0.006 *
Urea (19.3–42.3 mg/dL)	31.56 ± 10.25 (48)	31.00/11.00	32.40 ± 7.49 (45)	32.00/10.00	0.65
Creatinine (0.66–1.25 mg/dL)	0.68 ± 0.20 (58)	0.66/0.23	0.53 ± 0.16 (54)	0.50/0.21	<0.001 *
ALT (women < 35 U/L, men < 50 U/L)	30.22 ± 16.90 (58)	24.00/17.50	32.27 ± 16.78 (61)	29.00/22.00	0.45
AST (women < 36 U/L, men < 59 U/L)	26.71 ± 8.51 (59)	25.00/9.00	29.59 ± 10.18 (60)	27.00/13.25	0.11
Bilirubin (0.2–1.3 mg/dL)	0.62 ± 0.19 (40)	0.65/0.22	0.66 ± 0.43 (46)	0.57/0.39	0.66

Table 1. *Cont.*

Parameter (Reference Range and Units)	GO		Non-GO		*p*-Value
	Mean ± SD (N)	Median/IQR	Mean ± SD (N)	Median/IQR	
WBC (4–10 × 10^3/μL)	6.96 ± 2.21 (63)	6.49/2.87	6.71 ± 2.37 (64)	6.62/3.02	0.57
RBC (women 3.8–5.8 × 10^6/μL, men 4.5–6.5 × 10^6/μL)	4.58 ± 0.51 (62)	4.49/0.62	4.68 ± 0.50 (65)	4.60/0.65	0.23
HGB (women 12–15, men 13–18 g/dL)	13.39 ± 1.59 (62)	13.30/1.77	13.09 ± 2.00 (65)	13.00/1.70	0.21
HCT (women 36–45, men 40–54%)	39.62 ± 4.44 (62)	39.00/4.97	38.58 ± 3.46 (65)	38.70/3.80	0.25
PLT (150–400 × 10^3/μL)	238.37 ± 59.11 (62)	235.00/79.25	270.11 ± 72.74 (65)	259.00/89.00	0.02 *
MCV (women 78–93 fl, men 82–94 fl)	86.21 ± 6.35 (58)	86.95/7.25	82.93 ± 5.27 (60)	82.55/7.67	<0.001 *
MCHC (32–37 g/dL)	33.78 ± 1.08 (62)	34.00/1.10	33.73 ± 1.01 (65)	33.70/1.00	0.39
TC Total cholesterol (<200 mg/dL)	192.84 ± 53.39 (64)	189.00/82.75	161.96 ± 48.21 (54)	152.00/70.25	0.001 *
LDL cholesterol (<100 mg/dL)	117.28 ± 46.53 (64)	103.50/86.00	94.13 ± 39.74 (54)	85.00/31.75	0.006 *
HDL cholesterol (>40 mg/dL)	51.80 ± 15.84 (64)	50.00/21.00	47.94 ± 15.79 (53)	46.00/19.00	0.13
triglycerides (<150 mg/dL)	121.53 ± 54.28 (64)	111.50/77.50	106.93 ± 47.41 (54)	91.50/56.75	0.13
25(OH)D (>30 ng/mL)	27.05 ± 10.37 (51)	25.30/15.80	25.24 ± 15.21 (45)	22.60/9.40	0.12

* The difference is statistically significant, with $p < 0.05$.

Severity of thyrotoxicosis was significantly higher in the non-GO group than in the GO group, with a higher TSH mean and lower mean concentration of FT3 and FT4 in the GO group than in non-GO group (0.18 ± 0.59 vs. 0.10 ± 0.48 μIU/mL, 9.59 ± 8.89 vs. 12.85 ± 8.06 pg/mL and 2.64 ± 2.09 vs. 3.58 ± 1.96 ng/dL, respectively). In the GO group, the mean TRAb concentration was significantly higher than in the non-GO group, while thyroglobulin antibody (TgAb) levels were significantly lower in the GO group than in the non-GO group (347.22 ± 894.99 vs. 406.41 ± 640.99) (Table 1). A statistically significant difference was found for serum glucose concentration with higher levels in the non-GO group than in the GO group (98.26 ± 18.74 vs. 94.10 ± 24.76 mg/dL). Creatinine levels were significantly higher in the GO group than in the non-GO group (0.68 ± 0.20 vs. 0.53 ± 0.16 mg/dL). Among the blood count parameters, the mean corpuscular volume (MCV) was significantly higher in the GO group than in the non-GO group (86.21 ± 6.35 vs. 82.93 ± 5.27 fl), while platelet (PLT) levels were lower in the GO group than in the non-GO group (238,370 ± 59,110 vs. 270,110 ± 72,740/μL). Among the lipid parameters, both TC and LDL cholesterol levels were significantly higher in the GO group than in the non-GO group (192.84 ± 53.39 vs. 161.96 ± 48.21 mg/dL and 117.28 ± 46.53 vs. 94.13 ± 39.74 mg/dL, respectively) (Table 1).

No statistically significant differences were found for other laboratory parameters (Table 1).

3.2. Comparison of GO and Non-GO Groups with Respect to Gender, Environmental Risk Factors and Treatment

Smoking was significantly more common in the GO group than in the non-GO group. Thyroidectomy was also performed more often in the GO group. No significant differences were found with respect to gender, stress or radioiodine treatment (Table 2).

Table 2. Comparison of GO and non-GO groups in regard to gender, environmental risk factors and treatment.

Parameter	GO	Non-GO	*p*-Value
Male/Female ratio	14/57	15/76	0.59
Smoking (Yes/No)	32/37	13/71	<0.001 *
Stressful events (Yes/No)	46/20	46/27	0.27
^{131}I treatment (Yes/No)	21/50	17/72	0.12
Thyroidectomy (Yes/No)	16/55	2/87	<0.001 *

* The difference is statistically significant, with $p < 0.05$.

3.3. Correlation Analysis between TRAb and CAS Score

The analysis of correlation between the TRAb and CAS scores revealed a weak, although definitely positive, correlation (r = 0.201, $p = 0.161$). The logarithm of TRAb showed

a slightly higher correlation with CAS (r = 0.213, $p = 0.137$), while Spearman's rank correlation coefficient was 0.245, $p = 0.085$.

4. Discussion

The risk factors of GO are not entirely clear and include genetic predispositions and interactions between endogenous and environmental factors [12]. Although genetic susceptibility seems to play an important role, GO is triggered and modulated by non-genetic factors. In the present study, we compared both clinical and environmental factors of GO groups and non-GO groups in order to find potential, novel and non-genetic GO risk factors as well as to analyze factors, which were previously postulated to be GO-associated.

Smoking and high TRAb levels were postulated as risk factors in many papers, including the current EUGOGO guidelines [4,8]. Our results confirmed a strong relationship between smoking and GO. Smoking is a strong oxidative stress inducer. Reactive oxygen species (ROS) were demonstrated to play a role in GO development. They stimulate orbital fibroblast proliferation, synthesis of glycosaminoglycans and inflammatory mediators [13]. Increased concentrations of ROS were found not only in blood, but also in the urine of GO patients [13]. The beneficial effect of selenium supplementation in GO is related to its antioxidant properties [13,14]. Tsai et al. also demonstrated the presence of oxidative DNA damage markers (higher level of 8-OHdG–8-hydroxy-2′-deoxyguanosine) in the urine of GO patients and a positive correlation between oxidative DNA damage and the clinical activity of GO [15].

TRAb levels were also, as previously demonstrated [16,17], significantly higher in GO patients. Since TSHR overexpression was confirmed in GO orbital tissues [18,19], TRAb levels are considered as an independent GO risk factor and a GO biomarker [8]. Many studies demonstrated higher levels of TRAb in GO than in non-GO GD patients. Wiersinga et al., in a prospective observational study on patients with newly diagnosed GD, postulated TRAb as an independent baseline determinant for the development of GO [20]. Diana et al. observed that TRAB levels correlate well with clinical activity and severity of GO [21]. Nicoli et al. showed a statistically significant, direct correlation between serum TRAb levels and CAS in patients with recent onset and untreated GO [16]; our results also revealed a positive correlation; however, it had not reached the level of statistical significance. Lantz et al. showed an increased risk of GO in patients with TRAb > 6.3 IU/L at the time of GD diagnosis [22]. In our cohort, the difference was significant but the mean levels of TRAb were much higher than 6.3 IU/L in both groups (i.e., 17.59 vs. 13.65 IU/L in GO and non-GO groups, respectively). Therefore, the actual threshold of TRAb for the risk of GO development should be further evaluated as it may be much higher than previously postulated [22].

There is a strong correlation between smoking and TRAb levels. Roos et al. demonstrated that during treatment TRAb levels normalize faster in non-smokers than in smokers [23]. Several authors reported that GO patients had higher TRAb levels, were statistically older and tended to smoke more frequently with the duration of hyperthyroidism being shorter when compared to patients without GO [24,25]. Our study confirmed these observations, as our GO group were older, had higher TRAb levels with smoking being significantly more common among them.

Unstable hyperthyroidism was also demonstrated as a GO risk factor [4] and the association between GO risk and the level of stimulatory TRAb was proved to be the most important. In our cohort, most patients in both GO and non-GO groups had unstable hyperthyroidism at the time of diagnosis, so we did not find such a correlation. Moreover, we observed a significantly less severe thyrotoxicosis in the GO group when compared to the non-GO group. A similar finding of lower FT4 levels in GO patients was previously reported by Goh et al. [26]. It is well known that GD-related biochemical thyrotoxicosis is usually much more severe in younger patients. As stated above, our study confirmed previous observations [27,28] that an older age at the onset of the disease is a GO risk factor. GO patients were significantly older than non-GO patients, and as GO occurred

in some patients even after a two year onset of GD, we monitored our non-GO group during the >3-year follow up period in order to confirm that they were properly included in the non-GO group. Taking into account the above described findings, we supposed that the lower severity of thyrotoxicosis in the GO group may result from the older age of GO patients.

Thyroid antibody profiles in GO is a very interesting issue and it has been a subject in many studies. Expression of not only TSHR but also thyroglobulin (Tg) mRNA were found in GO orbital fibroblasts [29,30]. These reports suggested that Tg fibrocyte expression is a result of a substantial Tg promoter activity [29,30]. Although Khamisi et al. reported higher Tg levels in GO patients when compared to non-GO patients, they found TgAb levels to be lower in the GO group [31]. We have not analyzed Tg levels but TgAb concentrations were significantly lower in our GO patients. However, it should be underlined that the mean TgAb as well as TPOAb levels were elevated significantly above the reference range in both GO and non-GO groups. Similarly, other authors reported lower TgAb as well as TPO Ab in GO patients [26]. We also observed lower TPOAb levels but the difference did not reach a statistical significance.

Interestingly, we observed that serum glucose concentration was higher at the time of GD diagnosis in the non-GO group when compared with GO patients, which may be associated with a higher severity of thyrotoxicosis (higher FT3 and FT4 concentrations) in this group.

In addition, our study indicated significantly higher creatinine serum concentrations in the GO group than in non-GO group. We might postulate that this observation resulted mainly from the older age of the patients [32]. It is a plausible hypothesis but taking into account that urea levels did not significantly differ between GO and non-GO groups, and the mean and median urea values were actually lower in the GO group (although the difference were not statistically significant), this hypothesis seems questionable. Therefore, as higher creatinine levels in older age is most commonly associated with prerenal factors and increased urea levels, age does not seem to be the main cause of our finding of higher creatinine in GO patients. It should be underlined that we did not include patients with renal insufficiency, as their laboratory results would have been related mainly to renal disease. Therefore, our observation may indicate that higher (but still within the reference range) creatinine levels may constitute a novel GO risk factor. Obviously, further studies are necessary to confirm this observation and to find out whether there is any cut-off creatinine value, which can be considered as GO-related.

Moreover, significantly higher MCV in GO patients (when compared to the non-GO group) was demonstrated in our current study. Thyrotoxicosis was previously found to adversely affect hematopoiesis [33,34]. Krygier et al. demonstrated that thyrotoxic patients had lower but still normal MCV, which increased during treatment. They postulated that this phenomenon is inversely correlated with hepcidin levels [35]. Similarly, we observed lower MCV in our non-GO patients in whom thyrotoxicosis was more severe. However, oxidative stress may also play a role in this phenomenon as several authors observed that oxidative stress is associated with higher MCV levels [36]. Unfortunately, neither creatinine nor MCV levels had previously been analyzed as potential GO risk factors; therefore, our findings require further confirmation.

Our current research indicates significantly lower PLT levels in GO patients than in the group without GO. The autoimmune etiology of thrombocytopenia associated with Graves' disease and the overlapping of the thyroid and platelets autoimmunity was well explained by Cordiano et al. [34]. Gill et al. showed that treatment of Graves' disease resulted in a significant improvement in thrombocytopenia that was refractory to intravenous immunoglobulin and steroid treatment [37]. Thus, a significantly higher serum TRAb concentration and greater severity of the autoimmune process when compared to the group without GO may be a possible explanation for a significantly lower platelet concentration in GO patients.

In recent years, high serum total cholesterol (TC) and LDL cholesterol levels were demonstrated as an independent risk factors of GO [4,38,39]. Additionally, TC and LDL cholesterol were positively correlated with the GO CAS result [24,38]. No relationship between GO and high-density lipoprotein cholesterol (HDL) or triglycerides (TG) was observed [39]. In our cohort, we also observed higher TC and LDL concentrations in GO patients, with no differences in HDL and TG levels between GO and non-GO groups. Interestingly, we confirmed that there are significant differences in TC and LDL levels between GO and non-GO groups, even though in our groups mean and median TC values were within the reference range, and LDL mean and median values were only slightly above the upper reference value in the GO group. This finding underlines the significance of TC and LDL in GO development even in only mild LDL elevation. It was also demonstrated that cholesterol-lowering treatment improved the outcome in GO patients treated with intravenous glucocorticoid therapy (ivGC) [40,41]. Therefore, current guidelines recommend cholesterol lowering treatment in all GD patients [4]. Our present findings confirmed that even mild LDL elevation, or possibly even high normal LDL levels, are risk factors of GO development. Therefore, our study provides a further strong indication for cholesterol lowering therapy in GD in all patients even with a slight LDL elevation.

Recently, Heisel et al. postulated that serum 25-hydroxyvitamin D (25(OH)D) deficiency was an independent risk factor of GO [9]. The sizes of analyzed groups were similar to ours, but we have not observed such correlation. Interestingly, mean 25(OH)D concentrations were close to the lower normal limit in both the analyzed groups and in both studies. However, Heisel et al. observed lower levels in the GO group than in the non-GO group (24.8 vs. 29.4 ng/mL, $p = 0.006$) [8] while we observed even higher levels in the GO group (27 vs. 25 ng/mL) but the difference was not statistically significant. Since both studies did not reveal severe 25(OH)D deficiency in either of the analyzed groups, we believe that the differences within levels above 20 ng/mL are not associated with GO risk. The potential impact of severe vitamin deficiency (<10 ng/mL) on the risk of GO development requires further studies.

Topcu et al. showed a significantly higher number of negative stressful life events (SLEs) in GD patients when compared to the control group [42]. Kahaly et al., observed that six months prior to GO onset, 74 of 102 patients experienced a mean of 4 (range 0–13) SLEs [43]. In addition, patients with optic neuropathy had more stressful events than those without nerve involvement [43]. Additionally, the rates of anxiety and depression in GO were significantly higher than in healthy people [43]. However, it is unclear whether the anxiety and depression preceded GO, and more evidence is needed to understand the correlation between anxiety, depression and GO [44]. In our current study stressful events were common in both GO and non-GO groups and occurred in 70% and 63%, respectively. Although stressful events were more frequent in the GO group, the difference did not reach a statistical significance.

Although GD is more common in women, previous studies on the association between GO and gender provided inconsistent results. Many studies postulated a higher incidence in women when compared to men [45,46]. However, gender-related differences reduce in severe GO patients, and the patients with severe GO were more frequently men than women [47]. As our study was performed in a tertiary center, where patients mostly with moderate to severe GO are referred, we did not observe any correlation between GO risk and gender. In our center, we strictly follow the actual recommendations for GO prevention and radioiodine therapy is not applied in GD patients with a presence of any GO risk factor. This fact is the most probable explanation of the finding that we did not report any differences between our GO and non-GO groups with respect to the frequency of radioiodine therapy. Similarly, avoiding radioiodine treatment in patients at risk of GO resulted in a significantly higher frequency of thyroidectomies in the GO group than in the non-GO group.

The presented research has its strengths and limitations. It should be noted that our study included a selected group of patients who were referred to the tertiary center and, therefore, some group selection bias was possible. The statistical analysis was limited to a binary GO/non-GO scenario searching for significant differences between the groups, while correlation analysis, including CAS scores would probably reveal some more potentially interesting details. This direction will be considered in our future work. The strengths of the present study, on the other hand, lies in the strict inclusion criteria, long term follow-up of the non-GO group, a large number of analyzed parameters and unified laboratory assays for all the investigated patients.

5. Conclusions

Our present study confirmed that smoking, older age, higher TRAb and lower TgAb levels and higher TC and LDL concentrations are risk factors of GO development in patients with GD. We also further proved that in moderate to severe GO patients, gender-related differences attenuate. Moreover, our study suggests the existence of novel potential GO risk factors, including higher serum creatinine levels, higher MCV and lower PLT. These new, simple and easily accessible parameters may constitute valuable auxiliary markers in a GO risk assessment; however, their clinical significance for a GD/GO course requires further studies. We did not find any influence of vitamin deficiency on GO development in patients with 25(OH)D > 20 ng/mL. The present findings provides a set of GO risk factors, which can be used as a precise tool for an individual GO risk assessment, which can improve a personalized GO prevention and management.

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Abbreviations

AITD	autoimmune thyroid diseases
ALT	alanine aminotransferase
AST	aspartate aminotransferase
CAS	clinical activity score
CRP	C-reactive protein
ESR	erythrocyte sedimentation rate
EUGOGO	European Group of Graves' Ophthalmopathy
FT3	free triiodothyronine
FT4	free thyroxine
GD	Graves' disease
GO	Graves' orbitopathy

HCT	hematocrit
HDL	high-density lipoprotein
HGB	hemoglobin
HLA	human leukocyte antigens
LDL	low-density lipoprotein
mRNA	messenger RNA
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
PLT	platelet count
QoL	quality of life
RAI	radioactive iodine
ROS	reactive oxygen species
SLEs	stressful life events
TC	total cholesterol
TG	triglycerides
Tg	thyroglobulin
TgAb	thyroglobulin antibodies
TPOAb	thyroid peroxidase antibodies
TRAb	TSH-receptor antibodies
TSH	thyroid stimulating hormone (thyrotropin)
US	ultrasound
8-OHdG	8-hydroxy-2′-deoxyguanosine
25(OH)D	25-hydroxyvitamin D

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Komisja Bioetyczna przy Instytucie Centrum Zdrowia Matki Polki działając zgodnie z zasadami Dobrej Praktyki Klinicznej na posiedzeniu w dniu 20 października 2020 r. rozpatrywała wniosek dotyczący pracy:

„Czy obecność określonych alleli układu HLA może być markerem ryzyka wystąpienia orbitopatii Gravesa oraz ciężkości jej przebiegu?"

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3. Prof. dr hab. n. med. Andrzej Lewiński

Opinia Nr 62/2020

Komisja Bioetyczna przy Instytucie Centrum Zdrowia Matki Polki zapoznała się z ww projektem eksperymentu medycznego, przeanalizowała wniosek, wysłuchała opinii recenzenta o przedstawionym projekcie i wyniku przeprowadzonej dyskusji oraz tajnego głosowania, po rozważeniu kryteriów etycznych oraz celowości i wykonalności projektu pozytywnie zaopiniowała projekt eksperymentu medycznego.

Uchwałę podjęto jednogłośnie.
Uchwałę podjęto przy sprzeciwie —

Przewodnicząca:
Dr hab. med. Iwona Maroszyńska, prof. instytutu .....

Zastępca Przewodniczącej:
Prof. dr hab. n. farm. Daria Orszulak-Michalak .....

Członkowie:
Mec. Michał Araszkiewicz .....
Prof. dr hab. n. med. Tadeusz Biegański .....
Dr n. med. Paweł Czekalski .....
Dr hab. n. med. Piotr Grzelak, prof. instytutu .....
Mgr Grażyna Korybut .....
Dr n. med. Michał Krekora .....
Prof. dr hab. med. Jacek Rysz .....
Dr n. filozofii Wojciech Sztombka .....
Ks. dr hab. Jan Wolski .....
Dr hab. n. med. Marek Zadrożny, prof. instytutu .....
Prof. dr hab. n. med. Krzysztof Zeman .....

OŚWIADCZENIA WSPÓŁAUTORÓW PUBLIKACJI

Łódź, 02.02.2023

Mgr Bogusław Tymoniuk

Klinika Immunologii i Alergii

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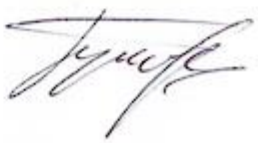
OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, iż wyrażam zgodę na wykorzystanie przez lek. Katarzynę Zawadzką-Starczewską w postępowaniu o nadanie tytułu doktora nauk medycznych publikacji „Actual Associations between HLA Haplotype and Graves' Disease Development" autorów: Katarzyna Zawadzka-Starczewska, Bogusław Tymoniuk, Bartłomiej Stasiak, Lewiński Andrzej, Magdalena Stasiak, opublikowanej w Journal of Clinical Medicine (2022 Apr 29;11(9):2492. doi: 10.3390/jcm11092492).



Łódź, 01.02.2023

Dr hab. inż. Bartłomiej Stasiak

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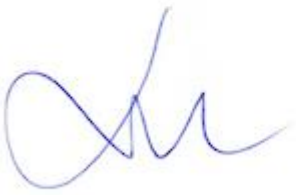

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Łódź, 02.02.2023

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CURRICULUM VITAE

Curriculum Vitae

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2015-2021	Specjalizacja w dziedzinie endokrynologii zakończona Państwowym Egzaminem Specjalizacyjnym w kwietniu 2021
Od 12.2022	Specjalizacja w dziedzinie chorób wewnętrznych

Języki obce: angielski (poziom B2), niemiecki (poziom podstawowy)