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MIKOŁAJA KOPERNIKA
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Wydział Lekarski
Collegium Medicum w Bydgoszczy

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Ocena stężenia witaminy D, melatoniny, biomarkerów stresu oksydacyjnego, stanu zapalnego i wykładników endokrynnej aktywności tkanki tłuszczowej u pacjentów z nowotworami głowy i szyi

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

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Serdecznie dziękuję

Pani Promotor, dr hab. n. med. Karolinie Szewczyk–Golec, prof. UMK,

za inspirację, wyrozumiałość oraz pomoc przy realizacji niniejszej pracy.

Słowa podziękowań kieruję do moich Najbliższych,

za nieustanne wsparcie, miłość i wiarę we mnie przez wszystkie te lata.

Pragnę również podziękować moim Współpracownikom,

którzy swoją wiedzą, doświadczeniem i życzliwością przyczynili się do powstania tej pracy.

Jarosław Nuszkiewicz

Gutta cavat lapidem non vi, sed saepe cadendo, sic homo doctus fit non vi, sed saepe studendo.

Spis treści

1. Wykaz stosowanych skrótów

AKT	Kinaza białkowa B	*Protein kinase B*
AMPK	Kinaza białkowa aktywowana 5' monofosforanem adenozyny	*5' adenosine monophosphate–activated protein kinase*
BMI	Indeks masy ciała	*Body mass index*
CAT	Katalaza	*Catalase*
EBV	Wirus Epsteina–Barr	*Epstein–Barr virus*
ELISA	Test immunoenzymatyczny	*Enzyme–linked immunosorbent assay*
ERK1/2	Kinazy regulowane sygnałem zewnątrzkomórkowym 1/2	*Extracellular signal–regulated kinases 1/2*
GIP	Glukozozależny peptyd insulinotropowy	*Glucose–dependent insulinotropic peptide*
GLP–1	Glukagonopodobny peptyd–1	*Glucagon–like peptide 1*
GLUT	Transporter glukozy	*Glucose transporter*
GPx	Peroksydaza glutationowa	*Glutathione peroxidase*
GSH	Glutation	*Glutathione*
GSSG	Disulfid glutationu	*Glutathione disulfide*
Hb	Hemoglobina	*Hemoglobin*
HNC	Nowotwór głowy i szyi	*Head and neck cancer*
HPV	Wirus brodawczaka ludzkiego	*Human papillomavirus*

iBMI	Grupa pacjentów o podwyższonym indeksie masy ciała	*Group of patients with increased body mass index*
ICD–11	Międzynarodowa Statystyczna Klasyfikacja Chorób i Problemów Zdrowotnych ICD–11	*International Classification of Diseases 11th Revision*
IGF–1	Insulinopodobny czynnik wzrostu	*Insulin–like growth factor 1*
IL–6	Interleukina 6	*Interleukin 6*
IQR (Q1; Q3)	Rozstęp międzykwartylowy (kwartyl dolny; kwartyl górny)	*Interquartile range (lower quartile, upper quartile)*
IU	Jednostka międzynarodowa aktywności enzymatycznej	*International unit of enzyme activity*
K_2EDTA	Sól disodowa kwasu etylenodiaminotetraoctowego	*Ethylenediaminetetraacetic acid disodium salt*
LSD1	Specyficzna dla lizyny demetylaza 1	*Lysine–specific histone demethylase 1*
MAPK	Kinazy aktywowane mitogenami	*Mitogen–activated protein kinases*
MDA	Malonylodialdehyd	*Malondialdehyde*
mTOR1	Ssaczy cel rapamycyny–1	*Mammalian target of rapamycin–1*
nBMI	Grupa pacjentów o prawidłowym indeksie masy ciała	*Group of patients with normal body mass index*

NFκB	Jądrowy czynnik transkrypcyjny kappa B	*Nuclear factor kappa–light–chain–enhancer of activated B cells*
OCG	Grupa starszych pacjentów	*Older cancer group*
PAI–1	Inhibitor aktywatora plazminogenu–1	*Plasminogen activator inhibitor–1*
PBS	Sól fizjologiczna buforowana fosforanami	*Phosphate–buffered saline*
PET/CT	Pozytonowa tomografia emisyjna połączona z wielorzędowym tomografem komputerowym	*Positron emission tomography/computed tomography*
PI3K	Kinaza 3–fosfatydyloinozytolu	*Phosphoinositide 3–kinases*
ROS	Reaktywne formy tlenu	*Reactive oxygen species*
SEM	Błąd standardowy średniej	*Standard error of the mean*
SIRT1	Cichy regulator informacji 1	*Silent information regulator 1*
SOD	Dysmutaza ponadtlenkowa	*Superoxide dismutase*
SOD–1	Zn/Cu dysmutaza ponadtlenkowa	*Zn/Cu Superoxide Dismutase*
TBA	Kwas tiobarbiturowy	*Thiobarbituric acid*
TBARS	Substancje reagujące z kwasem tiobarbiturowym	*Thiobarbituric acid reactive substances*
TCA	Kwas trichlorooctowy	*Trichloroacetic acid*
TNF–α	Czynnik martwicy nowotworów alfa	*Tumor necrosis factor alpha*

TNM	Klasyfikacja nowotworów złośliwych (akronim: Guz, Węzeł, Przerzut)	*Classification of Malignant Tumors (acronym: Tumour, Node, Metastasis)*
WHO	Światowa Organizacja Zdrowia	*World Health Organization*
YCG	Młodsza grupa pacjentów	*Younger cancer group*

2. Wykaz publikacji stanowiących rozprawę doktorską

Publikacja I:

Praca poglądowa

Autorzy: Nuszkiewicz Jarosław, Woźniak Alina, Szewczyk–Golec Karolina

Tytuł: *Ionizing radiation as a source of oxidative stress: the protective role of melatonin and vitamin D*

Czasopismo: International Journal of Molecular Sciences

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Autor korespondencyjny: Jarosław Nuszkiewicz, Karolina Szewczyk–Golec

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Autorzy: Nuszkiewicz Jarosław, Czuczejko Jolanta, Maruszak Marta, Pawłowska Marta, Woźniak Alina, Małkowski Bogdan, Szewczyk–Golec Karolina

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Czasopismo: International Journal of Molecular Sciences

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3. Wstęp

Nowotwory stanowią jedną z głównych globalnych przyczyn zgonów [1]. Określono, że w 2012 roku na całym świecie odnotowano 14,1 mln nowych zachorowań na nowotwory i 8,2 mln zgonów związanych z chorobami nowotworowymi [2]. Nowotwory głowy i szyi (HNCs, ang. *head and neck cancers*) są stosunkowo rzadką grupą chorób onkologicznych, ale stanowią istotny problem kliniczny i społeczny. Szacuje się, że rocznie na całym świecie HNCs diagnozuje się u ponad 500 000 osób [3]. Według najnowszych dostępnych danych, w 2020 roku w Europie odnotowano 151 000 nowych przypadków HNCs [4]. Szczególnie narażona na HNCs jest populacja południowoazjatycka, co może być związane z czynnikami społeczno–ekonomicznymi [5]. HNCs dotyczą różnych struktur zlokalizowanych w obszarze głowy i szyi, takich jak warga, jama ustna, język, dziąsła, gardło, elementy pierścienia gardłowego Waldeyera, krtań, zatoki przynosowe oraz ślinianki [6]. Etiologia HNCs jest złożona i obejmuje zarówno czynniki środowiskowe, jak i genetyczne, które mogą prowadzić do transformacji nowotworowej. Dotychczas zidentyfikowano kilka czynników ryzyka sprzyjających rozwojowi HNCs związanych ze stylem życia. Podrażnianie błon śluzowych jamy ustnej i gardła dymem papierosowym lub mocnym alkoholem, a także chroniczne, mechaniczne uszkodzenia tkanek poprzez źle dopasowane protezy lub uszkodzone zęby mogą prowadzić do kancerogenezy [7]. Zakażenie wirusem brodawczaka ludzkiego (HPV, ang. *human papillomavirus*) oraz wirusem Epsteina–Barra (EBV, ang. *Epstein–Barr virus*) mogą również przyczyniać się do rozwoju HNCs [8–10]. HNCs są dość jednorodne pod względem morfologii. Zdecydowana większość nowotworów tego typu obejmuje raki płaskonabłonkowe o różnym stopniu zróżnicowania, pochodzące z nabłonka błon śluzowych [8]. Symptomy HNCs zależą od pierwotnej lokalizacji guza. Częstymi objawami są ból i owrzodzenie dotkniętej tkanki, które wraz z rozwojem choroby mogą prowadzić do problemów z oddychaniem, połykaniem oraz mówieniem [11]. U niektórych pacjentów z HNC można zaobserwować powiększenie węzłów chłonnych na szyi [6]. W przypadku nowotworów głowy i szyi stosuje się klasyfikację zaawansowania klinicznego TNM (od angielskich słów: *tumour* – guz, *node* – węzeł, *metastasis* – przerzut), która określa stopień rozprzestrzeniania się nowotworu w organizmie za pomocą trzech cech [12]. Cecha T opisuje rozmiar guza, jego lokalizację oraz inwazję w obrębie prawidłowych tkanek. Cecha N odnosi się do rozmiaru przerzutu w węźle chłonnym oraz liczby zajętych węzłów chłonnych. Cecha M wskazuje na obecność przerzutów nowotworu w tkankach oddalonych od

pierwotnego ogniska, co pozwala określić stopień rozsiania choroby nowotworowej. Główne strategie terapeutyczne stosowane w leczeniu HNCs obejmują zabiegi operacyjne, radioterapię i w niektórych typach nowotworu chemioterapię, zazwyczaj łączone w terapii skojarzonej [13].

Stres oksydacyjny jest pojęciem odnoszącym się do zaburzenia równowagi między generowaniem reaktywnych form tlenu (ROS, ang. *reactive oxygen species*) a zdolnością systemów biologicznych do efektywnego ich neutralizowania lub naprawy powstałych uszkodzeń [14]. ROS są niestabilnymi, wysoce reaktywnymi cząsteczkami zawierającymi tlen, które mogą prowadzić do oksydacyjnego uszkodzenia biologicznych makromolekuł: lipidów, białek i DNA [15]. ROS obejmują zarówno wolne rodniki tlenowe, takie jak anionorodnik ponadtlenkowy ($O_2^{\bullet-}$) oraz rodnik wodorotlenowy ($OH^{\bullet}$), jak i związki tlenu, które nie są wolnymi rodnikami, na przykład nadtlenek wodoru (H_2O_2) [16]. Te ostatnie mają zdolność do przekształcania się w wolne rodniki tlenowe lub do indukowania ich tworzenia [16]. ROS powstają w wyniku procesów metabolicznych, głównie fosforylacji oksydacyjnej, a także na skutek ekspozycji na czynniki środowiskowe, takie jak promieniowanie jonizujące, zanieczyszczenie powietrza czy toksyny chemiczne [15]. W warunkach homeostazy, organizmy utrzymują równowagę między produkcją ROS a ich neutralizacją za pomocą enzymatycznych i nieenzymatycznych mechanizmów antyoksydacyjnych [17]. Do enzymów antyoksydacyjnych należą między innymi dysmutazy ponadtlenkowe (SODs, ang. *superoxide dismutases*; EC 1.15.1.1), katalaza (CAT, ang. *catalase*; EC 1.11.1.6) oraz peroksydazy glutationowe (GPxs, ang. *glutathione peroxidases*; EC 1.11.1.9), a do antyoksydantów nieenzymatycznych zalicza się witaminę A, C, E oraz glutation (GSH, ang. *glutathione*) i melatoninę [18]. W kontekście chorób nowotworowych, stres oksydacyjny może wpływać na inicjację, promocję oraz progresję choroby poprzez różne mechanizmy [19]. Mutacje w obrębie DNA, spowodowane oksydacyjną modyfikacją zasad azotowych, mogą prowadzić do aktywacji protoonkogenów, dezaktywacji genów supresorowych nowotworów oraz uszkodzenia systemów naprawy DNA [20]. Te zmiany genetyczne przyczyniają się do transformacji komórek, które uzyskują zdolność do niekontrolowanej proliferacji i unikania apoptozy – zjawisk charakterystycznych dla komórek nowotworowych [20]. Stres oksydacyjny może również wpływać na proces angiogenezy, czyli tworzenia nowych naczyń krwionośnych, które dostarczają składników odżywczych i tlenu do rosnącego guza [21]. Przewlekły stan zapalny, często towarzyszący stresowi oksydacyjnemu, może prowadzić do produkcji czynników angiogennych oraz wzrostu

naczyń krwionośnych, co z kolei sprzyja progresji choroby nowotworowej [21]. Warto zauważyć, że stres oksydacyjny może również wpływać na odpowiedź komórek nowotworowych na terapie przeciwnowotworowe, takie jak radioterapia czy chemioterapia [22,23]. Niektóre z tych terapii wykorzystują zjawisko stresu oksydacyjnego w celu wywołania uszkodzeń komórek rakowych, prowadząc do ich apoptozy [24].

Jednym z procesów będących następstwem nasilonego stresu oksydacyjnego jest peroksydacja lipidów [25]. Proces ten prowadzi do uszkodzenia błon komórkowych, zaburzeń funkcji komórek i może być jednym z czynników powodujących rozwój wielu schorzeń. W przebiegu tego procesu wyróżnia się trzy etapy: inicjację, propagację i terminację [26,27]. Pierwszym etapem peroksydacji lipidów jest inicjacja, podczas której ROS uszkadzają podwójne wiązania nienasyconych kwasów tłuszczowych tworząc rodnik lipidowy [28]. W kolejnym etapie, propagacji, rodnik lipidowy reaguje z cząsteczką tlenu tworząc nadtlenek lipidowy, który może z kolei reagować z innymi nienasyconymi kwasami tłuszczowymi, prowadząc do łańcuchowych reakcji utleniania [29]. Następnie, w procesie terminacji, rodnik lipidowy ulega dezaktywacji przez reakcję z innym rodnikiem lipidowym, białkiem lub antyoksydantem [30]. W wyniku peroksydacji lipidów powstają produkty końcowe – aldehydy, które mają zdolność do reagowania z białkami komórkowymi, lipidami oraz kwasami nukleinowymi [31]. Reakcje te prowadzą do modyfikacji tych cząsteczek i zaburzania ich funkcji. Jednym z głównych produktów peroksydacji lipidów jest organiczny związek chemiczny, malonylodialdehyd (MDA, ang. *malondialdehyde*) [32]. Powstaje on w wyniku degradacji nadtlenków lipidowych, które ulegają rozkładowi w trakcie peroksydacji nienasyconych kwasów tłuszczowych w błonach komórkowych [33]. MDA jest często wykorzystywany jako biomarker stresu oksydacyjnego i uszkodzeń spowodowanych przez ROS [34].

Melatonina (5–metoksy–N–acetylotryptamina) jest hormonem z grupy indoloamin, syntetyzowanym głównie przez szyszynkę zgodnie z rytmami okołodobowymi [35]. Stężenie melatoniny osiąga najwyższe wartości w godzinach nocnych, podczas gdy w ciągu dnia jest na niższym poziomie [36]. Hormon ten odgrywa kluczową rolę w regulacji cykli okołodobowych i sezonowych, wykazując jednocześnie szereg właściwości plejotropowych [37]. Melatonina jest efektywnym antyoksydantem, zarówno hydrofilowym, jak i lipofilowym [38]. Dzięki swoim właściwościom antyoksydacyjnym, związek ten może redukować stres oksydacyjny bezpośrednio neutralizując ROS, jak również pośrednio przez stymulowanie ekspresji genów

odpowiedzialnych za syntezę endogennych antyoksydantów enzymatycznych i nieenzymatycznych [39]. Melatonina ogranicza wzrost komórek nowotworowych poprzez hamowanie neoangiogenezy [40]. Ponadto zaobserwowano, że hormon ten wpływa na ekspresję genów związanych z kontrolą wzrostu komórek, prowadząc do zahamowania proliferacji komórek nowotworowych [41].

Witamina D pełni zasadniczą rolę w homeostazie wapnia i fosforu oraz kształtowaniu układu kostnego [42]. Aktywną postać witaminy D stanowi hormon steroidowy, kalcytriol (1,25–dihydroksycholekalcyferol) [43]. W ostatnich latach zainteresowanie badaczy skupiało się również na potencjalnym wpływie witaminy D na stres oksydacyjny [44]. Analogicznie do melatoniny, kalcytriol może zwiększać ekspresję oraz aktywność enzymów antyoksydacyjnych, takich jak SOD, CAT i GPx [45]. Ponadto stymuluje syntezę GSH, a działając immunomodulacyjnie ogranicza sekrecję cytokin prozapalnych [46,47]. Witamina D wpływa hamująco na wzrost i rozwój komórek nowotworowych oraz stymulująco na ich apoptozę [48]. Działanie to wynika z jej wpływu na ekspresję genów odpowiedzialnych za kontrolę cyklu komórkowego i różnicowanie komórek [49]. W ten sposób witamina D może hamować proliferację komórek nowotworowych i promować ich śmierć. Dodatkowo wykazano, że witamina D może wpływać na odpowiedź immunologiczną organizmu, stymulując układ odpornościowy do zwalczania komórek nowotworowych [50].

Osteopontyna jest glikofosfoproteiną, która jest szeroko rozpowszechniona w różnych tkankach i komórkach organizmu [51]. Pełni wiele istotnych funkcji, takich jak regulacja mineralizacji kości, modulacja odpowiedzi immunologicznej, stymulowanie gojenia się ran [51]. Ponadto, ma wpływ na angiogenezę oraz procesy adhezji, migracji i proliferacji komórek [51]. Modulacja odpowiedzi immunologicznej przez osteopontynę może wpływać na generowanie ROS oraz procesy zapalne, które nasilają stres oksydacyjny [52]. Osteopontyna może stymulować proliferację komórek nowotworowych, aktywując szereg szlaków sygnalizacyjnych odpowiedzialnych za regulację wzrostu komórek [53]. Działa także na adhezję komórek nowotworowych do macierzy pozakomórkowej, co jest kluczowe dla procesu inwazji i przerzutowania [54].

W ostatnich dekadach liczba przypadków otyłości na świecie gwałtownie wzrosła, stając się jednym z najpoważniejszych problemów zdrowotnych [55]. Otyłość definiowana jest jako nadmierne nagromadzenie tkanki tłuszczowej w organizmie, które przekracza możliwości

adaptacyjne ustroju [56]. Jednym ze sposobów diagnozowania otyłości jest wyliczenie indeksu masy ciała (BMI, ang. *body mass index*) [56]. Zgodnie z klasyfikacją Światowej Organizacji Zdrowia (WHO, ang. *World Health Organization*), wartość BMI ≥ 30 kg/m² pozwala na zdiagnozowanie otyłości, która może prowadzić do licznych schorzeń, w tym chorób nowotworowych [57]. Otyłość jest nierozerwalnie związana z nadmiernym generowaniem ROS [58]. Ponadto u osób otyłych obserwuje się niższy poziom antyoksydantów [59]. Nadmiar tkanki tłuszczowej, zwłaszcza tłuszczu trzewnego, prowadzi do przewlekłego stanu zapalnego o niskim nasileniu [60]. W wyniku tego procesu uwalniane są cytokiny prozapalne, które dodatkowo zwiększają poziom ROS i prowadzą do nasilenia stresu oksydacyjnego [61]. Adipocyty, komórki budujące białą tkankę tłuszczową, są źródłem nie tylko triacylogliceroli, ale także adipokin [62]. Adipokiny to hormony wydzielane głównie przez tkankę tłuszczową, które odgrywają istotną rolę w regulacji różnych procesów metabolicznych, takich jak metabolizm glukozy, wrażliwość na insulinę, apetyt i ilość przyjmowanych pokarmów lub odpowiedź immunologiczna [63]. W otyłości obserwuje się zaburzenia w równowadze między prozapalnymi a przeciwzapalnymi adipokinami. U osób otyłych dochodzi do zwiększonego wydzielania adipokin prozapalnych, m.in. leptyny, rezystyny, czynnika martwicy nowotworów alfa (TNF–α, ang. *tumor necrosis factor alpha*) czy interleukiny 6 (IL–6, ang. *interleukin 6*), które z kolei mogą prowadzić do przewlekłego stanu zapalnego, insulinooporności i zaburzeń w funkcjonowaniu naczyń krwionośnych [64]. Z drugiej strony, wydzielanie przeciwzapalnych adipokin, takich jak adiponektyna i omentyna–1, jest w otyłości obniżone, co dodatkowo wpływa negatywnie na homeostazę organizmu, uczestnicząc w patogenezie powikłań [65]. Jednym z głównych mechanizmów łączących otyłość z nowotworami jest przewlekły stan zapalny [66]. Ponadto, otyłość jest silnie związana z insulinoopornością i hiperinsulinemią, co może stymulować proliferację komórek nowotworowych poprzez aktywację szlaków sygnalizacyjnych: szlaku kinazy 3–fosfatydyloinozytolu (PI3K, ang. *phosphoinositide 3–kinase*) oraz szlaku kinazy białkowej aktywowanej przez mitogeny (MAPK, ang. *mitogen–activated protein kinase*) [67,68]. Insulina i insulinopodobny czynnik wzrostu (IGF–1, ang. *insulin–like growth factor 1*) mogą natomiast prowadzić do neoangiogenezy w przebiegu chorób nowotworowych [69].

4. Cele pracy

Cele główne:

1. Analiza ochronnego działania melatoniny i witaminy D przed uszkodzeniami oksydacyjnymi wywołanymi promieniowaniem jonizującym, mającym zastosowanie w diagnostyce i leczeniu nowotworów – na podstawie dostępnej literatury.
2. Ocena stężenia melatoniny i witaminy D, a także aktywności wybranych enzymów antyoksydacyjnych, stężenia markerów peroksydacji lipidów, wybranych adipokin, czynników regulujących homeostazę glukozy oraz wykładników stanu zapalnego u pacjentów z nowotworami głowy i szyi.
3. Analiza możliwych zależności między badanymi parametrami a wybranymi czynnikami antropometrycznymi u pacjentów z nowotworami głowy i szyi.

W tym celu zaplanowano:

1. Oznaczenie we krwi pacjentów z HNCs w różnych grupach wiekowych i w grupie osób zdrowych stężenia melatoniny i 25(OH)–witaminy D, aktywności enzymów antyoksydacyjnych: (SOD–1, CAT i GPx), a także stężenia markerów peroksydacji lipidów (stężenie MDA w erytrocytach i osoczu krwi) i osteopontyny jako czynnika związanego z kancerogenezą i regulacją poziomu witaminy D oraz przeprowadzenie analizy statystycznej pozwalającej na ocenę zależności między tymi parametrami.
2. Oznaczenie u pacjentów z HNCs i w grupie osób zdrowych stężenia wybranych adipokin oraz czynników regulujących metabolizm węglowodanów oraz przeprowadzenie analizy statystycznej pozwalającej na ocenę zależności między tymi parametrami w odniesieniu do wskaźnika masy ciała pacjentów.

5. Omówienie cyklu publikacji

5.1. Uczestnicy badania

Warunkiem zakwalifikowania pacjenta do badania było rozpoznanie pierwotnego, złośliwego nowotworu wargi, jamy ustnej lub gardła (zgodnie z Międzynarodową Klasyfikacją Chorób – 11. Rewizja [70] (ICD–11, ang. *International Classification of Diseases 11th Revision*)): 2B60–2B69, 2B6A–2B6D albo pierwotnego, złośliwego nowotworu krtani (zgodnie z ICD–11: 2C23) bądź raka *in situ* wargi, jamy ustnej lub gardła (zgodnie z ICD–11: 2E60.0). Kryteria wykluczenia obejmowały występowanie ostrych i przewlekłych chorób (zakaźnych, autoimmunologicznych, genetycznych i zapalnych) innych niż HNC i otyłość. Z grupy badanej wykluczono również pacjentów z rozsianą chorobą nowotworową. Ocena została przeprowadzona na podstawie wywiadu lekarskiego z pacjentem oraz analizy jego dokumentacji medycznej. Wszyscy pacjenci byli leczeni w Centrum Onkologii im. prof. Franciszka Łukaszczyka w Bydgoszczy. Uczestnicy zostali włączeni do badania w momencie skierowania na planowanie radioterapii za pomocą pozytonowej tomografii emisyjnej połączonej z wielorzędowym tomografem komputerowym (PET/CT, ang. *positron emission tomography/computed tomography*). Pacjentów poddano również badaniu histopatologicznemu. Analiza histopatologiczna wskazała, że w grupie badanej znajdowali się pacjenci z rakiem płaskonabłonkowym G1, rakiem płaskonabłonkowym G2, nierogowaciejącym rakiem płaskonabłonkowym G2 lub rogowaciejącym rakiem płaskonabłonkowym G2.

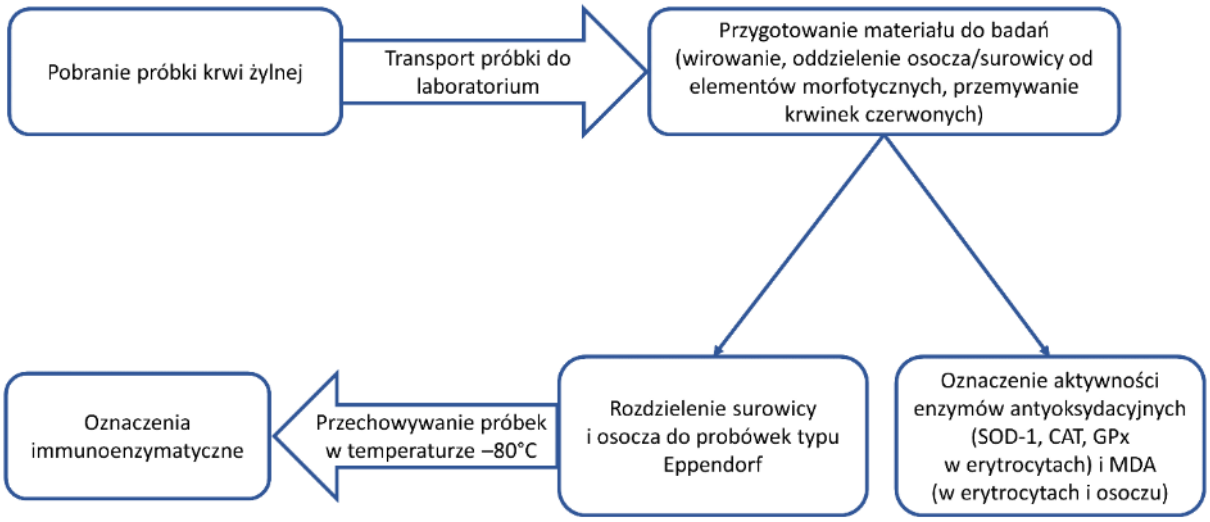
Grupę kontrolną stanowiły osoby zdrowe o parametrach antropometrycznych zbliżonych do pacjentów z nowotworami głowy i szyi. Kryteria wykluczenia z grupy kontrolnej obejmowały choroby przewlekłe lub ostre, takie jak nowotwór, cukrzyca, otyłość, choroby autoimmunologiczne i zaburzenia kardiometaboliczne.

Badanie zostało przeprowadzone po uzyskaniu akceptacji Komisji Bioetycznej Uniwersytetu Mikołaja Kopernika w Toruniu przy Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy (zgoda numer KB 221/2018). Wyrażenie zgody na udział w badaniu było dobrowolne i nie miało wpływu na przebieg leczenia.

5.2. Schemat badania

Materiał badany stanowiła krew żylna pobierana od pacjentów w godzinach 7:00–9:00 z żyły łokciowej pośrodkowej przez wykwalifikowany personel medyczny z Zakładu Medycyny Nuklearnej Centrum Onkologii im. prof. Franciszka Łukaszczyka w Bydgoszczy. Od każdego pacjenta pobierano krew do dwóch probówek polipropylenowych. Pierwsza probówka (Greiner Bio–One GmbH, Kremsmünster, Austria) o objętości 6 mL zawierała aktywator krzepnięcia w celu uzyskania surowicy krwi oraz żelowy separator. Druga probówka (Greiner Bio–One GmbH, Kremsmünster, Austria) o objętości 10 mL zawierała sól disodową kwasu etylenodiaminotetraoctowego (K_2EDTA), co umożliwiło uzyskanie osocza krwi. Próbki były niezwłocznie transportowane w warunkach obniżonej temperatury do laboratorium Katedry Biologii i Biochemii Medycznej, Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy, UMK w Toruniu. W laboratorium przeprowadzano wirowanie (6000 x g przez 10 min w 4°C) w celu oddzielenia surowicy krwi od elementów morfotycznych. Po odwirowaniu surowicę i osocze dzielono na porcje do probówek typu Eppendorf. Próbki przechowywano w temperaturze –80°C do dalszej analizy biochemicznej. Elementy morfotyczne krwi pozostałe po odwirowaniu przemywano trzykrotnie solą fizjologiczną buforowaną fosforanami (PBS, ang. *phosphate–buffered saline*) w stosunku 1:3 i każdorazowo odwirowywano (6000 x g przez 10 min w 4°C) w celu usunięcia leukocytów i trombocytów. Otrzymane tą metodą krwinki czerwone mieszano z roztworem PBS w celu uzyskania zawiesiny erytrocytów o indeksie hematokrytu 50%. Na Rycinie 1 przedstawiono schemat badania.

Wśród uczestników badania przeprowadzono badanie ankietowe. Pytania zawarte w kwestionariuszu dotyczyły uzależnień i innych czynników predysponujących do wystąpienia HNC oraz czynników mogących wpłynąć na uzyskane wyniki. Wzór kwestionariusza dla uczestnika badania przedstawiony został w załączniku do niniejszej rozprawy doktorskiej.



Rycina 1. Schemat przedstawiający poszczególne etapy badania. Legenda: CAT – katalaza; GPx – peroksydaza glutationowa; MDA – malonylodialdehyd; SOD–1 – Zn/Cu dysmutaza ponadtlenkowa.

5.3. Badania biochemiczne

Aktywność wybranych enzymów antyoksydacyjnych oznaczano w zawiesinie erytrocytów metodami kinetycznych pomiarów spektrofotometrycznych na aparaturze Varian Cary 100 UV–Vis (Varian Medical Systems Inc., Palo Alto, California, USA). Stężenie MDA oznaczano w osoczu krwi żylnej i zawiesinie krwinek czerwonych. Pomiary spektrofotometryczne wykonywano na aparaturze Cary 60 UV–Vis (Agilent Technologies Inc., Santa Clara, California, USA).

Aktywność Zn/Cu dysmutazy ponadtlenkowej (SOD–1; EC 1.15.1.1) oznaczano metodą Misra i Fridovicha [71]. Analiza opierała się na zahamowaniu utleniania adrenaliny do adrenochromu w zasadowym roztworze w temperaturze 37°C, co prowadziło do zmiany absorbancji przy długości fali 480 nm. Aktywność SOD–1 wyrażono w IU/g Hb.

Aktywność CAT (EC 1.11.1.6) wyznaczano metodą Beersa i Sizera [72], mierząc spadek absorbancji przy długości fali wynoszącej 240 nm. Reakcja enzymatyczna prowadzi do rozkładu nadtlenku wodoru na wodę i tlen, co powoduje zmianę absorbancji. Cały proces przebiega w kontrolowanej temperaturze wynoszącej 37°C. Aktywność CAT wyrażono w IU/g Hb.

Aktywność GPx (EC 1.11.1.9) oceniano stosując metodę opracowaną przez Paglia i Valentine'a [73]. W trakcie reakcji enzymatycznej GPx redukuje nadtlenek wodoru do wody, jednocześnie utleniając GSH do disulfidu glutationu (GSSG, ang. *glutathione disulfide*). GSSG jest substratem dla GPx, która redukuje go do GSH z jednoczesnym utlenieniem NADPH do $NADP^+$. Zmiana utlenienia koenzymu prowadzi do zmiany absorbancji światła przy długości fali 340 nm. Reakcja przebiega w temperaturze wynoszącej 37°C. Aktywność GPx wyrażono w IU/g Hb.

Stężenie MDA w erytrocytach i w osoczu krwi oznaczano metodą Buege'a i Austa [74] w modyfikacji Esterbauera i Cheesemana [75]. W tej metodzie próbki biologiczne (osocze i erytrocyty) inkubowane są w roztworze kwasu trichlorooctowego (TCA, ang. *trichloroacetic acid*), który działa jako środek denaturujący białka i stabilizujący MDA. Inkubacja przebiega w temperaturze pokojowej. Następnie, próbka jest poddawana reakcji z roztworem kwasu tiobarbiturowego (TBA, ang. *thiobarbituric acid*) i inkubowana w temperaturze 95°C przez 20 minut, co prowadzi do powstania kompleksu TBA–MDA. Stężenie MDA wyrażono jako stężenie substancji reagujących z kwasem tiobarbiturowym (TBARS, ang. *thiobarbituric acid reactive*

substances) i mierzono przy długości fali wynoszącej 532 nm w temperaturze pokojowej. Stężenie MDA w erytrocytach wyrażono w nmol/g Hb, a w osoczu krwi w nmol/mL.

Stężenie Hb oceniano metodą Drabkina [76] polegającą na przekształceniu hemoglobiny w cyjanometahemoglobinę za pomocą roztworu Drabkina. Po inkubacji próbki przez 20 minut w temperaturze pokojowej, mierzono absorbancję przy długości fali wynoszącej 540 nm. Na podstawie pomiarów absorbancji obliczono stężenie hemoglobiny w próbce krwi.

Stężenie melatoniny, 25(OH)–witaminy D, osteopontyny, omentyny–1, adipsyny, adiponektyny, peptydu C, greliny, glukozozależnego peptydu insulinotropowego (GIP, ang. *glucose–dependent insulinotropic peptide*), glukagonopodobnego peptydu–1 (GLP–1, ang. *glucagon–like peptide 1*), glukagonu, insuliny, leptyny, inhibitora aktywatora plazminogenu–1 (PAI–1, ang. *plasminogen activator inhibitor–1*), rezystyny oraz wisfatyny zostało oznaczone w surowicy krwi.

Pomiar stężenia melatoniny, 25(OH)–witaminy D, osteopontyny i omentyny–1 wykonany został za pomocą komercyjnie dostępnych zestawów analitycznych z zastosowaniem technik immunoenzymatycznych (ELISA, ang. *enzyme–linked immunosorbent assay*). Zastosowane testy obejmowały: zestaw do testu immunoenzymatycznego typu ELISA dla melatoniny (Cloud–Clone Corp., Houston, TX, USA), zestaw do testu immunoenzymatycznego typu ELISA w wersji kompetycyjnej dla 25(OH)–witaminy D (Immundiagnostik AG, Bensheim, Niemcy), zestaw do testu immunoenzymatycznego typu ELISA w wersji sandwich dla ludzkiej osteopontyny (BioVendor, Brno, Czechy) oraz zestaw do testu immunoenzymatycznego typu ELISA dla ludzkiej omentyny–1 (BioVendor, Brno, Czechy). Wszystkie analizy przeprowadzono zgodnie z instrukcjami producentów zestawów doświadczalnych. Zestawy te zawierały niezbędne odczynniki do analizy. Gęstość optyczna dla każdego zestawu badawczego była testowana za pomocą czytnika mikropłytek BMG Labtech CLARIOstar Multimode Microplate Reader (BMG LABTECH GmbH, Ortenberg, Niemcy). Stężenie melatoniny, 25(OH)–witaminy D, osteopontyny i omentyny–1 wyrażono odpowiednio w pg/mL, ng/mL, nmol/L i ng/mL.

Stężenie adipsyny i adiponektyny oznaczono z zastosowaniem komercyjnie dostępnego zestawu Bio–Plex Pro human diabetes adipsin and adiponectin immunoassays (Bio–Rad Laboratories Inc., Hercules, CA, USA), natomiast poziom peptydu C, greliny, GIP, GLP–1,

glukagonu, insuliny, leptyny, PAI–1, rezystyny oraz wisfatyny oznaczono za pomocą komercyjnie dostępnego zestawu Bio–Plex Pro human diabetes 10–plex immunoassay (Bio–Rad Laboratories Inc., Hercules, CA, USA). Wszystkie analizy wykonano zgodnie z wytycznymi producenta, a zestawy zawierały wymagane odczynniki do przeprowadzenia analizy. Metoda Bio–Plex Multiplex Immunoassay to technologia pozwalająca na jednoczesne badanie stężenia do 100 różnych białek, cytokin, chemokin oraz innych biomarkerów w jednej próbce [77]. Metoda ta oparta jest na technologii Luminex xMAP, która łączy wykorzystanie barwnych mikrokulek z detekcją przez cytometrię przepływową. W reakcji uczestniczą przeciwciała detekcyjne oraz przeciwciała sprzęgnięte z fluorochromem. Zestawy badawcze firmy Bio–Rad Laboratories Inc. wykorzystują pomiar fluorescencji do określenia poziomu poszczególnych analitów. Fluorescencję mierzono z zastosowaniem systemu Bio–Plex 200 (Bio–Rad Laboratories Inc., Hercules, CA, USA). Otrzymane wyniki wyrażono w pg/mL, ng/mL lub µg/mL w zależności od analitu.

5.4. Metody statystyczne

Analiza statystyczna została przeprowadzona za pomocą oprogramowania Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). W badaniu zastosowano test Shapiro–Wilka w celu oceny hipotezy dotyczącej rozkładu normalnego, test Levene'a do określenia jednorodności wariancji oraz współczynnik korelacji Pearsona do oceny związku pomiędzy badanymi parametrami. W przypadku analiz spełniających warunki rozkładu normalnego, użyto testu t–Studenta dla prób niezależnych, a wyniki przedstawiono jako średnią ± błąd standardowy średniej (SEM, ang. *standard error of the mean*). Gdy wyniki nie spełniały założenia o rozkładzie normalnym, zastosowano nieparametryczny test U Manna–Whitneya, a dane przedstawiono jako medianę oraz rozstęp międzykwartylowy (kwartyl dolny; kwartyl górny) (IQR–Q1; Q3). Różnice na poziomie $p < 0,05$ uznano za istotne statystycznie.

5.5. Publikacja I – artykuł poglądowy

Celem tego artykułu poglądowego było przeanalizowanie aktualnych danych naukowych na temat ochronnego działania melatoniny i witaminy D przed uszkodzeniami oksydacyjnymi wywołanymi promieniowaniem jonizującym. Omówiono obecny stan wiedzy, w tym możliwe molekularne mechanizmy działania. Promieniowanie jonizujące wykorzystywane jest zarówno w diagnostyce, jak i w terapii HNC. Radioterapia, obok leczenia chirurgicznego, stanowi główną formę terapii HNC.

W Publikacji I zwrócono uwagę na istotną rolę promieniowania jonizującego jako induktora stresu oksydacyjnego, który występuje w patogenezie i przebiegu wielu chorób. ROS powstają nie tylko podczas zabiegów medycznych wymagających użycia promieniowania jonizującego, ale również wtedy, gdy organizm jest narażony na światło słoneczne i promieniowanie tła obecne w środowisku naturalnym. Opisana w pracy endogenna synteza dwóch związków o potencjale antyoksydacyjnym, melatoniny i witaminy D, zależy od obecności światła (widzialnego lub z zakresu ultrafioletu). Liczne badania podkreślają rolę melatoniny jako przeciwutleniacza i jej ochronne działanie przed uszkodzeniami spowodowanymi promieniowaniem jonizującym. Hormon ten, zarówno bezpośrednio, jak i pośrednio neutralizuje ROS. W przypadku witaminy D wymagane są dalsze badania, które mogłyby wyjaśnić mechanizmy jej antyoksydacyjnego działania i sposób ochrony przed promieniowaniem jonizującym. W wyniku szerokiego wykorzystania promieniowania jonizującego w medycynie, coraz więcej osób narażonych jest na jego oddziaływanie w różnych dawkach. Szczególnie duże narażenie na promieniowanie jonizujące występuje podczas radioterapii, metody często stosowanej w leczeniu chorób nowotworowych. Z tego względu terapie wspomagające dla pacjentów, jak również dla personelu medycznego mają pierwszorzędne znaczenie. Syntetyczne związki radioochronne mają ograniczone zastosowanie, ponieważ często wywołują niepożądane skutki uboczne, zwłaszcza w dawkach wymaganych do osiągnięcia maksymalnej ochrony radiologicznej. Według danych przedstawionych w artykule, melatonina może być najlepszym kandydatem jako środek radioochronny u ludzi. Mniej wiadomo o radioprotekcyjnej roli witaminy D. Dotychczasowe wyniki są jednak obiecujące. Suplementacja obiema substancjami wydaje się również istotna w kontekście powszechnych we współczesnym społeczeństwie niedoborów melatoniny i witaminy D, które mogą przyczyniać się do nasilenia niepożądanych skutków ubocznych

medycznej ekspozycji na promieniowanie jonizujące. Ponadto stwierdzono, że obie substancje selektywnie uwrażliwiają komórki nowotworowe na promieniowanie, co czyni je obiecującymi adiuwantami do wzmacniania przeciwnowotworowego efektu radioterapii i poprawy wyników terapeutycznych. Dlatego w świetle istniejących badań melatonina i witamina D są warte rozważenia jako środki chroniące profesjonalistów narażonych na promieniowanie oraz pacjentów diagnozowanych lub leczonych promieniowaniem. Niemniej jednak potrzebne są dalsze badania w tej dziedzinie, zwłaszcza u ludzi. Odpowiednio zaplanowane badania kliniczne pozwolą na ustalenie odpowiednich dawek melatoniny i witaminy D, skutecznych w ochronie przed promieniowaniem jonizującym i bezpiecznych dla ludzi.

5.6. Publikacja II – wyniki

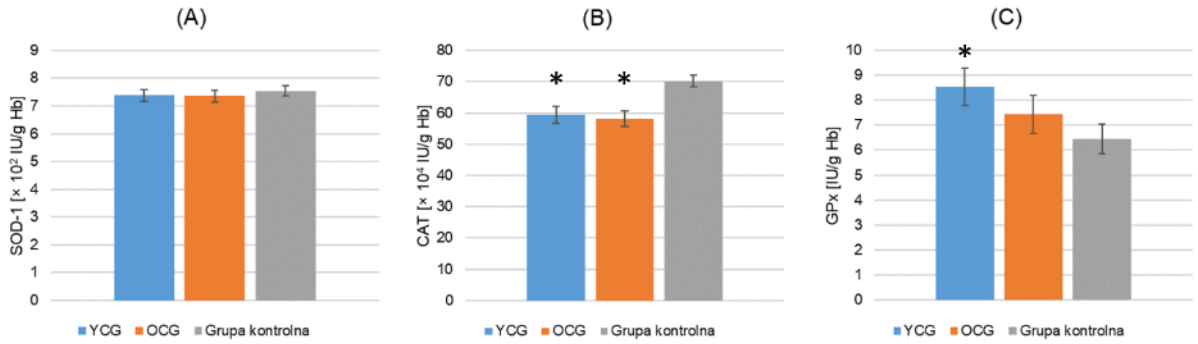
W badaniu wzięło udział 45 pacjentów z rozpoznanym HNC oraz 25 osób zdrowych. Pacjenci z HNC podzieleni zostali na dwie podgrupy ze względu na wiek: młodsza grupa z rakiem (YCG, ang. *younger cancer group*) i starsza grupa z rakiem (OCG, ang. *older cancer group*). Grupę YCG stanowiło 25 pacjentów w wieku 58,24 ± 1,29 lat, natomiast OCG – 20 pacjentów w wieku 69,70 ± 1,49 lat. Średni wiek w grupie kontrolnej wynosił 55,36 ± 1,17 lat. Szczegółowa charakterystyka antropometryczna uczestników tego badania przedstawiona została w Tabeli 1.

Tabela 1. Charakterystyka antropometryczna i kliniczna pacjentów z nowotworem głowy i szyi oraz zdrowych osób (grupa kontrolna) zakwalifikowanych do badania opisanego w Publikacji II.

Parametr	YCG	OCG	Grupa kontrolna
***n* (mężczyźni/kobiety)**	25 (15/10)	20 (14/6)	25 (11/14)
Wiek [lat]	58,24 ± 1,29 *	69,70 ± 1,49	55,36 ± 1,17 *
Masa ciała [kg]	72,53 ± 3,95	71,39 ± 3,52	71,02 ± 2,22
Wzrost [cm]	168,92 ± 1,84	168,55 ± 1,52	169,88 ± 1,72
BMI [kg/m^2]	25,09 ± 0,99	25,00 ± 1,02	24,50 ± 0,47
Suplementacja witaminy D (tak/nie)	5/20	4/16	8/17
Osoby palące tytoń (tak/nie)	7/18	5/15	4/21

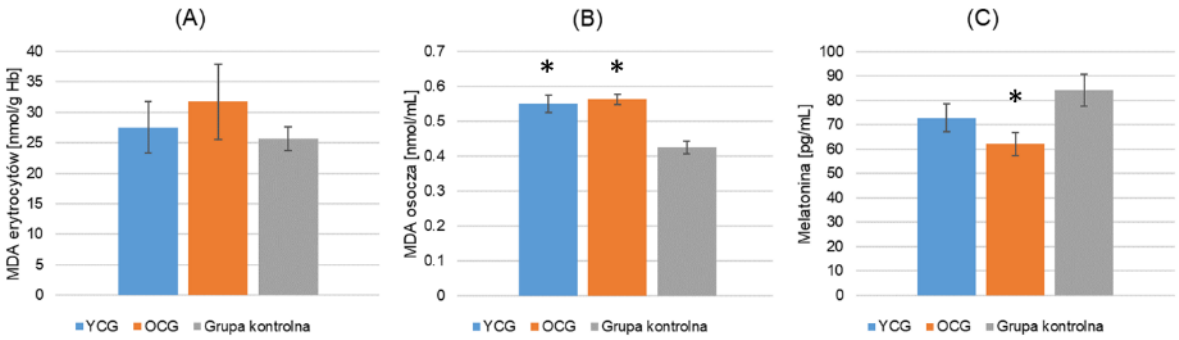
Wyniki przedstawiono jako średnia arytmetyczna ± SEM. Legenda: BMI – indeks masy ciała; OCG – starsza grupa z rakiem; SEM – błąd standardowy średniej; YCG – młodsza grupa z rakiem. * $p < 0,05$ w porównaniu z grupą OCG.

Aktywność SOD–1 była podobna we wszystkich grupach i wynosiła 738 ± 21 IU/g Hb w YCG, 735 ± 19 IU/g Hb w OCG i 755 ± 18 IU/g Hb w grupie kontrolnej. Statystycznie niższą aktywność CAT zaobserwowano w grupach YCG (59,34 ± 2,68 × 10^4 IU/g Hb) i OCG (58,11 ± 2,47 × 10^4 IU/g Hb) w porównaniu z grupą kontrolną (70,19 ± 1,87 × 10^4 IU/g Hb). Aktywność GPx w YCG wyniosła 8,54 ± 0,75 IU/g Hb i była istotnie wyższa w porównaniu z grupą kontrolną (6,45 ± 0,58 IU/g Hb). W OCG średnia aktywność GPx wynosiła 7,44 ± 0,76 IU/g Hb. Wyniki dotyczące aktywności enzymów antyoksydacyjnych przedstawiono na Rycinie 2.



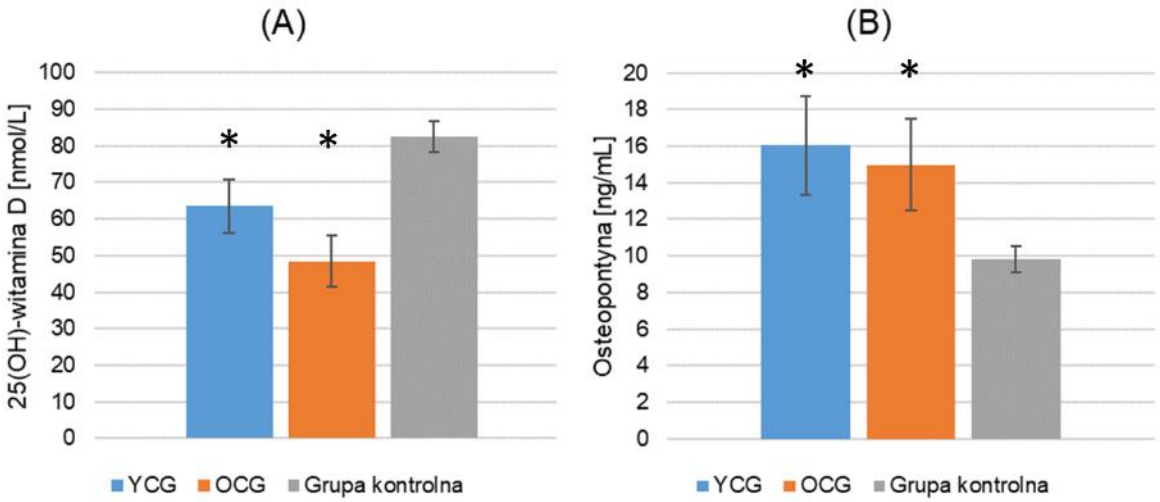
Rycina 2. Aktywność enzymów antyoksydacyjnych w erytrocytach pacjentów z rakiem wargi, jamy ustnej lub gardła w zależności od wieku i w grupie kontrolnej. Legenda: (A) – aktywność dysmutazy ponadtlenkowej Zn/Cu (SOD–1); (B) – aktywność katalazy (CAT); (C) – aktywność peroksydazy glutationowej (GPx); OCG – starsza grupa z rakiem; YCG – młodsza grupa z rakiem. Dane przedstawiono jako średnie ± SEM (błąd standardowy średniej). * $p < 0,05$, w porównaniu z grupą kontrolną.

Nie stwierdzono istotnych statystycznie różnic w stężeniu MDA w erytrocytach w YCG (27,52 ± 4,23 nmol/g Hb), OCG (31,74 ± 6,21 nmol/g Hb) i grupie kontrolnej (25,66 ± 1,98 nmol/g Hb). Istotnie wyższe stężenie MDA obserwowano w osoczu pacjentów z YCG i OCG, wynoszące odpowiednio 0,55 ± 0,02 i 0,56 ± 0,01 nmol/mL. W grupie kontrolnej stężenie MDA w osoczu wynosiło 0,42 ± 0,02 nmol/mL. Stężenie melatoniny w OCG wynosiło 62,12 ± 4,70 pg/mL i było istotnie niższe niż w grupie kontrolnej (84,33 ± 6,54 pg/mL). Stężenie melatoniny w YCG wynosiło 72,83 ± 5,80 pg/mL i nie zaobserwowano istotnych statystycznie różnic w stosunku do pozostałych grup badanych. Rycina 3 przedstawia wykresy stężenia MDA krwinek czerwonych, MDA osocza i melatoniny dla poszczególnych grup.



Rycina 3. Stężenie dialdehydu malonowego (MDA) i melatoniny u pacjentów z rakiem wargi, jamy ustnej lub gardła w zależności od wieku i w grupie kontrolnej. Legenda: (A) – stężenie MDA w erytrocytach; (B) – stężenie MDA w osoczu; (C) – stężenie melatoniny; OCG – starsza grupa z rakiem; YCG – młodsza grupa z rakiem. Dane przedstawiono jako średnie ± SEM (błąd standardowy średniej). * $p < 0,05$, w porównaniu z grupą kontrolną.

Stężenie 25(OH)–witaminy D w surowicy osób zdrowych wynosiło 82,57 ± 4,28 ng/mL. Znacznie niższe wartości obserwowano w YCG i OCG, wynoszące odpowiednio 63,55 ± 7,36 ng/mL i 48,42 ± 7,09 ng/mL. Poziom osteopontyny w grupach pacjentówz HNC był istotnie wyższy w porównaniu z grupą zdrową. Stężenie osteopontyny w YCG i OCG wynosiło odpowiednio 16,04 ± 2,69 nmol/L i 14,98 ± 2,48 nmol/L, podczas gdy w grupie kontrolnej 9,78 ± 0,72 nmol/L. Rycina 4 przedstawia wykresy stężenia 25(OH)–witaminy D i osteopontyny w badanych grupach.



Rycina 4. Stężenie 25(OH)–witaminy D i osteopontyny w surowicy krwi pacjentów z rakiem wargi, jamy ustnej lub gardła w zależności od wieku i w grupie kontrolnej. Legenda: (A) – stężenie 25(OH)–witaminy D; (B) – stężenie osteopontyny; OCG – starsza grupa z rakiem; YCG – młodsza grupa z rakiem. Dane przedstawiono jako średnie ± SEM (błąd standardowy średniej). * $p < 0,05$, w porównaniu z grupą kontrolną.

Uzyskane dane zostały również przeanalizowane pod kątem występowania korelacji. W YCG zaobserwowano istotne statystycznie ujemne korelacje między GPx a masą ciała (r = –0,51, p = 0,009), GPx a BMI (r = –0,52, p = 0,007) oraz CAT a witaminą D (r = –0,42, p = 0,036). Ponadto stwierdzono istotną statystycznie dodatnią korelację między MDA w osoczu a osteopontyną (r = 0,53, p = 0,007). W grupie kontrolnej zaobserwowano dodatnią korelację między SOD–1 a MDA erytrocytów (r = 0,44, p = 0,026) oraz ujemną korelację między masą ciała a MDA erytrocytów (r = –0,39, p = 0,049). W grupie OCG nie stwierdzono statystycznie istotnych korelacji.

5.7. Publikacja III – wyniki

W badaniu uczestniczyło 46 pacjentów ze zdiagnozowanym HNC oraz 23 zdrowe osoby, które tworzyły grupę kontrolną. Uczestnicy badania zostali podzieleni na dwie grupy po 23 pacjentów na podstawie BMI. Pacjenci z BMI < 25 kg/m^2 zostali przydzieleni do grupy o prawidłowym BMI (nBMI, ang. *group of patients with normal body mass index*). Grupa o podwyższonym BMI (iBMI, ang. *group of patients with increased body mass index*) składała się z pacjentów z HNC z BMI ≥ 25 kg/m^2. Klasyfikacji pacjentów do grup zależnych od BMI dokonano na podstawie rekomendacji WHO [78]. Wyniki analizy antropometrycznej oraz charakterystykę kliniczną uczestników badania przedstawiono w Tabeli 2.

Tabela 2. Charakterystyka antropometryczna i kliniczna pacjentów z rakiem głowy i szyi (grupy HNC) oraz zdrowych ochotników (grupa kontrolna).

Parametr	Nowotwory głowy i szyi				Grupa kontrolna		*p* nBMI *vs.* iBMI	*p* nBMI *vs.* grupa kontrolna
	nBMI		iBMI					
	Średnia	SEM	Średnia	SEM	Średnia	SEM		
***n* (mężczyźni/kobiety)**	23 (13/10)	–	23 (14/9)	–	23 (12/11)	–	0,770785	0,773350
Wiek [lat]	64,130	1,464	64,696	2,073	62,217	1,478	0,824801	0,362876
Masa ciała [kg]	61,957	1,787	90,957	2,556	62,652	1,524	< 0,000001	0,768436
Wzrost [cm]	169,130	1,464	170,957	1,172	168,304	1,202	0,335512	0,664882
BMI [kg/m^2]	21,601	0,445	31,089	0,755	22,066	0,358	< 0,000001	0,420516
Osoby palące tytoń (tak/nie)	11/12	–	12/11	–	5/18	–	0,774186	0,065521

Wyniki przedstawiono jako średnia arytmetyczna ± SEM. Legenda: BMI – indeks masy ciała; nBMI – grupa pacjentów o prawidłowym BMI; iBMI – grupa pacjentów o podwyższonym BMI, SEM – błąd standardowy średniej. $p < 0,05$ uznano za istotne statystycznie.

U pacjentów z grupy nBMI zaobserwowano statystycznie wyższe stężenie adiponektyny, omentyny–1 i greliny w surowicy krwi w porównaniu z uczestnikami z grupy iBMI. Osoby z grupy iBMI charakteryzowały się istotnie wyższymi stężeniami insuliny, leptyny, peptydu C, GLP–1, PAI–1, rezystyny i wisfatyny niż osoby z grupy nBMI. Nie stwierdzono istotnych różnic między grupami nBMI i iBMI w przypadku adipsyny, GIP i glukagonu. Wyniki analiz biochemicznych przeprowadzonych u pacjentów z HNC z grupy nBMI oraz iBMI przedstawiono w Tabeli 3 i w Tabeli 4.

Tabela 3. Parametry biochemiczne o statystycznie normalnym rozkładzie analizowane u pacjentów z rakiem głowy i szyi w odniesieniu do wskaźnika masy ciała (BMI).

Parametr	**Nowotwory głowy i szyi**				**Wartość *p***
	nBMI		**iBMI**		
	Średnia	**SEM**	**Średnia**	**SEM**	
Adiponektyna [µg/mL]	58,947	3,581	36,182	2,429	0,000004
Leptyna [pg/mL]	1941,866	129,804	4030,817	273,135	< 0,000001
Insulina [pg/mL]	334,688	25,264	684,051	42,573	< 0,000001

Legenda: BMI – indeks masy ciała; nBMI – grupa pacjentów o prawidłowym BMI; iBMI – grupa pacjentów o podwyższonym BMI; SEM – błąd standardowy średniej. $p < 0,05$ uznano za istotne statystycznie.

Tabela 4. Parametry biochemiczne o statystycznie nieparametrycznym rozkładzie analizowane u pacjentów z rakiem głowy i szyi w odniesieniu do wskaźnika masy ciała (BMI).

Parametr	Nowotwory głowy i szyi				Wartość *p*
	nBMI		iBMI		
	Mediana	IQR (Q1; Q3)	Mediana	IQR (Q1; Q3)	
Grelina [pg/mL]	282,341	229,697; 377,075	169,900	141,490; 196,700	0,000036
Omentyna–1 [ng/mL]	701,330	660,220; 779,030	456,000	399,300; 688,960	0,000048
Adipsyna [ng/mL]	677,935	475,431; 916,300	686,080	634,510; 815,832	0,775185
Rezystyna [pg/mL]	6846,331	5265,468; 7981,305	9089,872	7939,536; 12098,560	0,000033
Wisfatyna [pg/mL]	1394,150	1171,907; 1647,002	1939,650	1692,205; 2298,355	0,000007
Glukagon [pg/mL]	1557,810	1452,890; 1639,300	1643,660	1398,320; 1891,490	0,783612
Peptyd C [pg/mL]	611,900	512,850; 706,740	700,550	603,880; 834,100	0,019874
GLP–1 [pg/mL]	257,500	213,360; 277,600	297,200	277,200; 308,020	0,011522
PAI–1 [pg/mL]	3544,930	3122,099; 3956,693	4476,310	3924,160; 4979,300	0,004597
GIP [pg/mL]	315,300	290,650; 400,900	333,400	299,800; 388,600	0,660384

Legenda: BMI – indeks masy ciała; nBMI – grupa pacjentów o prawidłowym BMI; GIP – glukozozależny peptyd insulinotropowy; GLP–1 – glukagonopodobny peptyd–1; iBMI – grupa pacjentów o podwyższonym BMI; IQR (Q1; Q3) – rozstęp międzykwartylowy (kwartyl dolny; kwartyl górny); PAI–1 – inhibitor aktywatora plazminogenu typu 1. $p < 0,05$ uznano za istotne statystycznie.

W grupie nBMI zaobserwowano statystycznie istotnie wyższy poziom adipsyny, wisfatyny, glukagonu i PAI–1 w porównaniu z grupą kontrolną. Ponadto odnotowano statystycznie niższy poziom greliny w grupie nBMI w porównaniu z grupą kontrolną. Analiza wyników nie wykazała różnic w stężeniu omentyny–1, GIP, adiponektyny, peptydu C, GLP–1, insuliny, leptyny i rezystyny pomiędzy grupami nBMI i kontrolną. W Tabeli 5 i Tabeli 6 przedstawiono wyniki analizy laboratoryjnej dla pacjentów z nBMI i grupy kontrolnej.

Tabela 5. Parametry biochemiczne o statystycznie normalnym rozkładzie analizowane u pacjentów z nowotworem głowy i szyi (HNC) z prawidłowymi wartościami wskaźnika masy ciała (nBMI) oraz w grupie kontrolnej.

Parametr	HNC nBMI		Grupa kontrolna		Wartość *p*
	Średnia	SEM	Średnia	SEM	
Omentyna–1 [ng/mL]	727,701	18,184	721,247	20,433	0,814575
Adipsyna [ng/mL]	680,076	49,457	443,729	20,163	0,000063
Wisfatyna [pg/mL]	1412,926	69,342	1241,184	29,803	0,027802
GIP [pg/mL]	339,807	22,837	290,509	12,393	0,064363

Legenda: GIP – glukozozależny peptyd insulinotropowy; SEM – błąd standardowy średniej. $p < 0,05$ uznano za istotne statystycznie.

Tabela 6. Parametry biochemiczne o statystycznie nieparametrycznym rozkładzie analizowane u pacjentów z nowotworem głowy i szyi (HNC) z prawidłowymi wartościami wskaźnika masy ciała (nBMI) oraz w grupie kontrolnej.

Parametr	HNC nBMI		Grupa kontrolna		Wartość *p*
	Mediana	IQR (Q1; Q3)	Mediana	IQR (Q1; Q3)	
Grelina [pg/mL]	282,341	229,697; 377,075	343,510	295,610; 434,450	0,034942
Adiponektyna [µg/mL]	53,984	43,884; 75,405	45,673	39,945; 64,519	0,118805
Leptyna [pg/mL]	1899,600	1416,650; 2393,790	1715,204	1576,584; 2998,286	0,253290
Rezystyna [pg/mL]	6846,331	5265,468; 7981,305	6781,360	5736,944; 7695,312	0,741750
Glukagon [pg/mL]	1557,810	1452,890; 1639,300	1429,350	929,720; 1595,870	0,008941
Insulina [pg/mL]	297,780	232,930; 420,230	284,970	212,290; 500,420	0,792065
Peptyd C [pg/mL]	611,900	512,850; 706,740	684,038	431,604; 852,606	0,613355
GLP–1 [pg/mL]	257,500	213,360; 277,600	245,682	155,502; 291,314	0,333723
PAI–1 [pg/mL]	3544,930	3122,099; 3956,693	2926,950	2462,028; 3436,926	0,000661

Legenda: GLP–1 – glukagonopodobny peptyd–1; IQR (Q1; Q3) – rozstęp międzykwartylowy (kwartyl dolny; kwartyl górny); PAI–1 – inhibitor aktywatora plazminogenu typu 1. $p < 0,05$ uznano za istotne statystycznie.

Oceniono również korelację między stężeniami analizowanych biomarkerów. W grupie nBMI zaobserwowano statystycznie istotne ujemne korelacje między greliną a GIP (r = −0,6284; $p = 0,001$), GIP a PAI–1 (r = −0,6662; $p = 0,001$) oraz omentyną–1 i rezystyną (r = −0,4233; $p = 0,044$), natomiast istotną dodatnią korelację stwierdzono między greliną a PAI–1 (r = 0,6964; $p < 0,001$) oraz między insuliną a leptyną (r = 0,6359; $p = 0,001$). W grupie iBMI stwierdzono ujemną korelację między wiekiem a rezystyną (r = −0,4479, $p = 0,032$) oraz dodatnią korelację między PAI–1 a wisfatyną (r = 0,8253; $p < 0,001$), adipsyną i peptydem C (r = 0,7054; $p < 0,001$), jak również między insuliną a peptydem C (r = 0,6462; p = 0,001).

6. Dyskusja

6.1. Publikacja II

W Publikacji II zaobserwowano specyficzne zmiany w równowadze oksydacyjno–antyoksydacyjnej, w tym obniżenie aktywności CAT oraz wzrost aktywności GPx (w grupie YCG) i stężenia MDA w osoczu pacjentów z HNC w porównaniu z osobami zdrowymi. Dodatkowo zauważono, że aktywność GPx koreluje negatywnie z masą ciała i wskaźnikiem BMI w grupie YCG. Co interesujące, nie odnotowano istotnych statystycznie różnic w aktywności SOD–1. Wynik ten kontrastuje z wynikami innych badań. Biorąc pod uwagę różnice między badaniami, warto zaznaczyć, że dotychczas opublikowane wyniki dotyczyły bardzo małych grup pacjentów populacji azjatyckiej (głównie Indii) [79–87]. Dodatkowo w badanych grupach znaczny odsetek pacjentów stanowili palacze lub żujący tytoń. W licznych badaniach potwierdzono związek między uzależnieniem od tytoniu a wytwarzaniem ROS, prowadzącym do wzrostu poziomu stresu oksydacyjnego [88,89], więc czynnik ten mógł istotnie wpływać na uzyskiwane wyniki. Wiadomo również, że wiek pacjentów ma wpływ na równowagę oksydacyjno–antyoksydacyjną organizmu [90,91]. Warto wspomnieć, że aktywność SOD–1 jest zależna od poziomu cynku w diecie, natomiast GPx jest enzymem zależnym od selenu. Niedobory wynikające ze niezbilansowanej diety mogą zmniejszać aktywność SOD–1 i/lub GPx. Mniejsza aktywność GPx w OCG w porównaniu z YCG może świadczyć o niedoborze selenu w diecie starszych pacjentów z HNCs. H_2O_2 jest substratem, zarówno dla CAT, jak i GPx [92,93]. W niniejszym badaniu zaobserwowano niższą aktywność CAT i wyższą aktywność GPx u pacjentów z rakiem w porównaniu ze zdrową grupą kontrolną. Mniejsza aktywność CAT może być kompensowana wzrostem aktywności GPx. Zatem obrona antyoksydacyjna związana z glutationem wydaje się dominować u pacjentów. Podsumowując, wyniki niniejszego badania wskazują na zwiększoną generację ROS i zmniejszenie antyoksydacyjnych mechanizmów obronnych, które są charakterystyczne dla chorób nowotworowych [20]. Podwyższony poziom peroksydacji lipidów i MDA może być konsekwencją zaburzenia homeostazy oksydacyjno–przeciwutleniającej i może mieć udział w kancerogenezie.

Wielu naukowców wskazuje na ochronną rolę melatoniny w chorobach jamy ustnej i jamy nosowo–gardłowej głównie poprzez redukcję stresu oksydacyjnego [94–97]. Jednak w niniejszym badaniu istotne statystycznie niższe stężenie melatoniny u pacjentów z rakiem

wargi, jamy ustnej lub gardła w porównaniu z grupą osób zdrowych zaobserwowano jedynie w OCG. Podczas starzenia dochodzi do zmniejszenia syntezy i wydzielania melatoniny [98]. Może to wskazywać, że niedobór melatoniny nie jest szczególnie zaangażowany w patogenezę HNCs.

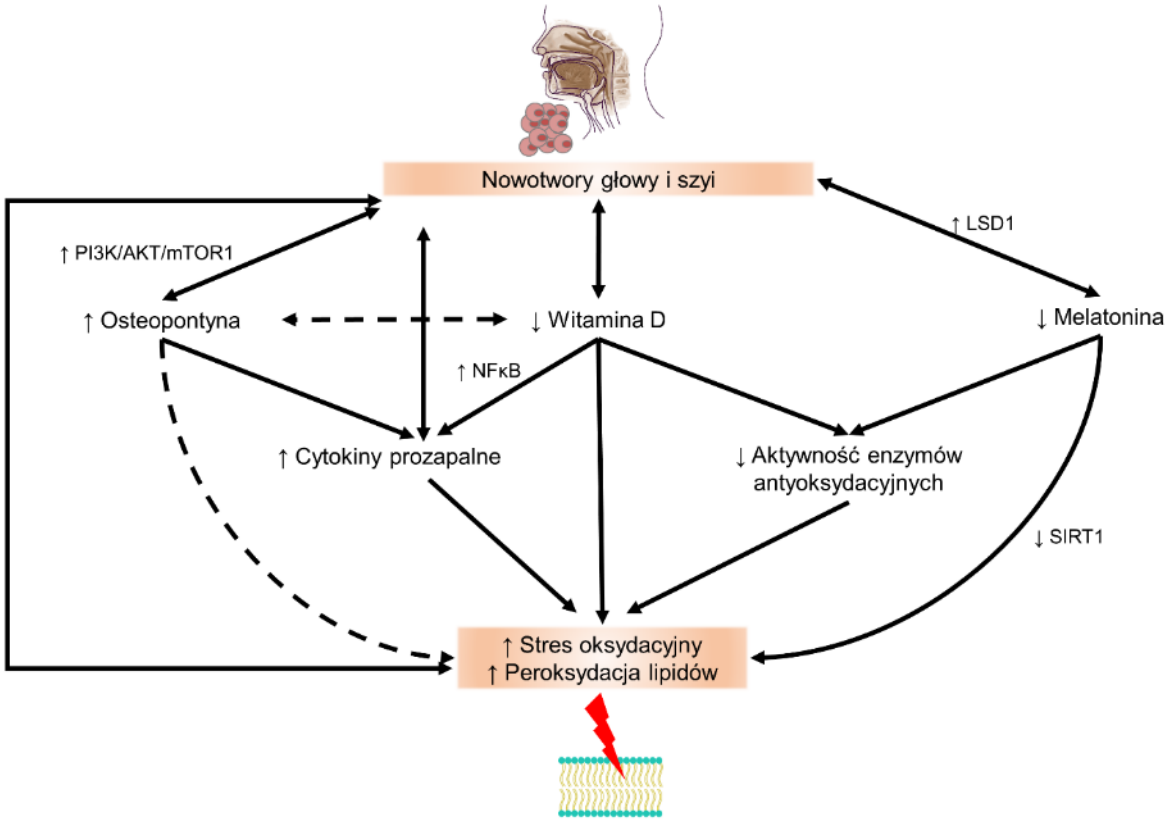
Liczne badania naukowe wskazują, że niedobór witaminy D koreluje ze śmiertelnością w przebiegu chorób nowotworowych [50,99,100]. Kalcytriol moduluje odpowiedź immunologiczną mikrośrodowiska guza poprzez inaktywację szlaku jądrowego czynnika transkrypcyjnego kappa B (NFκB, ang. *nuclear factor kappa–light–chain–enhancer of activated B cells*) [50]. Wartość referencyjna dla witaminy D w osoczu krwi wynosi 75–125 nmol/L [101]. W prezentowanym badaniu zaobserwowano, że w grupie chorych z HNCs stężenie witaminy D było poniżej zakresu referencyjnego.

Nadekspresja osteopontyny jest wiązana ze zwiększoną neoangiogenezą, proliferacją komórek nowotworowych, mobilnością, przeżyciem, inwazją i przerzutami [54]. Pozytywna korelacja między osteopontyną a peroksydacją lipidów, stwierdzona w prezentowanym badaniu, wskazuje na związek tego białka ze szkodliwymi procesami utleniania w błonach plazmatycznych. Niewątpliwie zależności te powinny być przedmiotem dalszych badań naukowych.

W oparciu o wyniki badania opisanego w Publikacji II można stwierdzić, że pacjenci z HNCs wykazują różnice w aktywności enzymów antyoksydacyjnych w porównaniu z osobami zdrowymi, co może wskazywać na zaburzenia równowagi redoks. Wyższe stężenie MDA w osoczu pacjentów z HNCs sugeruje zwiększoną peroksydację lipidów, co może być związane z procesem kancerogenezy. Należy również zwrócić uwagę na niższe stężenie melatoniny oraz 25(OH)–witaminy D u pacjentów z HNCs, co może wpływać na rozwój choroby oraz jej przebieg. Wysokie stężenie osteopontyny u pacjentów z HNC może być związane z kancerogenezą i wpływać na rozwój choroby. Różnice w wynikach między młodszą i starszą grupą pacjentów z HNC wskazują na konieczność uwzględnienia wieku pacjentów podczas opracowywania strategii leczenia. Podsumowując, wyniki badania wskazują na zaburzenia równowagi redoks i inne zmiany na poziomie biochemicznym u pacjentów z HNC, co może wpływać na rozwój i przebieg choroby. Badanie tych aspektów może pomóc w lepszym zrozumieniu patogenezy HNC oraz opracowaniu nowych strategii leczenia i monitorowania pacjentów.

Głównym ograniczeniem prezentowanego badania jest mała liczba uczestników. Należy jednak wskazać, że dotychczas nie przeprowadzono badań z udziałem pacjentów z rakiem wargi, jamy ustnej i gardła populacji europejskiej, w których oceniano mechanizmy obrony antyoksydacyjnej oraz rolę melatoniny, witaminy D i jednocześnie analizowano poziom osteopontyny.

Podsumowanie Publikacji II stanowi Rycina 5, która ukazuje postulowane związki pomiędzy analizowanym parametrami.



Rycina 5. Postulowane mechanizmy łączące melatoninę, witaminę D, stres oksydacyjny, enzymy antyoksydacyjne i osteopontynę w raku wargi, jamy ustnej i gardła. Legenda: LSD1 – specyficzna dla lizyny demetylaza 1; NFκB – jądrowy czynnik transkrypcyjny kappa B; PI3K/AKT/mTOR1 – kinaza 3–fosfatydyloinozytolu/kinaza białkowa B/ssaczy cel rapamycyny–1; SIRT1 – cichy regulator informacji 1. Przerywane strzałki — domniemane interakcje.

6.2. Publikacja III

Zarówno w przebiegu otyłości, jak i kancerogenezy obserwowano zmiany profilu metabolicznego tkanki tłuszczowej [102–104]. Może to prowadzić do zwiększonej syntezy i wydzielania wielu związków bioaktywnych, takich jak hormony, adipokiny, cytokiny zapalne i czynniki wzrostu [105–107]. Do tej pory odkryto ponad 600 adipokin. Większość opisanych adipokin odgrywa kluczową rolę w utrzymaniu homeostazy węglowodanowo–lipidowej [108]. Czynniki te wydzielane przez białą tkankę tłuszczową przyczyniają się do inicjacji i progresji kilku typów nowotworów poprzez stymulację metabolicznego przeprogramowania komórek [109]. Adipokiny wpływają na metabolizm nowotworu i prowadzą do wzrostu, proliferacji, migracji, inwazji, przejścia nabłonkowo–mezenchymalnego komórek nowotworowych, angiogenezy, przerzutów i rozwoju oporności wielolekowej [110–113]. Przewlekłe zapalenie o niskim stopniu złośliwości związane z otyłością kształtuje mikrośrodowisko guza, wpływając na plastyczność komórek poprzez przejście nabłonkowo–mezenchymalne, odróżnicowanie, polaryzację komórek odpornościowych, reaktywne formy tlenu, cytokiny i mechanizmy epigenetyczne [114,115]. Rak wątroby, pęcherza moczowego, płuca, jelita grubego i żołądka są silnie związane z przewlekłym stanem zapalnym [116,117]. Liczne badania koncentrowały się na roli stanu zapalnego w kancerogenezie oraz farmakologicznej redukcji stanu zapalnego jako potencjalnej terapii przeciwnowotworowej [114,118,119]. Komórki nowotworowe ze względu na szybką proliferację charakteryzują się znacznym zapotrzebowaniem na glukozę [120,121]. Nasilony metabolizm glukozy w komórkach nowotworowych zależy między innymi od ich lokalizacji, zwiększonej ekspresji białek transportujących glukozę z rodziny transporterów glukozy (GLUT, ang. *glucose transporter*), enzymów takich jak fosfoglukomutaza czy heksokinaza, stopnia proliferacji komórek oraz unaczynienia guza [122]. HNCs nie były przedmiotem licznych badań, zwłaszcza w aspekcie homeostazy adipokin oraz czynników regulujących gospodarkę węglowodanowo–lipidową. Prezentowane badanie może zatem stanowić cenny wkład w aktualny stan wiedzy w tym zakresie.

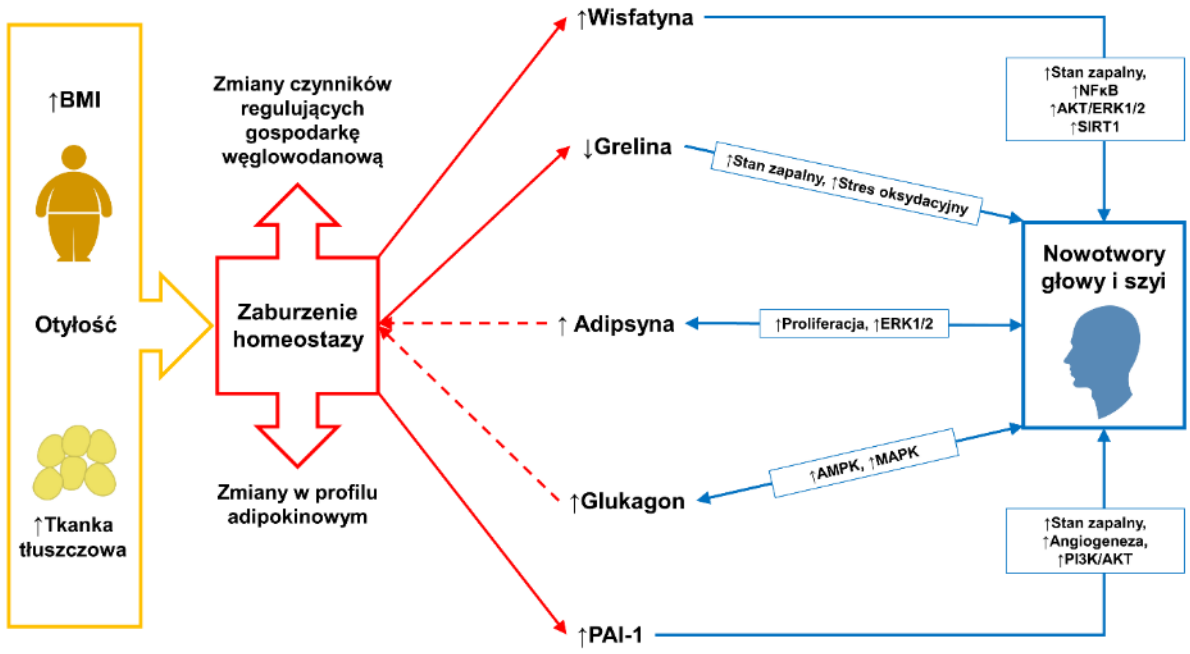
Różnice w stężeniu omentyny–1, adiponektyny, leptyny, rezystyny, insuliny, peptydu C i GLP–1 obserwowane między grupami nBMI i iBMI w prezentowanym badaniu mogą być związane z różnicą w BMI i objętości białej tkanki tłuszczowej. Zgodnie z wynikami przedstawionych badań wpływ wyżej wymienionych parametrów na kancerogenezę HNC wydaje się wątpliwy. Analiza statystyczna wykazała jednak istotne różnice w poziomie adipsyny

i glukagonu między grupą nBMI a grupą kontrolną zdrowych osób. Zatem parametry te mogą być związane z kancerogenezą HNC. Z kolei stężenia wisfatyny i PAI–1 w surowicy były najwyższe w grupie iBMI, a najniższe w grupie kontrolnej. Odwrotną zależność zaobserwowano w przypadku greliny. Udział tych biomarkerów w przebiegu HNC i otyłości pozostaje niepewny. Grelina, wisfatyna i PAI–1 mogą towarzyszyć otyłości i uczestniczyć w kancerogenezie HNC.

Analiza wyników badania opisanego w Publikacji III wskazuje na istotne różnice w stężeniach hormonów oraz czynników wzrostu i regulacyjnych między pacjentami z HNC o różnym BMI. Wyniki te sugerują, że otyłość może wpływać na procesy metaboliczne i hormony biorące udział w regulacji masy ciała, co z kolei może wpłynąć na rozwój i przebieg choroby nowotworowej. Różnice w stężeniach tych biomarkerów między grupami nBMI i iBMI oraz między pacjentami i grupą kontrolną wskazują na możliwe związki między zaburzeniami hormonalnymi a patogenezą HNC. Można przypuszczać, że uwzględnienie BMI u pacjentów z HNC może być istotne dla opracowywania strategii terapeutycznych oraz monitorowania stanu ich zdrowia.

Przedstawione badanie posiada ograniczenia, takie jak niewielka liczba uczestników. Niemniej jednak, nie przeprowadzono wcześniej badań z udziałem pacjentów z HNC, w których jednocześnie analizowano szeroki zakres parametrów związanych z endokrynną funkcją tkanki tłuszczowej, homeostazą metabolizmu węglowodanów oraz stanem zapalnym.

Podsumowanie Publikacji III stanowi Rycina 6, która ukazuje postulowane zależności pomiędzy analizowanym parametrami.



Rycina 6. Możliwe interakcje między zaburzoną funkcją tkanki tłuszczowej w otyłości a rakiem głowy i szyi. Legenda: AKT/ERK1/2 – kinaza białkowa B/kinazy regulowane sygnałem zewnątrzkomórkowym 1/2; AMPK – kinaza białkowa aktywowana 5' monofosforanem adenozyny; BMI – indeks masy ciała; MAPK – kinazy aktywowane mitogenami; NFκB – jądrowy czynnik transkrypcyjny kappa B; PAI–1 – inhibitor aktywatora plazminogenu typu 1; PI3K/AKT – kinaza 3–fosfatydyloinozytolu/kinaza białkowa B; SIRT1 – cichy regulator informacji 1. Przerywane strzałki — domniemane interakcje.

7. Wnioski

1. Analiza aktualnej literatury naukowej wskazuje, że zarówno melatonina, jak i witamina D mają potencjał jako substancje chroniące przed uszkodzeniami wywołanymi promieniowaniem jonizującym. Melatonina wykazuje silne właściwości przeciwutleniające i potencjalnie może być stosowana jako czynnik radioprotekcyjny u ludzi. Rola witaminy D jako substancji chroniącej przed negatywnymi skutkami eskpozycji na promieniowanie jonizujące wymaga dalszych badań. Można przypuszczać, że działanie witaminy D w tym aspekcie jest związane z redukcją stresu oksydacyjnego.
2. Z analizy aktualnej literatury naukowej wynika, że zarówno melatonina, jak i witamina D wykazują selektywne działanie radiosensybilizujące na komórki rakowe, co czyni je obiecującymi adiuwantami w radioterapii.
3. Istotnie niższe stężenie melatoniny zaobserwowano jedynie w grupie starszych pacjentów z HNC, co może wynikać z jej obniżonej produkcji wraz z wiekiem. Można sugerować, że niedobór melatoniny nie jest zaangażowany w patogenezę HNCs. Niewątpliwie określenie roli melatoniny w HNC wymaga dalszych badań.
4. Stwierdzone niskie stężenie witaminy D u pacjentów z nowotworami głowy i szyi wskazuje na możliwą rolę niedoborów witaminy D w rozwoju HNCs.
5. Wykazano zaburzenie równowagi oksydacyjno–antyoksydacyjnej oraz nasilenie peroksydacji lipidów w kancerogenezie HNC. Osłabienie enzymatycznej obrony antyoksydacyjnej było bardziej zaznaczone w grupie starszych pacjentów z HNC. Różnice między młodszą i starszą grupą pacjentów z HNCs wskazują na konieczność uwzględnienia wieku pacjentów w strategiach leczenia.
6. Wysokie stężenie osteopontyny u pacjentów z HNC może być związane z kancerogenezą i wpływać na rozwój choroby. Wykazana dodatnia korelacja pomiędzy stężeniem osteopontyny a ilością MDA w osoczu może wskazywać na związek osteopontyny z nasileniem stresu oksydacyjnego.
7. U pacjentów z HNC występują zaburzenia równowagi hormonalnej tkanki tłuszczowej, co wpływa na zmianę metabolizmu węglowodanów i profilu markerów stanu zapalnego.
8. Grelina, wisfatyna i PAI–1 wydają się być szczególnie zaangażowane w patomechanizmy rozwoju i/lub progresji HNC. Niezbędne jest przeprowadzenie dalszych badań klinicznych

na większej grupie pacjentów z HNC, aby przeanalizować udział tych parametrów w chorobie nowotworowej i ich potencjalne zastosowanie jako biomarkerów HNC.

9. Zaobserwowane wyższe stężenie adipsyny i glukagonu u pacjentów z HNC niezależnie od ich BMI w porównaniu z grupą kontrolną wskazuje na potencjalne znaczenie tych parametrów w rozwoju i/lub progresji nowotworów głowy i szyi.
10. Otyłość, która nie jest typowym czynnikiem ryzyka predysponującym do wystąpienia HNC, może wpływać negatywnie na zmiany metaboliczne u pacjentów z tym rodzajem nowotworu.

8. Streszczenie w języku polskim

Doktorant: mgr Jarosław Nuszkiewicz

Tytuł rozprawy: *Ocena stężenia witaminy D, melatoniny, biomarkerów stresu oksydacyjnego, stanu zapalnego i wykładników endokrynnej aktywności tkanki tłuszczowej u pacjentów z nowotworami głowy i szyi*

Promotor: dr hab. n. med. Karolina Szewczyk–Golec, prof. UMK

Streszczenie:

Nowotwory głowy i szyi (HNCs, ang. *head and neck cancers*) to rzadka grupa chorób onkologicznych. Pomimo znacznego rozwoju medycyny, HNCs stanowią istotny problem kliniczny i społeczny. Rocznie na świecie diagnozuje się ponad 500 000 nowych przypadków tych nowotworów. HNCs dotyczą różnych struktur w obszarze głowy i szyi, a ich etiologia jest złożona, obejmując czynniki środowiskowe i genetyczne. Głównymi czynnikami predysponującymi do wystąpienia HNC są palenie papierosów oraz spożywanie alkoholu. Objawy HNCs zależą od lokalizacji guza i obejmują ból, owrzodzenie tkanki, problemy z oddychaniem, połykaniem i mówieniem, a także powiększenie węzłów chłonnych na szyi u niektórych pacjentów. Podstawowe metody leczenia HNCs to chirurgia i radioterapia, która wymaga zastosowania promieniowania jonizującego. W przebiegu HNCs dochodzi do zaburzenia równowagi między wytwarzaniem reaktywnych form tlenu (ROS, ang. *reactive oxygen species*) a zdolnością organizmu do ich neutralizacji. W sytuacji homeostazy organizm zachowuje równowagę między wytwarzaniem ROS a ich neutralizacją za pomocą enzymatycznych i nieenzymatycznych mechanizmów antyoksydacyjnych. Enzymy antyoksydacyjne obejmują dysmutazy ponadtlenkowe (SODs, ang. *superoxide dismutase*), katalazę (CAT, ang. *catalase*) i peroksydazy glutationowe (GPxs, ang. *glutathione peroxidases*), natomiast do antyoksydantów nieenzymatycznych zalicza się witaminy A, C, E, glutation (GSH, ang. *glutathione*) i melatoninę. W ostatnich latach naukowcy wskazują na antyoksydacyjną rolę witaminy D. Jednym z głównych mechanizmów łączących otyłość z nowotworami jest przewlekły stan zapalny, który może stać się źródłem ROS. W przebiegu otyłości dochodzi do zaburzenia syntezy i sekrecji adipokin, hormonów białej tkanki tłuszczowej. U pacjentów z otyłością zmieniony zostaje profil adipokinowy. Adipokiny prozapalne promują stres oksydacyjny i wpływają na metabolizm węglowodanów.

Celem niniejszej rozprawy doktorskiej była ocena stężenia melatoniny i witaminy D, a także aktywności wybranych enzymów antyoksydacyjnych, stężenia markerów peroksydacji lipidów, wybranych adipokin, czynników regulujących homeostazę glukozy oraz wykładników stanu zapalnego u pacjentów z HNCs. Dodatkowo, badanie miało na celu określenie związku pomiędzy powyższymi parametrami a wiekiem i wskaźnikiem masy ciała (BMI, ang. *body mass index*) pacjentów w przebiegu HNCs.

Warunkiem zakwalifikowania pacjenta do badania było rozpoznanie pierwotnego, złośliwego HNC. Materiał badany stanowiła krew żylna pobierana od pacjentów z HNCs oraz od zdrowych osób stanowiących grupę kontrolną. Z krwi żylnej uzyskiwano zawiesinę krwinek czerwonych do oznaczania aktywności SOD–1, CAT, GPx i stężenia malonylodialdehydu (MDA, ang. *malondialdehyde*) oraz osocze (celem oznaczenia stężenia MDA w osoczu). W surowicy metodami immunoenzymatycznymi i Bio–Plex Multiplex Immunoassay oznaczono poziom melatoniny i 25(OH)–witaminy D, osteopontyny, omentyny–1, adipsyny, adiponektyny, peptydu C, greliny, glukozozależnego peptydu insulinotropowego (GIP, ang. *glucose–dependent insulinotropic peptide*), glukagonopodobnego peptydu–1 (GLP–1, ang. *glucagon–like peptide 1*), glukagonu, insuliny, leptyny, inhibitora aktywatora plazminogenu–1 (PAI–1, ang. *plasminogen activator inhibitor–1*), rezystyny oraz wisfatyny. Uzyskane wyniki opracowano statystycznie. Różnice na poziomie $p < 0,05$ uznano za istotne statystycznie.

Podstawę rozprawy doktorskiej stanowią trzy publikacje. Publikacja I (praca poglądowa), opisuje rolę melatoniny i witaminy D jako antyoksydantów redukujących negatywne skutki ekspozycji na promieniowanie jonizujące, wykorzystywane w diagnostyce i terapii przeciwnowotworowej.

Publikacja II (praca oryginalna) stanowi opis badania z udziałem 45 pacjentów z rozpoznanym HNC oraz 25 osób zdrowych osób jako grupa kontrolna (CG, ang. *control* group; 55,36 ± 1,17 lat). Pacjenci z HNC zostali podzieleni na dwie grupy wiekowe: młodszą grupę z rakiem (YCG, ang. *younger cancer group*; $n = 25$; 58,24 ± 1,29 lat) i starszą grupę z rakiem (OCG, ang. *older cancer group*; $n = 20$; 69,7 ± 1,49 lat). Aktywność SOD–1 była zbliżona we wszystkich analizowanych grupach. Natomiast aktywność CAT była statystycznie niższa w obu grupach z HNC (YCG i OCG) w porównaniu z CG. Aktywność GPx była istotnie wyższa w YCG w porównaniu z CG. Nie zaobserwowano istotnych statystycznie różnic w stężeniu MDA w erytrocytach pomiędzy grupami badanymi, ale stężenie MDA w osoczu było znamiennie

wyższe u pacjentów z YCG i OCG niż w CG. W OCG stężenie melatoniny było istotnie niższe niż w CG. Stężenie 25(OH)–witaminy D w surowicy osób zdrowych było wyższe niż u pacjentów z YCG i OCG. W obu grupach pacjentów stężenie osteopontyny było istotnie wyższe niż w CG.

W badaniu opisanym w Publikacji III wzięło udział 46 pacjentów z HNC oraz 23 osoby zdrowe jako CG. Pacjenci z HNC zostali podzieleni na dwie podgrupy po 23 osoby na podstawie BMI: grupa z prawidłowym BMI (nBMI, ang. *normal BMI*; BMI < 25 kg/m^2) i grupa o podwyższonym BMI (iBMI, ang. *increased BMI*; BMI ≥ 25 kg/m^2). U pacjentów z grupy nBMI stwierdzono znamiennie wyższe stężenie adiponektyny, omentyny–1 oraz greliny w surowicy w porównaniu z iBMI. W iBMI zaobserwowano istotnie wyższy poziom insuliny, leptyny, peptydu C, GLP–1, PAI–1, rezystyny i wisfatyny niż w nBMI. Nie odnotowano istotnych statystycznie różnic między grupami nBMI i iBMI w odniesieniu do adipsyny, GIP i glukagonu. W nBMI stwierdzono znacząco wyższe wartości stężenia adipsyny, wisfatyny, glukagonu i PAI–1, a także istotnie niższy poziom greliny w porównaniu z CG. Przeprowadzona analiza nie wykazała różnic w stężeniu omentyny–1, GIP, adiponektyny, peptydu C, GLP–1, insuliny, leptyny i rezystyny między grupami nBMI i CG.

Wyniki opisane w Publikacji II mogą wskazywać na zaburzenie równowagi redoks u osób z HNCs. Wyższe stężenie MDA w osoczu pacjentów z HNCs wskazuje na zwiększoną peroksydację lipidów, co może być związane z kancerogenezą. Ponadto, u pacjentów z HNC zaobserwowano niższe stężenie melatoniny oraz 25(OH)–witaminy D oraz wyższe stężenie osteopontyny, co może wpływać na rozwój i przebieg choroby. Różnice w wynikach między młodszą i starszą grupą pacjentów z HNC wskazują na konieczność uwzględnienia wieku pacjentów w strategiach leczenia. W badaniu opisanym w Publikacji III zaobserwowano istotne różnice w stężeniu adipokin oraz czynników regulujących metabolizm glukozy między pacjentami z HNC o różnym BMI i zdrową grupą kontrolną. Wyniki te sugerują, że otyłość może wpływać na procesy metaboliczne i hormony biorące udział w regulacji masy ciała, co z kolei może mieć znaczenie dla rozwoju i przebiegu HNC. Różnice w stężeniu biomarkerów między grupami nBMI, iBMI oraz grupą kontrolną wskazują na możliwe związki między zaburzeniami hormonalnymi a patogenezą HNC.

Słowa kluczowe: adipokiny; antyoksydanty; metabolizm węglowodanów; nowotwory głowy i szyi; otyłość; peroksydacja lipidów; stan zapalny; stres oksydacyjny

9. Streszczenie w języku angielskim (Abstract in English)

PhD candidate: Jarosław Nuszkiewicz, MSc

Title of the dissertation: *Assessment of the concentration of vitamin D, melatonin, biomarkers of oxidative stress, inflammation and endocrine activity of adipose tissue in patients with head and neck cancer*

Supervisor: Karolina Szewczyk–Golec, PhD, NCU Prof.

Abstract:

Head and neck cancers (HNCs) are a rare group of oncological diseases. Despite significant advancements in medicine, HNCs remain a considerable clinical and social problem. More than 500,000 new cases of HNCs are diagnosed annually worldwide. HNCs affect various structures in the head and neck area, and their etiology is complex, involving environmental and genetic factors. The main predisposing factors for HNC include smoking and alcohol consumption. The symptoms of HNCs depend on the tumor's location and include pain, tissue ulceration, trouble breathing, difficulty swallowing and speaking, and enlarged lymph nodes in the neck for some patients. The primary treatment methods for HNCs include surgery and radiotherapy, which involve the use of ionizing radiation. During HNCs, there is an imbalance between the generation of reactive oxygen species (ROS) and the cells' ability to neutralize them. In a state of homeostasis, the body maintains a balance between the production of ROS and their scavenging through enzymatic and non–enzymatic antioxidant mechanisms. Antioxidant enzymes include superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidases (GPxs), while non–enzymatic antioxidants comprise vitamins A, C, E, glutathione (GSH), and melatonin. In recent years, scientists have highlighted the antioxidant role of vitamin D. One of the main mechanisms linking obesity and cancer is chronic inflammation, which may become a source of ROS. During obesity, the synthesis and secretion of adipokines, hormones of white adipose tissue, are disturbed. In obese patients, the adipokine profile is altered. Pro–inflammatory adipokines promote oxidative stress and affect carbohydrate metabolism.

The aim of this doctoral dissertation was to determine the level of melatonin and vitamin D, as well as the activity of selected antioxidant enzymes, the concentration of lipid peroxidation markers, selected adipokines, factors regulating glucose homeostasis, and

inflammatory markers in HNC patients. Additionally, the study aimed to establish the relationship between the above–mentioned parameters and the patients' age and body mass index (BMI) in the course of HNC.

The condition for qualifying a patient for the study was the diagnosis of primary, malignant HNC. The study material consisted of the venous blood samples collected from patients with HNC and from healthy controls. From the venous blood sample, a suspension of red blood cells for the determination of SOD–1, CAT, GPx activities and malondialdehyde (MDA) concentration and plasma for the determination of plasma MDA level were obtained. Using immunoassay methods and Bio–Plex Multiplex Immunoassay, the levels of melatonin and 25(OH)–vitamin D, osteopontin, omentin–1, adipsin, adiponectin, C–peptide, ghrelin, glucose–dependent insulinotropic peptide (GIP), glucagon–like peptide–1 (GLP–1), glucagon, insulin, leptin, plasminogen activator inhibitor–1 (PAI–1), resistin, and visfatin were determined in the serum. The results obtained from the laboratory tests were statistically analyzed. Differences at the level of $p < 0.05$ were considered statistically significant.

The foundation of the doctoral dissertation consists of three publications. Publication I (a review) describes the role of melatonin and vitamin D as antioxidants reducing the negative effects of exposure to ionizing radiation, used in cancer diagnosis and therapy.

Publication II (an original article) is a description of a study involving 45 patients diagnosed with HNC and 25 healthy people as a control group (CG, 55.36 ± 1.17 yrs). HNC patients were divided into two age groups: the younger cancer group (YCG, $n = 25$; 58.24 ± 1.29 yrs) and the older cancer group (OCG, $n = 20$; 69.7 ± 1.49 yrs). SOD–1 activity was similar in all analyzed groups. In contrast, CAT activity was statistically lower in both HNC groups (YCG and OCG) compared to CG. GPx activity was significantly higher in YCG compared to CG. There were no statistically significant differences in the concentration of MDA in erythrocytes between the study groups, but the concentration of MDA in plasma was significantly higher in patients with YCG and OCG than in CG. In OCG, the concentration of melatonin was significantly lower than in CG. The concentration of 25(OH)–vitamin D in the serum of healthy subjects was higher than in patients with YCG and OCG. In both groups of patients, the concentration of osteopontin was significantly higher than in CG.

The study described in Publication III (an original article) involved 46 patients with HNC and 23 healthy people as a control group (CG). HNC patients were divided into two subgroups of 23 people each based on their BMI, namely the normal BMI (nBMI; BMI < 25 kg/m^2) and the increased BMI (iBMI; BMI ≥ 25 kg/m^2). Patients in the nBMI group had significantly higher concentrations of adiponectin, omentin–1, and ghrelin in serum compared to iBMI. Significantly higher levels of insulin, leptin, C–peptide, GLP–1, PAI–1, resistin, and visfatin were observed in iBMI than in nBMI. There were no statistically significant differences between the nBMI and iBMI groups with regard to adipsin, GIP, and glucagon. Significantly higher levels of adipsin, visfatin, glucagon, and PAI–1, as well as a significantly lower level of ghrelin were found in nBMI compared to CG. The analysis showed no differences in the concentration of omentin–1, GIP, adiponectin, C–peptide, GLP–1, insulin, leptin, and resistin between nBMI and CG.

The results described in Publication II may indicate a redox imbalance in people with HNC. Higher plasma concentrations of MDA in the HNC patients indicate increased lipid peroxidation, which may be associated with carcinogenesis. In addition, lower levels of melatonin and 25(OH)–vitamin D and higher levels of osteopontin were observed in HNC patients, which may affect the development and course of the disease. Differences in the results between the younger and older groups of HNC patients indicate the need to take into account the age of patients in treatment strategies. In the study described in Publication III, significant differences in the concentrations of adipokines and factors regulating glucose metabolism were observed between HNC patients with different BMI and a healthy control group. These results suggest that obesity may influence metabolic processes and hormones involved in body mass regulation, which in turn may affect the development and course of HNC. Differences in biomarker concentrations between the nBMI, iBMI, and control groups indicate possible relationships between hormonal disorders and the pathogenesis of HNC.

Keywords: adipokines; antioxidants; carbohydrate metabolism; head and neck cancer; inflammation; lipid peroxidation; obesity; oxidative stress

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11. Spis rycin

12. Spis tabel

13. Bibliografia

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14. Załączniki

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14.1.1. Publikacja I

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Review

Ionizing Radiation as a Source of Oxidative Stress—The Protective Role of Melatonin and Vitamin D

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Abstract: Ionizing radiation (IR) has found widespread application in modern medicine, including medical imaging and radiotherapy. As a result, both patients and healthcare professionals are exposed to various IR doses. To minimize the negative side effects of radiation associated with oxidative imbalance, antioxidant therapy has been considered. In this review, studies on the effects of melatonin and vitamin D on radiation-induced oxidative stress are discussed. According to the research data, both substances meet the conditions for use as agents that protect humans against IR-induced tissue damage. Numerous studies have confirmed that melatonin, a hydro- and lipophilic hormone with strong antioxidant properties, can potentially be used as a radioprotectant in humans. Less is known about the radioprotective effects of vitamin D, but the results to date have been promising. Deficiencies in melatonin and vitamin D are common in modern societies and may contribute to the severity of adverse side effects of medical IR exposure. Hence, supporting supplementation with both substances seems to be of first importance. Interestingly, both melatonin and vitamin D have been found to selectively radiosensitise cancer cells, which makes them promising adjuvants in radiotherapy. More research is needed in this area, especially in humans.

Keywords: ionizing radiation; melatonin; oxidative stress; radioprotection; reactive oxygen species; vitamin D

1. Introduction

The field of radiology dates back to 1895 when the German scientist Wilhelm Konrad Roentgen discovered X-rays [1]. Since then, ionizing radiation (IR) has found wide application in medicine, both in diagnostics and in therapies [2–5]. The use of medical imaging, especially roentgenodiagnostics and computed tomography, and radiotherapy exposes both patients and medical professionals to the harmful side effects of radiation [2,3]. IR occurs naturally in the environment, having accompanied humanity since its dawn. Its sources are natural radioisotopes found in soil and cosmic rays reaching the Earth's surface [6–8]. This radiation is called background radiation and its value changes with natural conditions [7,9]. The highest background radiation dose values of circa 0.26 Gy/year are observed in Ramsar (Iran) [10]. This dose is 10–100 times higher than the average one, but no greater incidence of cancer or other IR-related diseases is observed in this region [10]. This is due to radiation hormesis, which is an evolutionary adaptation to the presence of background radiation and the development of appropriate repair systems [11].

The mechanism of deleterious IR action is strongly associated with increasing oxidative stress in irradiated tissues [12]. IR is capable of penetrating the cells of living organisms, where it induces the ionization of both organic and inorganic compounds [13,14]. Due to the high water content in cells,

radiolysis of water molecules by IR is the main process contributing to the increased formation of reactive oxygen species (ROS) [15,16]. ROS rapidly react with macromolecules, including proteins, nucleic acids and lipids, leading to cell dysfunction and apoptotic cell death [12]. As a result of augmented oxidative stress, not only direct negative side effects, but also ROS-related diseases may develop. Therefore, it is especially important to identify effective and safe prophylactic compounds to protect people from IR damage [4]. Undoubtedly, the substances considered in this type of supporting therapy should demonstrate an ability to counteract excessive oxidative stress.

Recently, attention has been paid to radioprotective properties of two hormones, whose synthesis depends on the specific light wavelengths, namely melatonin and vitamin D [17,18]. Both substances are endogenous compounds, but their deficiencies have been widely described in modern societies [19,20]. Melatonin as a strong direct and indirect antioxidant has been considered a radioprotector since the beginning of the 21st century [21–23]. However, animal model and in vitro studies have not been translated into human use yet [23]. Vitamin D, originally associated with bone homeostasis, has been found to perform many regulatory functions, affecting, among other things, the oxidative–antioxidant balance of the body [24–26]. Thus, its use to prevent irradiation side effects has also been taken under consideration, but data are limited and require more research.

Taking into account the relevance of the problem, the aim of the current review was to provide new scientific data on the protective effects of melatonin and vitamin D against oxidative damage caused by ionizing radiation. The current state of knowledge, including possible molecular mechanisms of action, is discussed. We hope that our review will be an impetus for further research on the use of both hormones in preventing deleterious side effects of ionizing radiation, especially in the field of human studies.

2. Ionizing Radiation as a Source of Reactive Oxygen Species

IR is a form of energy transfer that is able to cause ionization of a material medium while interacting with it [7]. This energy can be transferred by means of electromagnetic waves, including X radiation, gamma radiation and a small range of ultraviolet (UV) radiation with short wavelength and high energy, or through alpha and beta particles [27,28]. Each type of radiation differs in its energy, penetration and biological effects of the exposure. Alpha particles, consisting of two protons and two neutrons, have a short range due to their high mass [29]. There are two types of beta radiation. Beta minus radiation consists of electrons, while beta plus radiation consists of positrons, which are the antimatter counterpart of the electron [30]. Both X and gamma rays are characterized by high penetration and a plate made of lead is needed as an effective shield against them [31]. UV radiation capable of causing ionization has a wavelength in the range of 100–280 nm (UVC) and is absorbed by the atmosphere [28,32].

An important parameter used in dosimetry characterizing IR is linear energy transfer (LET), which determines the average amount of energy lost per unit of length transferred by radiation quanta [33]. High LET values are characteristic of alpha particles, neutrons and cosmic rays (heavy ions) [34]. Alpha particles, compared to other types of radiation, are characterized by shallow penetration, so the radiation energy is deposited at a shorter distance [35]. Neutron radiation and heavy ions, characteristic of cosmic radiation, have a greater range and penetrate deeper than alpha particles [34]. Low LET, typical of beta and gamma types of radiation, involves deposition of energy over a longer distance, causing less damage per distance unit [36].

High LET alpha radiation interacts mainly with molecules on the surface of the tissue by destroying its structure [36]. The most common source of alpha radiation in the environment is one of the natural radon isotopes, namely radon-222 [37]. Despite limited tissue penetration, alpha particles have high relative biological effectiveness. They can cause significant damage, especially in tissues sensitive to alpha particles due to their shallowness, such as bronchial epithelium. This makes radon, as an inhaled residential gas, a significant cause of lung cancer [37]. Characterized by higher penetration, low LET radiation is mainly responsible for the generation of ROS by ionization of atoms [35,38].

It should be noticed that most environmental, occupational and medical IR sources expose people to simultaneous action of different types of radiation. The interaction of low and high LET radiation may lead to increased and more complex biological damage [35].

IR, absorbed by tissues and cells, affects their functioning and structure to various extents, depending on the dose and type of radiation [13,14]. In affected cells, ROS are generated mainly through the radiolysis of water molecules (decay by the action of radiation quanta) or the excitation of water molecules and their decay [15,16,39]. IR can also indirectly influence the oxidative–antioxidant homeostasis by damaging different biomolecules [12]. The altered molecules, such as DNA or proteins responsible for stabilizing the DNA structure, become more susceptible to damage caused by ROS [40,41]. In addition, antioxidants or genes encoding for enzymatic antioxidants can be damaged, which directly increases the oxidative stress [40,41]. A meta-analysis carried out by Einor et al. [42], based on 41 studies concerning various biological matrices, proved that IR, even at low doses, generates ROS.

In biological systems, the state in which the amount of ROS and reactive nitrogen species (RNS) exceeds the physiological ability to maintain homeostasis is called oxidative stress [43,44]. ROS, which are products of excitation and one-, two- and three-electron reduction of the oxygen molecule, are characterized by much greater reactivity than the oxygen in the ground state [45,46]. ROS are a broad concept, including ions, atoms, as well as molecules and radicals such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) [46,47]. The hydroxyl radical is the most dangerous for tissues due to its high reactivity and the ability to oxidize many cell components, such as lipids, proteins, carbohydrates and deoxyribonucleic acids [48,49]. As a result of lipid peroxidation, reactive lipid derivatives are formed, which are capable of oxidative damage to other biomolecules [50]. Depending on the fatty acid that undergoes oxidation, trans-4-hydroxy-2-nonenal (4-HNE) and/or malondialdehyde (MDA) are formed as one of the end products, used as markers of the lipid peroxidation level [44]. Oxidative modifications of the protein structure have been observed in many pathophysiological conditions, including the ageing process, as well as apoptosis [51,52]. They lead to a loss of spatial conformation and biological properties, impeded degradation and accumulation of modified protein products, such as protein carbonyl derivatives [51,52]. Oxidative stress causes damage to both mitochondrial and nuclear DNA, which may result in mutation and carcinogenesis. The marker of DNA damage is 8-oxoguanine, a chemical derivative of guanine [53,54]. Oxidative stress is associated with many diseases, including epidemiologically significant diseases of affluence such as cancer [55], cardiovascular disease [56], obesity [57], neurodegenerative diseases [58] and allergic diseases [59].

Oxygen metabolism and the prevalence of ROS have forced living organisms to develop appropriate counteraction mechanisms to minimize the negative effects of oxidative stress [60]. The antioxidant defense system consists of endogenous and exogenous elements. Antioxidant enzymes, which include superoxide dismutases (SODs), catalase (CAT), glutathione peroxidases (GPxs) and glutathione reductase (GR), the enzyme necessary for the proper functioning of GPx, are a part of the endogenous primary enzymatic defense [61,62]. In addition to antioxidant enzymes, reduced glutathione (GSH), a cofactor for GPx, proteins (ferritin, transferrin, ceruloplasmin, albumin), uric acid, melatonin and vitamin D take part in the prevention of excessive oxidative stress [17,63–65]. Carotenoids, vitamins A, C, and E, selenium, and polyphenols are the main exogenous antioxidants [66,67]. The cooperation of both endogenous and exogenous antioxidants maintains the oxidative and antioxidant balance, preventing the negative effects of oxidative stress but enabling ROS to perform physiologically important functions as mediators of intercellular communication [68].

In numerous studies, the effect of ionizing radiation on the oxidative stress level has been examined [39,69–71]. Different radiation qualities and doses have been used in the experiments during recent years [72–75]. According to Kang et al. [72], a dose of 2 Gy γ-irradiation at a dose rate of 1.1 Gy/min affected ROS generation in murine splenocyte cell culture. The level of oxidative stress was determined by a method using 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which penetrates

inside the cells and is hydrolysed by intracellular esterase into 2′,7′-dichlorofluorescin (DCFH). DCFH reacts with ROS and is converted to highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). Fluorescence was assessed 24 h after irradiation and a significant increase in ROS levels was observed as a result of radiation. Similar observations were made by Shaban et al. [75] in a study whose purpose was to investigate the effect of gamma radiation at a dose of 2, 4, 6, 8 and 10 Gy (delivered in four fractions at one-day intervals at a dose rate of 0.5 Gy/min) in male Albino Sprague-Dawley rat testis. The authors examined blood samples and histopathologically evaluated the irradiated tissues. After exposure to IR, increases in MDA, nitric oxide and calcium ion levels were observed, while SOD and CAT activities and GSH concentration decreased. Karimi et al. [73] also described the relationship between gamma radiation at a dose of 15 Gy (at a dose rate of 0.985 Gy/min) and oxidative stress after irradiation of rat lenses. Two days after the exposure to IR, the animals were sacrificed and an increase in MDA concentrations and a decrease in GSH levels were detected in the tested lenses. Rezaeyan et al. [74] irradiated the adult male Sprague-Dawley rat chest area. The applied X-ray at a dose of 18 Gy in one fraction increased oxidative stress 24 h after the exposure through increased MDA levels and decreased SOD activities. It can be summarized that exposure to high doses of IR leads to increased ROS production, enhanced lipid peroxidation and reduced enzymatic antioxidant defense in a dose-, dose-rate- and LET-dependent manner, while low doses of low LET radiation may upregulate antioxidant defense, including the stimulation of GSH synthesis [39]. It has been proven that IR affects ROS and RNS cell metabolism, activating different signaling pathways and disrupting the normal redox system [69,71]. These changes lead to the dysregulation of the activities of cyclooxygenases, lipoxygenases, nitric oxide synthases, and nicotinamide adenine dinucleotide phosphate oxidases, accompanied by mitochondrial dysfunction [69,71]. It is also worth noting that the response to IR is tissue-dependent, with acute damage but fast regeneration for tissues with rapid turnover [70].

3. Melatonin—A Circadian Rhythm Regulator with Antioxidant Properties

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthesized and secreted mainly by the pineal gland present in the brain of vertebrates [76]. Extrapineal sources of melatonin are localised in bone marrow, skin, platelets, lymphocytes, retina, the gastrointestinal tract, and the Harderian gland [77,78]. It was first isolated from the bovine pineal gland by Aaron Lerner in 1958 [79] and since then researchers have explored new aspects of this hormone.

The pineal gland is an unpaired structure localized between thalamic bodies in the quadrigeminal cistern [80]. In an adult human, this small neuroendocrine gland reaches 5–9 mm in length, 1–5 mm in width, and 3–5 mm in thickness and weighs about 100–180 mg [81]. The substrate for melatonin biosynthesis in pinealocytes is the amino acid containing an indole ring, tryptophan [76,80]. With the tryptophan hydroxylase enzyme (TPH), the tryptophan molecule is converted to 5-hydroxytryptophan (oxitriptan). Then aromatic L-amino acid decarboxylase (AAAD), using pyridoxal phosphate (PLP) as a coenzyme, catalyzes the reaction in which serotonin is formed [82]. 5-hydroxytryptamine (serotonin), a neurotransmitter colloquially called the happiness hormone, is an intermediate for aralkylamine *N*-acetyltransferase (AANAT), which in the presence of acetyl coenzyme A (acetyl CoA) leads to the biosynthesis of N-acetylserotonin (normelatonin) [83]. The last stage of melatonin biosynthesis takes place with the participation of the enzyme acetylserotonin O-methyltransferase (ASMT) and S-adenosyl methionine (SAM), a coenzyme in methylation reactions [84].

Biosynthesis and secretion of melatonin by pinealocytes are regulated by the presence of electromagnetic radiation in the visible light range, especially light with a wavelength of 460–480 nm, which is perceived as blue light [85]. The highest secretion of melatonin is observed between 3:00 a.m. and 4:00 a.m. (with normal circadian rhythms) [86]. The plasma melatonin concentrations during these hours range from 18.5 to 180 pg/mL [87]. Night work and the use of computer screens or smartphones at night, typical of the modern society, lead to reduced melatonin synthesis [20]. In humans, the endogenous master clock, which controls many physiological processes and behavior patterns, is located in the hypothalamic suprachiasmatic nucleus (SCN) [88]. Light reaching intrinsically

photosensitive retinal ganglion cells is received by a photopigment sensitive to blue light called melanopsin [88,89]. The signal is transmitted via the retinohypothalamic tract to SCN located above the optic chiasm [80,89]. The SCN has direct connections to other hypothalamic nuclei and the pineal gland [90]. In this way, information sent by SCN regulates melatonin synthesis. Melatonin secreted into the circulatory system affects SCN by feedback, and other tissues by regulating their chronobiology [91]. The *Clock*, *Bmal1*, *Cry1-2*, *Per1-2* genes, whose expression is modulated by melatonin, play an important role in regulating SCN [88,92,93]. The *Bmal1* and *Clock* gene transcription products combine together to form heterodimers, which attach to the promoter region of the *Per* and *Cry* genes to initiate their transcription [94]. In the absence of light, greater transcription of the *Bmal1* and *Clock* genes is observed [94]. In the cytoplasm, PER and CRY proteins combine into a heterodimer, which inhibits further transcription of the genes responsible for their synthesis [92]. Additionally, melatonin is known to attenuate *Cry1* gene expression [93]. The combination of molecular clocks based on the promotion and inhibition of specific gene transcription, and regulation based on external stimuli, namely the presence or lack of blue light, allow the circadian rhythms to function properly. Melatonin acts as a regulator and synchronizer of these processes.

Melatonin is an endocrine, paracrine and autocrine hormone, so it has an effect on tissues distant from the synthesis site, on neighbouring cells, and directly on the cells that synthesize it [82,95]. The action of melatonin occurs through membrane G protein-coupled receptors (MT1, MT2, MT3), but also through nuclear receptors (RZR/RORα) and calmodulin [96,97]. The number of tissues in which MT1 and MT2 receptors have been detected demonstrates the broad spectrum of the compound's activity, including the liver, kidneys, retina, ovaries, testes, mammary glands, gallbladder, immune cells, cardiovascular system, exocrine pancreas, duodenal enterocytes, brain (hypothalamus, SCN, pituitary), blood vessels, gastrointestinal tract, adipocytes, and skin [98–100]. MT1 (MTNR1A), consisting of 350 amino acid residues, couples to pertussis toxin-sensitive G_i and toxin-insensitive $G_{q/11}$ proteins, inhibits cAMP response element-binding protein (CREB) phosphorylation, forskolin-stimulated cAMP and protein kinase A signaling, and increases potassium conductance through Kir internally rectifying channels [98,99]. MT2 (MTNR1B), consisting of 362 amino acid residues, inhibits cGMP formation and forskolin-stimulated cAMP production, reduces calcium-dependent dopamine release in the retina and activates protein kinase C (PKC) in the SCN [98,101].

The effect of melatonin is not limited to regulating circadian and seasonal rhythms. Melatonin also modulates the functioning of the immune system [102] and has anti-inflammatory properties [100,103–105]. Reduced concentration of melatonin is observed in many pathophysiological conditions and its supplementation may affect the course of disorders, such as neurodegenerative diseases, including Alzheimer's disease [106,107], primary headache disorders [108], obesity [105,109], diabetes mellitus type 2 [110,111] and hypertension [105,112].

Numerous studies indicate strong antioxidant properties of melatonin [17,77,113–116]. The molecule can cross the blood–brain barrier and its activity is not limited to the central nervous system (CNS) but it also affects other tissues distant from the site of synthesis [117]. The melatonin is soluble in both water and lipid environments, so it can act as an antioxidant in the aqueous environment inside the cells and in body fluids, as well as in plasma membranes of cells and cell organelles [118]. Research into the antioxidant properties of melatonin has confirmed that this hormone and its metabolites neutralize numerous ROS and RNS molecules, including H_2O_2, $^1O_2$, $O^{2\bullet-}$, peroxynitrite (ONOO-), as well as very reactive $OH^\bullet$ [17,119]. Melatonin metabolism products such as N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), N1-acetyl-5-methoxykynuramine (AMK) and 3-hydroxymelatonin (3-OHM) are also ROS and RNS scavengers [77,120,121]. The antioxidant properties of melatonin are due to its chemical structure, specifically the aromatic indole ring rich in delocalized electrons, a source of electrons in ROS and RNS neutralization reactions [122]. Melatonin may also indirectly affect the oxidative–antioxidant balance, stimulating the expression of genes encoding for some antioxidant enzymes. This effect is observed in the case of SODs, GPxs and GR [123,124]. The effect of melatonin and its chemical derivatives on the oxidoreductive balance is shown in Figure 1.

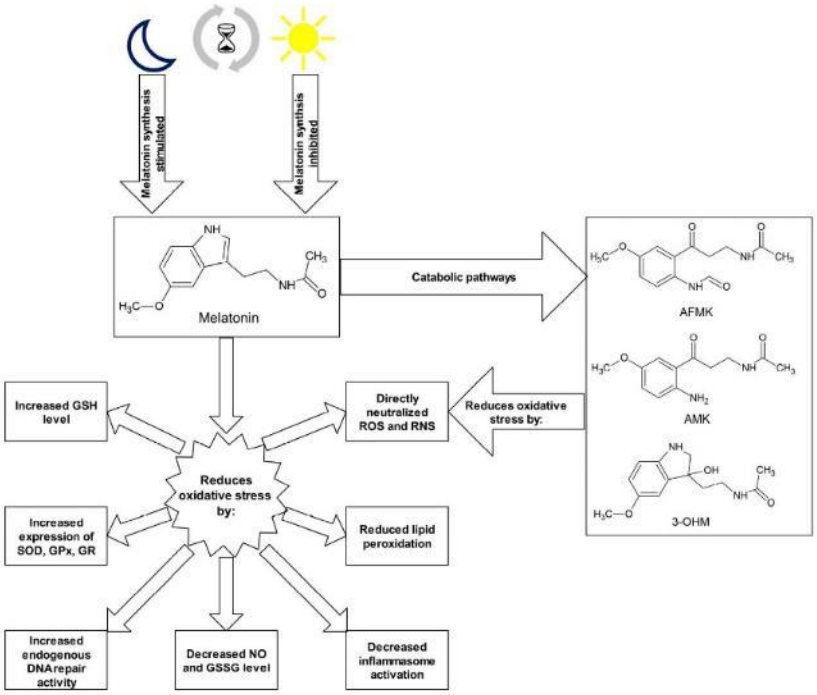

Figure 1. Melatonin and its metabolites as antioxidants. Abbreviations used: 3-OHM—3- hydroxymelatonin, AFMK—N1-acetyl-N2-formyl-5-methoxykynuramine, AMK—N1-acetyl-5-methoxykynuramine, GPx—glutathione peroxidase, GR—glutathione reductase, GSH—glutathione, GSSG—glutathione disulphide, NO—nitric oxide, RNS—reactive nitrogen species, ROS—reactive oxygen species, SOD—superoxide dismutase.

The study of the effect of melatonin on organisms exposed to IR has been described mainly in animal models [23]. Numerous studies have been conducted on the effect of melatonin pretreatment in the irradiation of specific parts of the body [125–130], indicating a reduction in lipid peroxidation, an improvement in enzymatic and non-enzymatic antioxidant defense, stimulation of the DNA damage response, and a reduction in the inflammatory state and histopathological changes. According to Fernandez-Gil et al. [125], melatonin may protect small intestine cells from toxic products formed during radiation therapy used in the oral mucosa. In this experiment, the researchers used adult male Wistar rats, which were given 3% melatonin gel (the total melatonin dose was 45 mg/day for 21 days). The rats were anesthetized prior to irradiation with a dose of 7.5 Gy/day (X-ray) for five consecutive days, where only the oral cavity was irradiated. Melatonin was applied in the oral cavity, starting 48 h before the first irradiation. After the sacrifice of animals, small intestine samples were taken for further analysis. Oral cavity irradiation resulted in small intestinal damage associated with oxidative stress. An increase in lipid peroxidation and nitrite/nitrate was observed, compared to non-irradiated controls. In the melatonin treated group, a significant increase in the activities and protein levels of GPx, GR, SOD2 and a substantial decrease in inflammasome activation in the small intestine were described, compared to the irradiated but non melatonin-treated group. In the Gurses et al. [126] study, Wistar rats were given a 50 mg/kg dose of melatonin (injected intraperitoneally) 15 min prior to irradiation of the anatomical area surrounding the heart. A dose of 18 Gy was used in one fraction. Six months after exposure to radiation, the rats were sacrificed, and histopathological preparations were performed to assess changes in the study and control groups. The use of melatonin prevented the development of vasculitis, and also reduced myocyte necrosis and cardiac fibrosis.

According to the experiments concerning whole body irradiation, melatonin administered both before and after IR exposure increased the survival rate of examined animals, reduced symptoms of acute irradiation disease, decreased histopathological changes, and improved the oxidative–antioxidant balance in the organism [130–136]. In a mouse model study, Vasin et al. [136] examined the effect of the whole body exposure to IR at a dose of 9.5–10 Gy at a power of 0.077–0.171 Gy/min given in one fraction. Melatonin was dissolved in water at a concentration of 5 mg/L and was administered from 3 to 30 days after the irradiation. The daily dosage of melatonin was changed with the onset of acute radiation sickness to 0.9–1.0 mg/kg and 1.2 mg/kg during recovery. The group of mice treated with melatonin showed less severe symptoms of acute radiation sickness and significantly higher survival was observed within 30 days of irradiation, compared to the control group. At the peak of radiation sickness (12 days after the irradiation), the average number of leukocytes in the group of mice supplemented with melatonin was higher than in the control group by 40%. Similar results were noted in a study conducted by Haddadi et al. [127] on adult male Wistar rats that received melatonin (100 mg/kg b.w.) intraperitoneally 30 min before irradiation and 5 mg/kg per day after irradiation for a maximum of 22 weeks. The animals were anesthetized and sacrificed at 4 and 24 h of irradiation, as well as 1, 3, 8, 16, 20, and 22 weeks after the treatment. The total radiation dose was 22 Gy at a dose rate of 1.8 Gy/min. The authors of the study indicate that the survival of animals from the melatonin-treated group was higher than the control group. In addition, in the melatonin group, lower expression of vascular endothelial growth factor (VEGF) and fewer histopathological changes were shown.

Research data on human studies is very limited. Vijayalaxmi et al. [137] performed an in vitro study on the effect of melatonin on radiated human peripheral blood samples and obtained very promising results. Approximately 15 min before the administration of 300 mg melatonin orally, volunteers gave a blood sample. The next blood collection took place 1 and 2 h after melatonin supplementation. Every blood sample was exposed in vitro to 1 Gy of gamma radiation at a dose rate of 1.087 Gy/min. The lymphocytes were examined to determine the amount of primary DNA damage. A significant increase in melatonin concentration in both serum and leukocytes was observed as early as 1 h after the administration of melatonin. The extent of primary DNA damage was reduced in both blood samples taken 1 and 2 h after melatonin administration, compared to the blood taken before melatonin supplementation. It is worth emphasizing that no negative effects of such a high dose of melatonin (300 mg) were observed. The dose of melatonin given to the subjects in the study is 30 times higher than the safe dose of the substance (10 mg/day) recommended in the treatment of sleep disorders, so further research is crucial to determining the appropriate amount of melatonin needed to protect people from the side effects of irradiation. Table 1 presents the summary of studies on the effects of melatonin on organisms exposed to ionizing radiation.

Table 1. Research on the impact of ionizing radiation on the generation of reactive oxygen species and the radioprotective role of melatonin.

Subjects	Melatonin Dosage (Route of Administration)	Time of Melatonin Administration	Radiation Dosage (Irradiation Area)	Outcomes	Reference
Adult male Sprague-Dawley rats	10 and 20 mg/kg (IP injection)	Immediately before and after irradiation	X-ray radiation of 8 Gy (whole body)	Melatonin reduced the levels of MDA and increased the GSH concentration.	[134]
Adult female Sprague-Dawley rats	30 and 5 mg/kg (IP injection)	30 min prior to irradiation and on the following days of experiment	Gamma radiation of 5 and 8 Gy (total cranial)	Melatonin decreased the formation of late side effects of radiation. Melatonin administration during radiotherapy protected ocular lenses against radiation-induced oxidative injuries.	[129]

Table 1. *Cont.*

Subjects	Melatonin Dosage (Route of Administration)	Time of Melatonin Administration	Radiation Dosage (Irradiation Area)	Outcomes	Reference
Adult male Wistar rats	100 and 5 mg/kg (IP injection)	30 min before irradiation and once a day per after irradiation	Gamma radiation 22 Gy (cervical segment of the spinal cord)	Melatonin increased survival rate and decreased histopathological changes.	[127]
Adult male Wistar rats	50 mg/kg (IP injection)	15 min prior to irradiation	18 Gy (anatomical area of the heart position)	Melatonin prevented the development of vasculitis, reduced myocyte necrosis and cardiac fibrosis.	[126]
Adult male Wistar rats	10, 20, and 10 mg/kg (IP injection)	Before irradiation, just after irradiation and 24h after irradiation	Gamma radiation 8 Gy, twice (whole body and abdominopelvic)	Melatonin administration inhibited primary spermatocyte degeneration.	[130]
Adult male Wistar rats	0.2 mg/day (IP injection)	Once a day for 14 days before irradiation	Gamma radiation 8 Gy (whole body)	Melatonin had a protective effect on suprarenal gland.	[131]
Adult male Wistar rats	5 and 10 mg/kg (IP injection)	30 min before irradiation	Gamma radiation 6 Gy (whole body)	Melatonin decreased hepatic MDA and nitric oxide (NO) levels.	[135]
Adult male Wistar rats	45 mg/day (PO)	Once a day for 21 days before irradiation	X-ray 7.5 Gy/day for five consecutive days (oral cavity)	Melatonin increased the activities and protein levels of GPx, GR, SOD2 and strongly decreased inflammasome activation.	[125]
Adult both sexes Wistar rats	100 mg/kg (IP injection)	For 5 days post radiation	Total dose of 7.2 Gy in two fractions (whole body)	Melatonin reduced MDA level, rates of oedema, necrosis, neuronal degeneration, and vasodilatation.	[133]
Adult male mice	From 0.9–1.0 to 1.2 mg/kg (PO)	From the third day after irradiation	Gamma radiation 9.5–10 Gy (whole body)	Melatonin reduced symptoms of acute radiation sickness, increased survival rate and leukocyte level.	[136]
Adult male Swiss albino mice	0.1 mg/kg/day (PO)	15 consecutive days prior to radiation	Gamma radiation 6, 8 and 10 Gy (whole body)	Melatonin reduced lipid peroxidation, glutathione disulphide (GSSG) level, deficit in the body and organ weight. Melatonin increased GSH level and survival rate.	[132]
Young adult male squirrels	250 mg/kg (SC injection)	Once a day for four weeks before irradiation	X-ray radiation of 2.06 Gy (abdominal, near the splenic region)	Long term melatonin treatment protected the splenocytes and modulated endogenous DNA repair activity.	[128]
In vitro, human blood	300 mg (PO)	1 h before irradiation of blood sample	Gamma radiation 1 Gy (blood sample)	Melatonin reduced primary DNA damage.	[137]

Abbreviations used: IP injection—intraperitoneal injection, PO—oral administration, SC injection—subcutaneous injection.

To complete the picture of the relationship between melatonin and IR, it should be emphasised that melatonin has been found to radiosensitize cancer cells in a selective manner [69,138–140]. Melatonin's ability to sensitize cancer cells to irradiation, along with its radioprotective properties, makes it an ideal adjuvant in radiotherapy [69]. In the case of neck squamous cell carcinoma (HNSCC cell lines), melatonin (0.1, 0.5, 1.0, and 1.5 mM melatonin combined with 8 Gy irradiation) was described to enhance radiation cytotoxicity by stimulating mitochondrial ROS generation, apoptosis and autophagy [138]. It was also observed that melatonin (pretreatment with 1 mmol/L melatonin for 2 h) effectively inhibited cellular proliferation of the human colorectal carcinoma cell line HCT 116, and decreased colony formation rate and cell migration counts following IR exposure (gamma rays, 0–8 Gy) [140]. This effect was associated with activation of the caspase-dependent apoptotic pathway,

cell cycle arrest in G2/M, and an impaired DNA double-strand break repair. Moreover, in the study, it was shown that melatonin in combination with IR treatment significantly suppressed tumor cell growth in colorectal tumor xenografts. Analogous results have been confirmed in breast cancer [139]. What remains unestablished is the use of melatonin combined with IR in patients, including the effects of the treatment, the time-lapse between melatonin administration and radiotherapy, as well as the optimal dosage of melatonin in humans exposed to IR. Further patient-based studies, such as pre-clinical and randomized control trials are needed to explain all uncertainties.

4. Vitamin D—Function and Antioxidant Effect

Vitamin D is a group of organic chemical compounds belonging to the group secosteroids, among which calcitriol (1,25-dihydroxycholecalciferol) performs the highest biological (hormonal) activity [26,141]. Vitamin D is currently at the center of research interest for many scientists due to its widespread deficiency, reaching about 30–50% on a global scale, especially in older age groups [19,142]. Many scientists have been involved in the research on the discovery and description of vitamin D properties. The largest contribution was made by Sir Edward Mellanby [143], Elmer McCollum [144] and Adolf Windaus [145], who in 1928 received the Nobel Prize for their work on vitamin D [146]. Vitamin D comes from both external sources and from the body's own synthesis [147]. Two forms of vitamin D are taken with food, namely cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) [148]. Fatty fish (such as salmon, mackerel, herring), meat, egg yolks, milk and butter are sources of cholecalciferol, while fungi, yeast and some plants contain ergocalciferol [149,150]. Vitamin D taken from food sources is only a fraction of the daily requirement for this compound [151]. The first stage of calcitriol biosynthesis is the transformation of 7-dehydrocholesterol in the skin under the influence of UV radiation at a wavelength of approximately 290–315 nm (UVB) [152,153]. For that reason, vitamin D is sometimes called the "sunshine vitamin". Excessive exposure to UV radiation does not cause the formation of toxic amounts of previtamin D because it photoisomerises into two biologically inert products, lumisterol and tachysterol [154]. Previtamin D undergoes spontaneous isomerisation to provitamin D (cholecalciferol) under the influence of body temperature [155]. Then, cholecalciferol both formed in the skin and originating from dietary sources binds to a specific transport protein, vitamin D-binding protein (DBP), and is transported to the liver [156]. Hydroxylation with cytochrome P450 CYP2R1 enzymes occurs in the liver. The product of this reaction, 25-hydroxyvitamin D, binds to DBP and is transported to the kidney for subsequent hydroxylation by the enzyme CYP27B1 [157]. The end product of this pathway is the hormonally active form of vitamin D, calcitriol, which is stored mainly in adipose tissue [158,159]. The vitamin D receptor (VDR) belongs to a subfamily of nuclear receptors that act as transcription factors [160]. VDR is heterodimerized with the retinoid-X receptor (RXR), which causes a change in its spatial conformation. The resulting heterodimer binds to appropriate promoter sites of vitamin D-dependent genes [161]. VDR occurs in almost all cells and tissues, including the skeletal system, cells involved in immune modulation, brain, heart, skin, gonads, prostate, breast and gut [162]. Originally, calcitriol was considered to be associated only with calcium-phosphate metabolism by cooperating with parathyroid hormone and the skeletal system, stimulating the absorption of dietary calcium from the gastrointestinal tract, promoting renal tubular reabsorption of calcium, and inducing the release of calcium from bones [163]. However, the role of vitamin D is known to be much greater and its deficiency is associated not only with diseases of the skeletal system, such as osteomalacia or osteoporosis in adults and rickets in children [164], but also with depression [165], cancer [152], adverse cardiovascular risk profile [166], obesity [24], type 2 diabetes mellitus [25] and autoimmune thyroid disease [167]. The reference vitamin D concentration range is 30–50 ng/mL (75–125 nmol/L) [168,169]. It should be added that this is the level of 25-hydroxyvitamin D, not calcitriol, that is tested because of lower test costs, higher analyte stability and good correlation with the concentration of the hormonally active form in the organism [168,169].

Vitamin D is thought to have antioxidant properties although involved mechanisms have not been fully described yet and further research is required [18,170]. Vitamin D, acting through its

nuclear receptors, can stimulate the expression of genes coding for antioxidant enzymes such as SODs and GPxs [171]. It has also been confirmed that after exposure of the skin to UV radiation, calcitriol and its precursors increase p53 levels, which reduces intracellular ROS [172]. In addition, calcitriol has been shown to induce the synthesis of metallothioneins, which are ROS scavengers [172]. Tang et al. [173] reported that MDA levels negatively correlated with serum vitamin D levels in patients with non-segmental vitiligo. Furthermore, the researchers pointed out that vitamin D protected human melanocytes against ROS by activation of Wnt/β-catenin signaling. In addition, Jain et al. [174] showed a positive link between vitamin D and GSH concentrations, as well as a reduction in the levels of pro-inflammatory cytokines (monocyte chemoattractant protein 1 and interleukin 8), which lead to reduced ROS generation. In this study, U937 monocyte cells were treated with calcitriol at the concentration of 0, 10, and 25 nM for 24 h. Similar results were described by Dzik et al. [175]. In their study, patients, qualified for lumbar spine surgery utilizing static or dynamic implants, were supplemented with 25-hydroxyvitamin D at a daily dose of 3200 IU (equal to 80 µg) for 5 weeks. Vitamin D supplementation to appropriate serum levels reduced oxidative stress in skeletal muscle. The patients with prior vitamin D deficiency showed increases in Cu/ZnSOD and GPx activities in paraspinal muscles after supplementation. Chen et al. [176] tested 10α-hydroxylase knockout mice ($1\alpha(OH)ase^{-/-}$) supplemented with calcitriol at a dose of 1 µg/kg. The authors noted that low calcitriol levels were associated with higher oxidative stress. In addition, calcitriol regulated the expression of nuclear factor-erythroid-2-related factor 2 (Nrf2), which controls antioxidant and detoxification enzymes. In response to reduced ROS levels, SOD2 activity decreased. Sepehrmanesh et al. [177] confirmed that vitamin D supplementation led to a significant increase in GSH concentrations. Patients with major depressive disorder were supplemented with 25-hydroxyvitamin D at a weekly dose of 50,000 IU (equal to 1.25 mg) for 8 weeks. In addition to the increase in the GSH level, there was also a significant increase in total antioxidant capacity (TAC). On the other hand, Barzegari et al. [178] did not observe significant changes in SOD, CAT, and GPx activities, as well as in the MDA and TAC levels, despite a 8-week calcitriol supplementation at 50,000 IU once a week. The study was based on a double-blind, randomized, placebo-controlled clinical trial, involving 50 patients with type 2 diabetes and nephropathy. Undoubtedly, further studies on the antioxidant function of vitamin D are required. The main mechanisms of vitamin D action as an antioxidant are shown in Figure 2.

Considering the broad spectrum of vitamin D action in the organism, it has been identified as a potential protective agent against radiation-induced damage [179]. However, the analysis of the available literature indicates very limited research data on the radioprotective role of vitamin D in the context of IR action. It was observed that the administration of vitamin D_3 (0.7 µg of vitamin D_3 or 28 IU/100 g body mass) to chronically irradiated Wistar rats (0.01 Gy per day for 30 days) induced the normalization of carbohydrate metabolism by improving the activities of glycolytic enzymes in erythroid and myeloid bone marrow cells [180]. In the in vitro study of Müller et al. [181], it was found that the cell growth and clonogenic survival of irradiated keratinocytes (cell line HaCaT), pretreated with calcitriol, were significantly increased when compared to the untreated cells after IR exposure. In the experiment, exponentially growing HaCaT cells were irradiated with X-rays (total dose of 0 to 7.5 Gy was delivered with a dose rate of 1 Gy/min). To assess the vitamin D effect, the HaCaT cells were incubated with 10 nmol/L $1\alpha,25(OH)_2D_3$ for 48 h before irradiation. It was demonstrated that vitamin D improved cell growth and survival, as well as inhibited the radiation-induced up-regulation of adhesion molecule expression on human keratinocytes. These results were confirmed by Langberg et al. [182], who proved that treatment with calcitriol (100 nmol/L 24 h before and for 24–48 h after IR) inhibited caspase-dependent and -independent programmed cell death occurring within 48 h of irradiation, increased the colony formation capacity, attenuated radiation-induced increase in matrix metalloproteinase-9 and mRNA levels in irradiated (4 Gy with a dose rate 2 Gy/min) HaCaT keratinocytes. The same cell line was also the subject of the Trémezaygues et al. [183] experiment. It was observed that the pretreatment of HaCaT-keratinocytes with $1,25(OH)_2D_3$ (100 nmol/L) over 48 h differentially modulated harmful effects of IR (1–5 Gy) in a dose- and time-dependent manner,

indicating a protective effect of vitamin D against relatively low IR (1–2 Gy). A study on the cell culture of human umbilical vein endothelial cells (HUVEC), conducted by Marampon et al. [184], showed the protective effect of vitamin D against damage caused by ROS generated under the influence of IR. Cell cultures were preincubated in a solution with the addition of vitamin D in concentrations of 25, 50, 75 and 100 nmol/L for 24 h, then transferred to the growth medium and irradiated with X-rays (total dose of 0 to 8 Gy was delivered with a dose rate of 1.3 Gy/min). Vitamin D preincubation reduced the amount of ROS by the protection of proliferating and quiescent cells via the regulation of the mitogen-activated protein kinase (MAPK) pathway, prevented apoptosis by activating signal-regulated kinases (ERKs) in proliferating HUVEC, and inhibited p38, associated with ageing in quiescent cells.

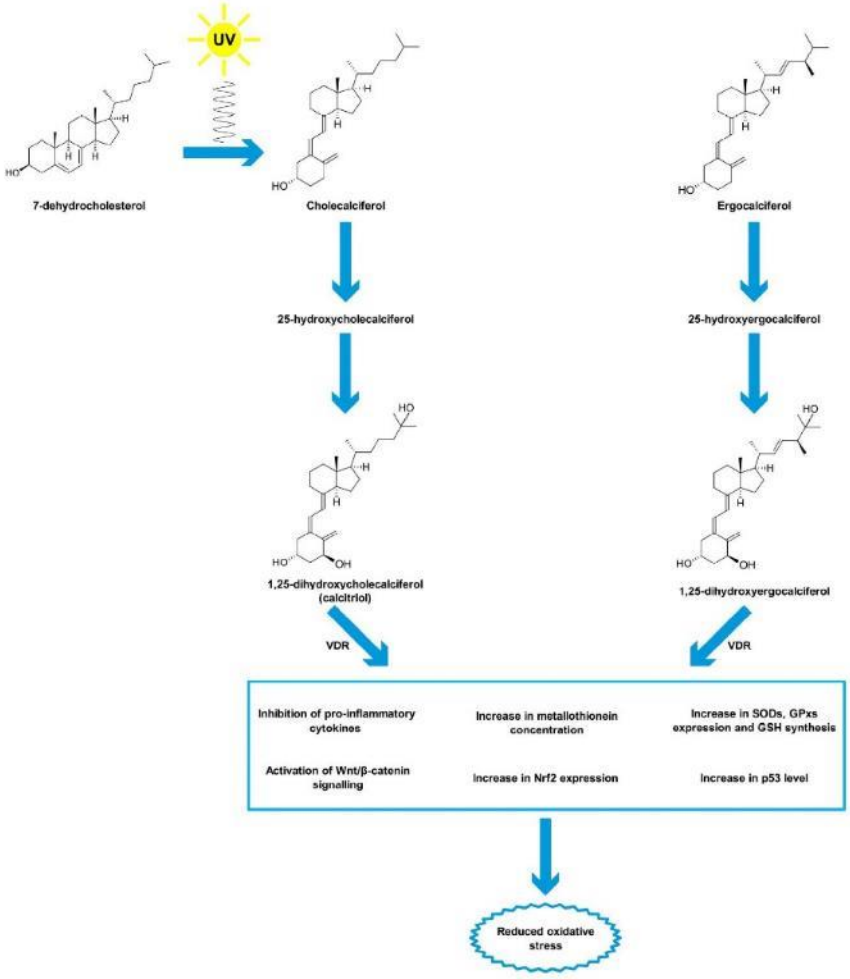

Figure 2. Antioxidant properties of vitamin D. Abbreviations used: GPxs—glutathione peroxidases, GSH—glutathione, Nrf2—nuclear factor-erythroid-2-related factor 2, SODs—superoxide dismutases, VDR—vitamin D receptor.

As with melatonin, vitamin D and its analogues have been found to selectively radiosensitize cancer cells, including breast and non-small cell lung tumor cells [185–189], which makes it a promising adjuvant in radiotherapy, enhancing the treatment effect and reducing side effects. In irradiated (5 times of 2 Gy administered over a period of 3 days) MCF-7 breast tumor cells, pretreatment with a hormonally

active form of vitamin D (100 nmol/L 1,25$(OH)_2D_3$ for 72 h) promoted autophagy, sensitized the cells to IR and suppressed the proliferative recovery occurring after radiation alone [185]. This effect was not observed in the BT474 breast tumor cell line with low-level expression of VDR, suggesting a receptor-mediated action of calcitriol. Moreover, similar responses were not detected in a model of normal human fibroblasts [187]. The promotion of an enhanced response to radiation by 1,25-D_3 in non-small cell lung cancer cells has been found to be mediated by VDR, tumour protein p53 and AMPK pathways [188]. Normal human bronchial cells and cardiomyocytes were not radiosensitized by vitamin D in this study [188].

Interestingly, it was found that chronic exposure to IR affected the vitamin D_3 active form level and caused modifications of enzymes involved in vitamin D metabolism [190]. In accordance with this study, Kaminskyi et al. [191] described significantly lower vitamin D concentrations among the populations of radiologically contaminated regions of Chernivtsi oblast due to the Chornobyl catastrophe, compared to those in the uncontaminated Ukrainian cities of Charnivtsi and Vyzhnytsia. Therefore, the deficiency of vitamin D in patients during radiotherapy or in medical professionals chronically exposed to low IR doses should be taken into consideration in further research on the supplementary treatment.

5. Conclusions

This review points to the important role of ionizing radiation as an inducer of oxidative stress, which occurs in the pathogenesis and the course of many diseases. ROS are not only generated during medical procedures that require the use of IR but also when the organism is exposed to sunlight and background radiation present in the environment. The endogenous synthesis of two compounds with antioxidant potential described in this paper, namely melatonin and vitamin D, depends on the presence of light (visible or UV). Numerous studies emphasize the role of melatonin as an antioxidant and its protective effects against IR damage. This hormone both directly and indirectly neutralizes ROS. In the case of vitamin D, further experiments are required that could align its antioxidant mechanisms and protection against IR, as previous publications show conflicting findings. As a result of ever-growing use of IR in medicine, more and more people are being exposed to IR at different doses, including several dozen Gy during radiotherapy. Thus, supportive therapies for both patients and medical professionals are of first importance. Synthetic radioprotective compounds have a limited use because they often induce some undesirable side effects, especially at doses required to achieve maximal radioprotection. According to the research data presented in the review, melatonin could be the best candidate for a radioprotectant in people. Less is known about vitamin D. However, the results have been promising so far. The supporting supplementation with both substances seems to be also important in the context of common deficiencies in melatonin and vitamin D in modern societies, which may contribute to the severity of adverse side effects of medical IR exposure. Moreover, both substances have been found to selectively radiosensitize cancer cells, which makes them promising adjuvants for enhancing the anticancer effect of radiotherapy and improving therapeutic outcomes. Thus, in light of existing studies, melatonin and vitamin D are worth considering as agents for protecting professionals exposed to radiation and patients diagnosed or treated with radiation. Nevertheless, more research is needed in this area, especially in humans. Most importantly, appropriate doses of melatonin and vitamin D, effective in protecting against radiation and safe for people, should be established and tested in clinical trials.

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Abbreviations

$^1O_2$	singlet oxygen
3-OHM	3-hydroxymelatonin
4-HNE	trans-4-hydroxy-2-nonenal
AAAD	aromatic L-amino acid decarboxylase
AANAT	aralkylamine *N*-acetyltransferase
AFMK	N1-acetyl-N2-formyl-5-methoxykynuramine
AMK	N1-acetyl-5-methoxykynuramine
ASMT	acetylserotonin O-methyltransferase
CAT	catalase
CNS	central nervous system
CREB	cAMP response element-binding protein
DBP	vitamin D-binding protein
DCF	2′,7′-dichlorodihydrofluorescein
DCFH	2′,7′-dichlorofluorescin
DCFH-DA	2′,7′-dichlorofluorescin diacetate
ERKs	signal-regulated kinases
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
HUVEC	human umbilical vein endothelial cells
IP	intraperitoneal injection
IR	ionizing radiation
LET	linear energy transfer
MAPKs	mitogen-activated protein kinase pathway
MDA	malondialdehyde
Nrf2	nuclear factor-erythroid-2-related factor 2
$O_2^{\bullet-}$	superoxide anion radical
$OH^{\bullet}$	hydroxyl radical
ONOO-	peroxynitrite
PKC	protein kinase C
PLP	pyridoxal phosphate
PO	oral administration
RNS	reactive nitrogen species
ROS	reactive oxygen species
RXR	retinoid-X receptor
SAM	S-adenosyl methionine
SC	subcutaneous injection
SCN	suprachiasmatic nucleus
SOD	superoxide dismutase
TAC	total antioxidant capacity
TPH	tryptophan hydroxylase
VDR	vitamin D receptor

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14.1.2. Publikacja II

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Research Article

Parameters of Oxidative Stress, Vitamin D, Osteopontin, and Melatonin in Patients with Lip, Oral Cavity, and Pharyngeal Cancer

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Lip, oral cavity, and pharyngeal cancers (LOCP) constitute a group of rare neoplasms with unfavorable prognosis. So far, not much is known about the role of vitamin D and oxidative stress in the pathogenesis of LOCP in the European population. The aim of the study was to determine the concentrations of vitamin D, osteopontin, melatonin, and malondialdehyde (MDA) as markers of oxidative stress and/or inflammation, as well as the activities of antioxidant enzymes in the course of LOCP. The vitamin D, melatonin, and osteopontin concentrations in blood serum, the MDA levels in erythrocytes and blood plasma, and the activities of superoxide dismutase (SOD-1), catalase (CAT), and glutathione peroxidase (GPx) in erythrocytes were measured in blood samples taken from 25 LOCP patients of middle age (YCG), 20 LOCP elderly patients (OCG), and 25 healthy middle-aged volunteers. In both cancer groups, decreases in vitamin D and CAT, as well as increases in osteopontin and blood plasma MDA, were observed. An increase in GPx activity in YCG and a decrease in melatonin level in OCG were found. The results indicate the vitamin D deficiency and disturbed oxidant-antioxidant homeostasis in LOCP patients. Osteopontin seems to be associated with LOCP carcinogenesis and requires further research.

1. Introduction

Lip, oral cavity, and pharyngeal cancers (LOCP) belong to the most common head and neck cancers worldwide. Moreover, scientific analyses indicate that the incidence of this type of neoplasm will increase in the future. According to data, in 2012, 529,500 new cases of LOCP were detected worldwide, which corresponds to 3.6% of all cancers [1, 2]. Mortality in 2012 from this group of neoplasms was estimated at 292,300 cases, which corresponds to 3.6% of deaths due to neoplastic diseases [1, 2]. Projections for 2035 show a 62% increase in the number of cases to around 856,000 cases annually [1]. Cancers of lip, oral cavity, and pharynx are considered together because they are characterized by similar risk factors. Neoplasms belonging to this group affect male much more often than female, and the age group 50-70 years is particularly vulnerable [3–5]. In addition, this type of cancer is especially common in south-central Asia [1]. The main risk factors for the development of LOCP cancer include smoking [6], alcohol consumption [7, 8],

infections caused by Epstein-Barr virus (EBV) [9], and *human papillomavirus* (HPV) [10]. Early diagnosis and treatment initiation significantly increases patient survival; unfortunately, most cases are detected in the advanced stage of the disease, which lowers the 5-year survival rate to about 40% [3].

Pathogenesis of lip, oral cavity, and pharyngeal cancers is still not clear and is believed to be multifactorial in origin. Few studies indicate the participation of extracellular matrix and fibroblast changes, immune system, and oxidative stress in the pathogenesis of oral submucous fibrosis, leading to cancer of the oral cavity [11]. Additionally, molecular pathogenesis of head and neck cancer is associated with deletion in region located at chromosome 9p21–22 containing p16 tumor suppressor gene [12]. An inherent element of carcinogenesis and neoplastic disease is the increased generation of reactive oxygen species (ROS) [13–18]. Moreover, cancer cells synthesize and secrete cytokines that modulate inflammation and significantly increases ROS generation [19]. The disturbance of homeostasis by ROS generated in exceeding of physiological capacity of adaptation leads to oxidative stress [20–22]. Although the disease has a specific localization, systemic symptoms of oxidative stress are observed in patients [23, 24]. ROS are a group of chemical molecules which are characterized by the presence of nonpair electrons and high chemical reactivity [25]. The most important ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$) [20, 25]. Due to high chemical reactivity, ROS modify proteins, lipids, and genetic material [26, 27]. The effect of lipid peroxidation is damage to cell membranes, and the main markers of this process is malondialdehyde (MDA) and 4-hydroxynonenal [28]. Antioxidants play an important role in maintaining the redox balance [29]. Endogenous antioxidants include enzymes such as superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidases (GPxs) [30]. The antioxidant defense is also constituted by small endo- and exogenous biomolecules such as vitamins A, C, and E, melatonin, and glutathione (GSH) [31, 32]. The role of vitamin D as an antioxidant remains ambiguous due to inconclusive research results [33].

Vitamin D is a biomolecule with pleiotropic properties. Calcitriol (1,25-dihydroxycholecalciferol) plays the most important role among the group of compounds called vitamin D [34]. Chemical compounds belonging to this group can be absorbed with food, most often in the form of cholecalciferol and ergocalciferol [35, 36]. Another source of vitamin D is the endogenous synthesis under the influence of ultraviolet radiation (UV) and hydroxylases found in the liver and kidney. The substrate for this process is 7-dehydrocholesterol [37]. Despite endogenous synthesis and the presence of vitamin D in food products, vitamin D deficiencies affect a significant part of the population worldwide [38–40]. Vitamin D is involved in the regulation of calcium-phosphate homeostasis, which is of particular importance for the functioning of the skeletal system [41]. Calcitriol, acting through the vitamin D receptor (VDR), reduces oxidative stress by increasing the level of SODs, GPxs, and GSH expression [33, 42]. Moreover, it was observed that vitamin D reduced the secretion of proinflammatory cytokines, decreasing the level of oxidative stress [43]. The role of vitamin D and its derivatives in cancer is still under investigation. The results of the studies conducted so far are not unequivocal. Some researchers point to a significant role of vitamin D deficiency on cancer mortality, while no effect on morbidity [44–46]. On the contrary, some studies do not link cancer with vitamin D levels [47].

Research indicates a positive correlation between the concentration of vitamin D and osteopontin [48, 49]. Osteopontin is a glycoprotein secreted by osteoblasts and osteoclasts involved in shaping the correct bone structure [50]. The presence of this glycoprotein is not limited to the skeletal system. Osteopontin was found in many tissues and body fluids such as brain astrocytes, kidney, smooth muscle, saliva, and milk [50–52]. Tumor cells of lung, gastric, prostate, ovarian, and colorectal cancer were also found to secrete osteopontin [50]. In the course of neoplastic diseases, an increase in the concentration of osteopontin was observed along with an increase in the level of proinflammatory cytokines [53–55]. Osteopontin was found to be a modulator of the immune response [54]. The relationship between osteopontin and oxidative stress has not been analyzed frequently. The results of the research indicate that the concentration of osteopontin positively correlates with the markers of increased oxidative stress [56–59].

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthesized and secreted by pinealocytes in the circadian rhythm [60, 61]. Gastrointestinal tract, lymphocytes, ovaries, skin, and retina are sources of extrapineal melatonin independent of the circadian rhythms [62]. The melatonin molecule contains an indole ring that neutralizes ROS directly [63, 64]. Moreover, research indicates that melatonin decreases the level of ROS by activating the silent information regulator 1 (SIRT1) pathway [65]. Melatonin may also indirectly affect the oxidant–antioxidant balance, stimulating the expression of genes encoding antioxidant enzymes, such as SODs and GPxs [66]. In addition to its direct and indirect action, melatonin inactivates ROS through its metabolites, namely, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK) [67]. The pleiotropic role of melatonin as endo- and paracrine hormone has been analyzed in carcinogenesis [68, 69]. Scientists indicated an oncostatic role of melatonin in breast, ovarian, prostate, oral, gastric, and colorectal tumors [70]. One of the mechanisms of the oncostatic action of melatonin seems to be based on the reduction of ROS levels [71, 72]. Moreover, melatonin was found to hinder angiogenesis and increase apoptosis of cancer cells [73]. However, little is known about the role of melatonin in LOCP cancer.

So far, only a few studies on the activity of antioxidant enzymes and lipid peroxidation markers in patients with LOCP have been conducted. The results described in the literature are not unequivocal. A decrease in SOD, CAT, and GPx activities with an increase in MDA concentration has been most frequently reported [74–76]. Still, according to some other research, no changes in the activity of antioxidant enzymes in the course of LOCP have been observed

[77]. The relationship between the activity of antioxidant enzymes and vitamin D and osteopontin and melatonin has not been studied. Examining the mechanisms related to vitamin D, osteopontin, melatonin, and oxidative stress in the course of LOCP seems to be important for a better understanding of the pathophysiology of this type of cancer, as well as for finding new methods of treatment and prevention. Thus, the aim of this study was to determine the activities of selected antioxidant enzymes, as well as the concentrations of vitamin D, osteopontin, melatonin, and MDA in the course of lip, oral cavity, and pharyngeal cancer.

2. Materials and Methods

2.1. Participants. The study involved 45 patients diagnosed with *carcinoma* in situ of lip, oral cavity, or pharynx according to the International Classification of Diseases–11th Revision (ICD-11)–2E60.0 [78]. The patients were divided into two groups according to their age, namely, younger cancer group (YCG) and older cancer group (OCG). The classification of patients into age groups was based on the United Nations report, which stated that old age begins after the age of 65 [79]. The participants were treated at the Oncology Center, Prof. Franciszek Łukaszczyk Memorial Hospital, Bydgoszcz, Poland. The patients were referred for planning radiotherapy using positron emission tomography–computed tomography (PET/CT) after FDG ([18F]-fluorodeoxyglucose) administration. The patients with G1 squamous cell carcinoma, G2 squamous cell carcinoma, and nonkeratinizing G2 squamous cell carcinoma in histopathological analysis were included in the study. The patients with other grade and type of tumor were excluded from the study. The control group consisted of 25 healthy volunteers. The criteria of exclusion from the control group were associated conditions known to be caused by or to result in oxidative stress or involving disruption of the oxidant-antioxidant equilibrium (cancer, diabetes, cardiovascular, and infectious diseases). A survey was conducted among the people qualified for the study. The questions concerned tobacco addiction and vitamin D supplementation. The characteristics of the study and control groups are presented in Table 1. The study was approved by the Bioethics Committee of the Nicolaus Copernicus University in Toruń functioning at Collegium Medicum in Bydgoszcz, Poland (consent no. KB 221/2018).

2.2. Study Design. The patients were eligible for the study on the day of planning for radiotherapy. Blood samples were collected by qualified medical personnel in the morning (between 8:00 AM and 9:00 AM) after overnight fasting from median cubital vein just prior to the administration of the radiopharmaceutical. Every blood sample was collected into two polypropylene tubes. First tube (vol. 6 mL) contained a clotting activator to obtain blood serum, and another tube (vol. 10 mL) was covered with K_2EDTA to obtain blood plasma. The tubes were immediately transported under reduced temperature condition to the laboratory for centrifugation (6,000 g for 10 min at 4°C). After centrifugation, blood serum and plasma were separated and stored at -80°C for further analysis. The blood morphotic elements remaining after centrifugation were washed three times with a phosphate-buffered saline (PBS) at a ratio of 1 : 3 and each time centrifuged (6,000 g for 10 min at 4°C) to remove leukocytes and thrombocytes. The red blood cells obtained in this method were mixed with the PBS solution to obtain erythrocytic suspension with a 50% hematocrite index.

2.3. Biochemical Analysis. The activity of selected antioxidant enzymes was determined in erythrocytic suspension with the use of spectrophotometric methods. Activity of Zn/Cu-superoxide dismutase (SOD-1; EC 1.15.1.1) was assayed according to the Misra and Fridovich method [80]. Analysis was based on the inhibition of adrenaline oxidation to adrenochrome in alkaline solution at 37°C, which induced a change in the absorbance at 480 nm. Activity of SOD-1 was expressed in IU/g Hb. CAT (EC 1.11.1.6) activity was determined with the use of the Beers and Sizer method [81] by measuring the decrease in the absorbance at 240 nm of a solution of hydrogen peroxide decomposed by the enzyme at 37°C. CAT activity was expressed in IU/g Hb. Activity of cytosolic glutathione peroxidase (GPx; EC 1.11.1.9) was assessed using the method of Paglia and Valentine [82]. The principle of the method for measuring GPx activity is based on the ability of the enzyme to reduce hydrogen peroxide with a simultaneous oxidation of GSH as a coenzyme at 37°C, measured at 340 nm. Activity of GPx was expressed in IU/g Hb. Erythrocytic and plasma MDA concentrations were determined with the method of Buege and Aust [83] in the modification of Esterbauer and Cheeseman [84]. The MDA concentration was expressed as the concentration of thiobarbituric acid-reactive substances (TBARS), measured at 532 nm at room temperature. The MDA concentration in erythrocytes was expressed in nmol/g Hb and in blood plasma in nmol/mL. Hemoglobin (Hb) concentration was evaluated using the Drabkin method [85]. Hemoglobin and selected hemoglobin derivatives under the influence of potassium ferricyanide are oxidized to methemoglobin. The absorbance is measured at 540 nm at room temperature.

Serum concentrations of melatonin, vitamin D, and osteopontin were determined with commercially available enzyme immune assay kits. The kits were used accordingly: an enzyme-linked immunosorbent assay kit for melatonin (Cloud-Clone Corp., Houston, TX, USA), a competitive enzyme-linked immunosorbent assay kit for 25(OH)-vitamin D (Immundiagnostik AG, Bensheim, Germany), and a sandwich enzyme-linked immunosorbent assay kit for human osteopontin (BioVendor, Brno, Czech Republic). The measurements were made according to manufacturer's instructions. The enzyme immune assay kits used in the study contain the reagents necessary for the study, standard concentration analytes, blank, and control samples. The principle of the assay is to bind the antigen by specific anti-human monoclonal antibodies that coat the wells of microplates found in the kits. The antigen concentration was determined from the calibration curve. The concentrations of melatonin, vitamin D, and osteopontin were expressed in pg/mL, ng/mL, and nmol/L, respectively.

Table 1: Anthropometric and clinical characteristic of patients with lip, oral cavity, or pharyngeal cancer and healthy volunteers (control group). Each value is mean ± S.E.M. YCG: younger cancer group; OCG: older cancer group, $^*p < 0.05$ vs. OCG.

Parameter	YCG	OCG	Control group
n (male/female)	25 (15/10)	20 (14/6)	25 (11/14)
Age [yrs]	58.24 ± 1.29*	69.7 ± 1.49	55.36 ± 1.17*
Body mass [kg]	72.53 ± 3.95	71.39 ± 3.52	71.02 ± 2.22
Height [cm]	168.92 ± 1.84	168.55 ± 1.52	169.88 ± 1.72
BMI [kg/m^2]	25.09 ± 0.99	25.00 ± 1.02	24.50 ± 0.47
Current smoker (y/n)	7/18	5/15	4/21
Vitamin D supplementation (y/n)	5/20	4/16	8/17

2.4. Statistical Analysis. Statistical analysis was performed using the Statistica 13.3 (TIBCO Software Inc.). The results were presented as means ± S.E.M. Statistical analysis included Student's t-test for independent samples, for the comparison of study group and control group, Shapiro-Wilk test to test hypothesis of normal distribution, Levene's test to asses homogeneity of variances. Pearson's correlation coefficient was used to quantify the relationship between the parameters measured. The level of significance was set at $p < 0.05$.

3. Results

Anthropometric and clinical characteristic of patients with lip, oral cavity, or pharyngeal cancer and healthy group were presented in Table 1. No significant differences were found between YCG and control group. There was a statistically significant difference in the age of the patients between the YCG, control group, and OCG.

The SOD-1 activity was similar in all groups and amounted to 738 ± 21 IU/g Hb in YCG, 735 ± 19 IU/g Hb in OCG, and 755 ± 18 IU/g Hb in control group. The statistically lower CAT activity was observed in YCG ($59.34 \pm 2.6810^4 \times$ IU/g Hb) and OCG ($58.11 \pm 2.4710^4 \times$ IU/g Hb) groups compared to the control group ($70.19 \pm 1.8710^4 \times$ IU/g Hb). The activity of GPx in YCG was 8.54 ± 0.75 IU/g Hb and was significantly higher compared to the control group (6.45 ± 0.58 IU/g Hb). In OCG, the mean GPx activity was 7.44 ± 0.76 IU/g Hb. The results concerning the activity of antioxidant enzymes are presented in Figure 1.

There were no statistically significant differences in the concentration of erythrocytic MDA in YCG (27.52 ± 4.23 nmol/g Hb), OCG (31.74 ± 6.21 nmol/g Hb), and control group (25.66 ± 1.98 nmol/g Hb). Significantly higher concentrations of MDA were observed in the plasma of YCG and OCG patients, amounting to 0.55 ± 0.02 and 0.56 ± 0.01 nmol/mL, respectively. In the control group, the plasma MDA level was 0.42 ± 0.02 nmol/mL. The melatonin level in OCG was 62.12 ± 4.70 pg/mL and was significantly lower than in the control group (84.33 ± 6.54 pg/mL). The concentration of melatonin in YCG was 72.83 ± 5.80 pg/mL , and no statistically significant differences were observed in relation to the other study groups. Figure 2 shows the concentration of MDA and melatonin in the graphs.

The concentration of 25(OH)-vitamin D in the serum of the healthy people was 82.57 ± 4.28 ng/mL. Considerably lower values were observed in YCG and OCG, amounting to 63.55 ± 7.36 ng/mL and 48.42 ± 7.09 ng/mL, respectively. The levels of osteopontin in the LOCP patient groups were significantly higher compared to the healthy group. The concentration of osteopontin in YCG and OCG was 16.04 ± 2.69 nmol/L and 14.98 ± 2.48 nmol/L, respectively, while in the control group, it was 9.78 ± 0.72 nmol/L. Figure 3 shows the graphs of 25(OH)-vitamin D and osteopontin concentration in the study groups.

The obtained data were also tested for the presence of correlations. In YCG, statistically significant negative correlations were observed between GPx and body mass ($r = -0.51$, $p = 0.009$), GPx and BMI ($r = -0.52$, $p = 0.007$), and CAT and vitamin D ($r = -0.42$, $p = 0.036$), whereas a significant positive correlation was found between plasma MDA and osteopontin ($r = 0.53$, $p = 0.007$) (see Figure 4). In the control group, a positive correlation between SOD-1 and erythrocytic MDA ($r = 0.44$, $p = 0.026$) and a negative correlation between body mass and erythrocytic MDA ($r = -0.39$, $p = 0.049$) were observed (Figure 5). No statistically significant correlations were found in OCG.

4. Discussion

As mentioned earlier, LOCP are relatively rare compared to other neoplastic diseases. Due to the small number of patients with such a diagnosis, there are few studies in which the mechanisms of antioxidant defense and the concentration of melatonin, osteopontin, and vitamin D were analyzed [74–77, 86–99]. Figure 6 presents putative mechanisms linking oxidative stress, antioxidant enzymes, vitamin D, osteopontin, and melatonin in LOCP carcinogenesis.

In the present study, specific modifications in the oxidant-antioxidant homeostasis, including a decrease in CAT and increases in GPx (in YCG) and blood plasma MDA, were found in the LOCP patients when compared to the healthy people. Moreover, GPx activity was found to negatively correlate with body mass and BMI in the YCG. Surprisingly, no statistically significant differences in SOD-1 activity were observed. This result is in contrast with the findings of other studies. In the study conducted by Gurudath et al. [86], a decrease in SOD-1 activity in patients with cancer of oral cavity was indicated. The study group

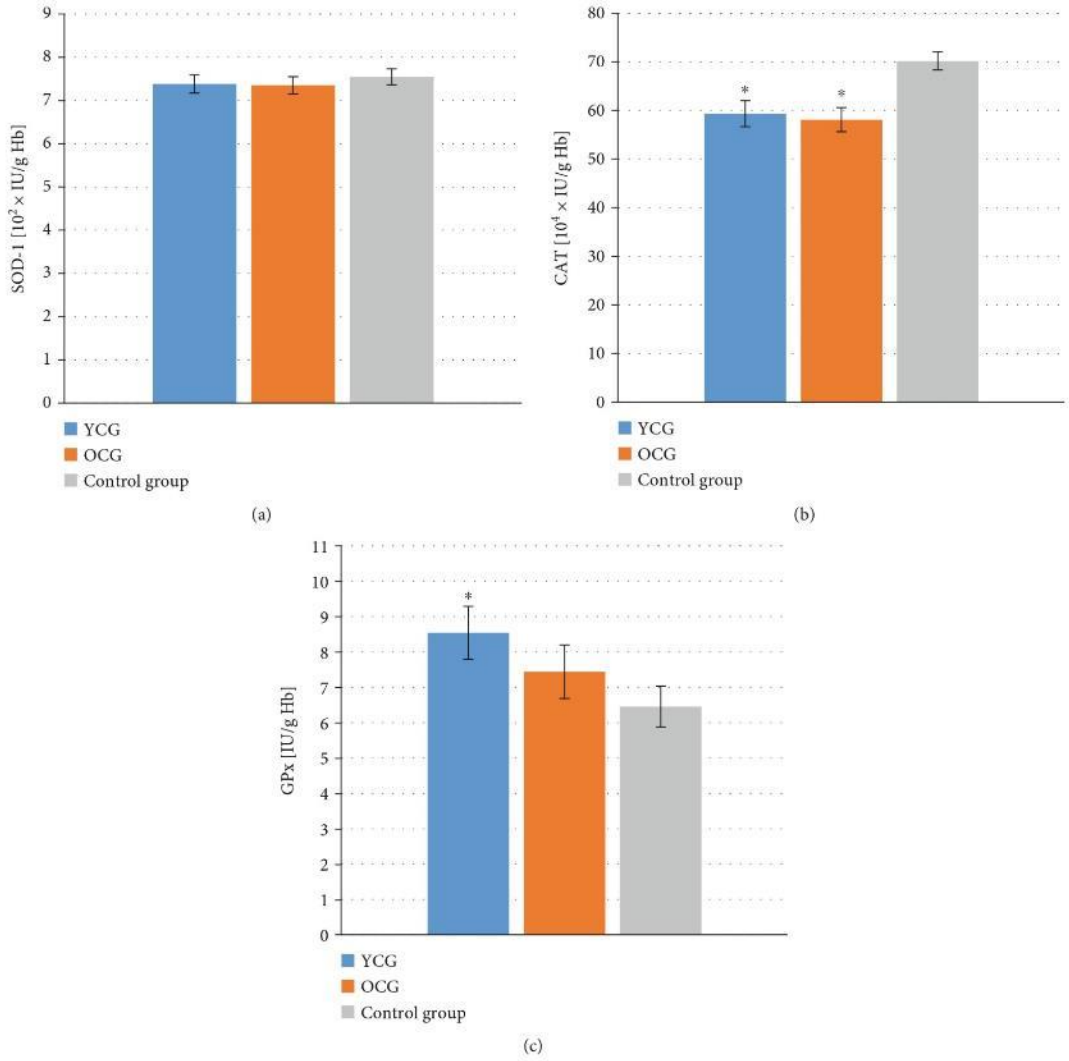

FIGURE 1: Activity of antioxidant enzymes in the erythrocytes of patients with lip, oral cavity, or pharyngeal cancer depending on age and in the healthy group. (a) Zn/Cu-superoxide dismutase (SOD-1) activity, (b) catalase (CAT) activity, (c) cytosolic glutathione peroxidase (GPx) activity. YCG: younger cancer group—mean age 58.24 ± 1.29 yrs; OCG: older cancer group—mean age 69.70 ± 1.49 yrs. Data are presented as the means ± S.E.M. $^*p < 0.05$ vs. control group.

consisted of 25 patients with oral cancer. Only smokers and tobacco chewers were included. Average age of the examined subjects was 53 yrs. The control group consisted of 25 healthy people; no information was provided on smoking or chewing tobacco in this group. Also, in the study of Subapriya et al. [87], the activity of SOD in group of 12 patients with oral precancerous lesions or oral cancer was tested. All subjects included in the study were between 35 and 60 yrs old and smoked or chewed tobacco. The cancer patients showed significantly lower SOD activity compared to the control group. Sabitha and Shyamaladevi [76] analyzed 12 blood samples obtained from patients with stage III oral cancer. The authors did not provide the age or information about the addictions of people participating in the study. Also, in that study, a significant decrease in SOD activity was observed in the course of cancer. Similarly, in the research by Manoharan et al. [75] decreased SOD activity was observed. A group of 46 men with oral cancer aged 40 to 60 years old was examined. The control group was free from smoking and chewing tobacco; no such information was provided in the context of the study group. Decreased activity of SOD in red blood cells was also reported by Patel et al. [77]. The age range for oral cancer patients was 22-75 years with a median of 45 years. A group of 126 patients, 113

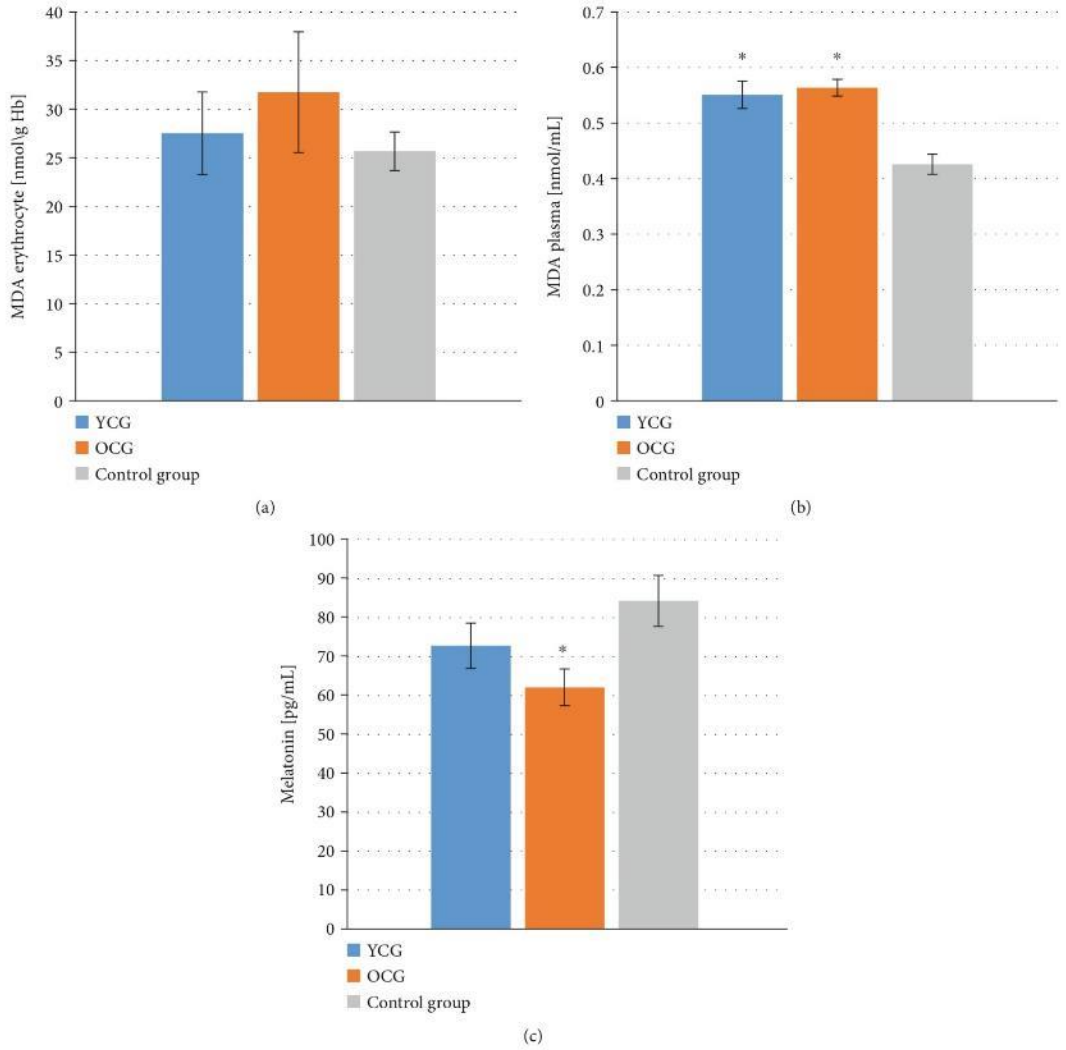

FIGURE 2: Concentration of malondialdehyde (MDA) and melatonin in patients with lip, oral cavity, or pharyngeal cancer depending on age and in the healthy group. (a) Erythrocytic MDA concentration, (b) plasma MDA concentration, (c) melatonin concentration. YCG: younger cancer group—mean age 58.24 ± 1.29 yrs; OCG: older cancer group—mean age 69.70 ± 1.49 yrs. Data are presented as the means ± S.E.M. $^{*}p < 0.05$, vs. control group.

of whom smoked or chewed tobacco, was tested. Different results were observed in the study of Huo et al. [74]. In their study, a group of 25 patients of both sexes aged 40 to 45 diagnosed with oral squamous cell carcinoma were investigated. Only smokers and tobacco chewers were included in the study. A healthy control group was free of tobacco chewing and smoking habits. The activity of SOD and CAT, as well as the level of erythrocytic MDA, was tested. SOD activity was higher in the group with neoplastic disease. In the case of observed in the present study lower CAT activity in the LOCP patients, similar results were reported by Huo et al. [74], Subapriya et al. [87], Sabitha and Shyamaladevi [76], and Manoharan et al. [75]. On the contrary, Patel et al. [77] did not observe any statistically significant changes in catalase activity in oral cancer patients. In the present study, we noted significantly higher GPx activity in the middle-aged LOCP patients than in the control group. The different results were described by Gurudath et al. [86], Subapriya et al. [87], Sabitha and Shyamaladevi [76], and Manoharan et al. [75]. The authors of these studies observed that GPx activity decreased in the course of cancer. In our study, erythrocytic MDA showed no statistically significant variability between the studied groups, unlike MDA level in blood plasma, which was higher in the cancer patients. In the other studies, increases in the level of MDA in plasma or serum and red blood cells in patients with cancers of

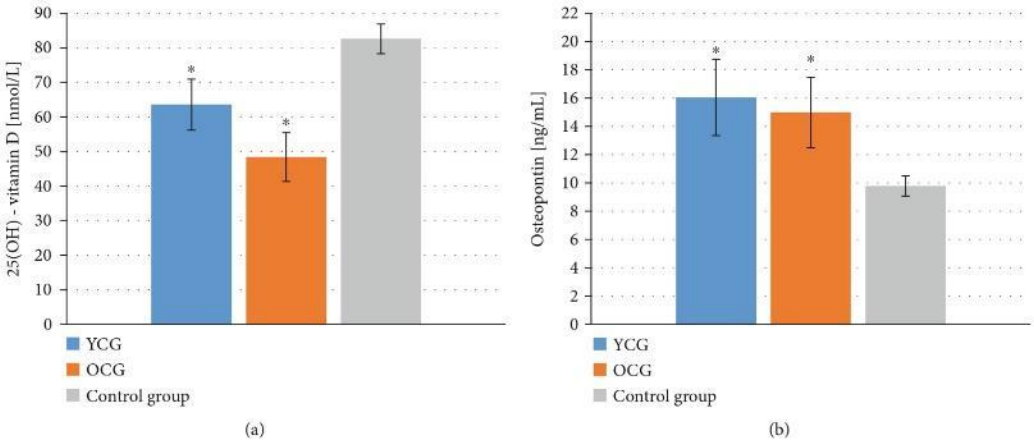

FIGURE 3: Concentration of 25(OH)-vitamin D and osteopontin in the blood serum of patients with lip, oral cavity, or pharyngeal cancer depending on age and in the healthy group. (a) 25(OH)-vitamin D concentration, (b) osteopontin concentration. YCG: younger cancer group—mean age 58.24 ± 1.29 yrs; OCG: older cancer group—mean age 69.70 ± 1.49 yrs. Data are presented as the means ± S.E.M. $^*p < 0.05$ vs. control group.

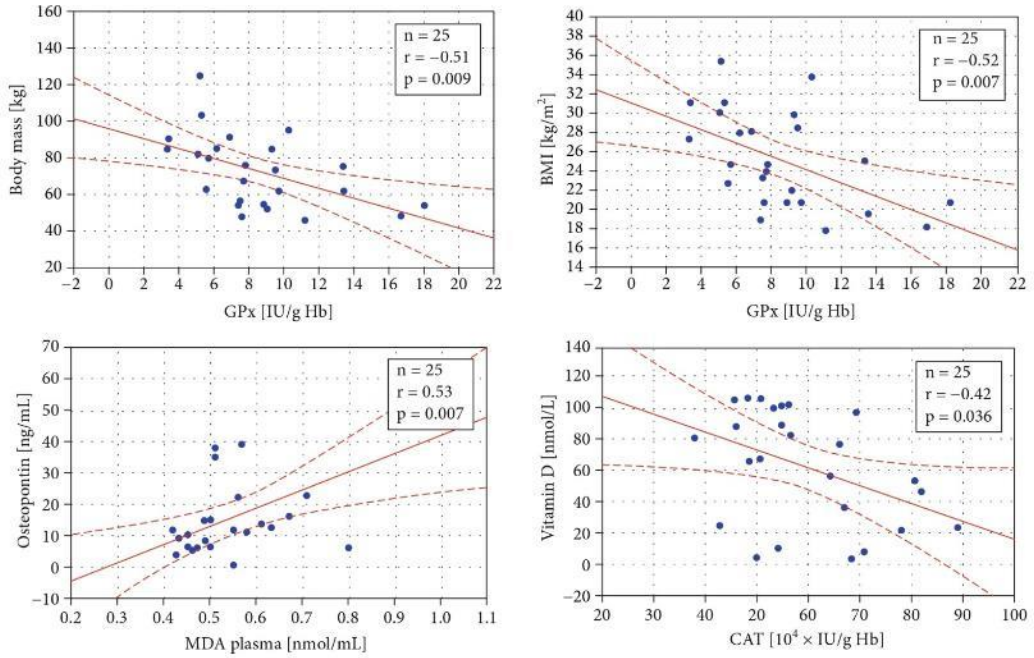

FIGURE 4: Statistically significant correlations in the younger cancer group (mean age 58.24 ± 1.29 yrs) between body mass and glutathione peroxidase (GPx) activity, body mass index (BMI) and GPx, osteopontin and plasma malondialdehyde (MDA) level, and vitamin D concentration and catalase (CAT) activity. The regression line is marked with a solid line, while the confidence intervals of 0.95 are marked with a dashed line.

the oral cavity and pharynx were unanimously indicated [74–76, 88–90].

Considering the differences between the studies, it is worth noting that the analyzed studies were conducted on small groups of patients of the Asian population (mainly India) [74–77, 86–90]. Additionally, in the study groups, a significant proportion of patients were smokers or chewing tobacco. The relationship between tobacco addiction and ROS generation, which leads to an increase in the level of oxidative stress, was confirmed in numerous studies [100,

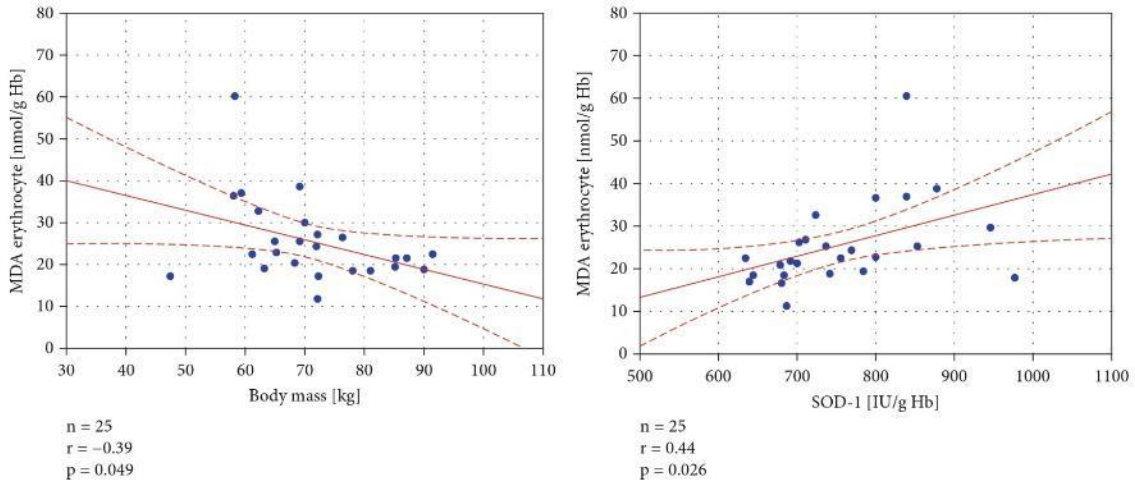

FIGURE 5: Statistically significant correlations in the healthy, control group between erythrocyte malondialdehyde (MDA) level and body mass, erythrocyte MDA concentration, and Zn/Cu-superoxide dismutase (SOD-1) activity. The regression line is marked with a solid line, while the confidence intervals of 0.95 are marked with a dashed line.

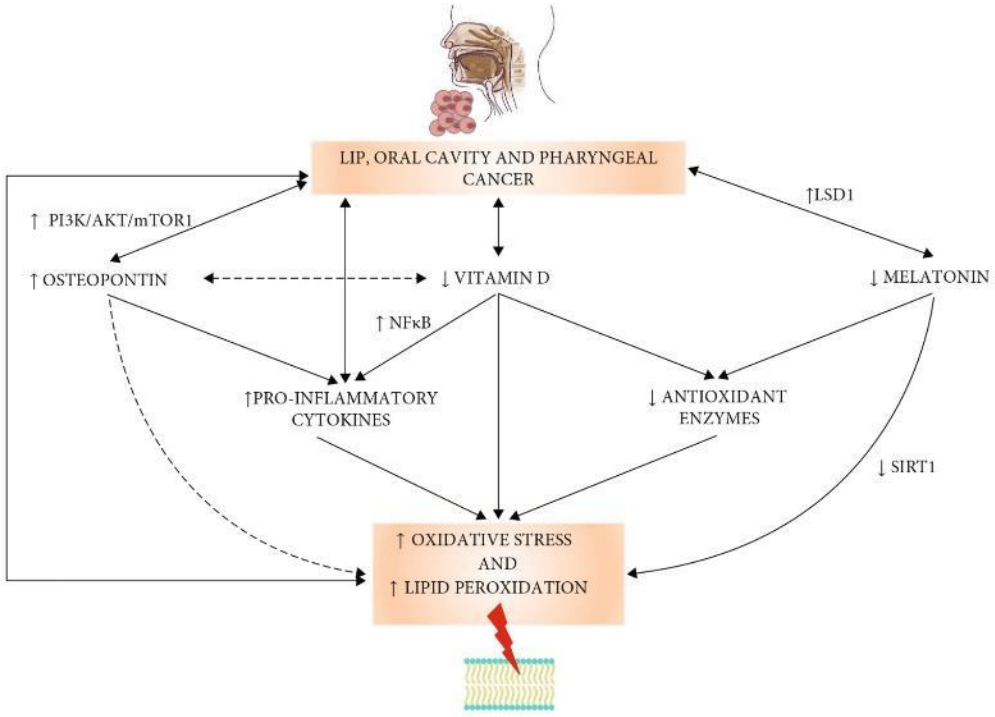

FIGURE 6: Putative mechanisms linking oxidative stress, antioxidant enzymes, vitamin D, osteopontin, and melatonin in lip, oral cavity, and pharyngeal cancer. Abbreviations used: LSD1: lysine-specific demethylase; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K/AKT/mTOR1: phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin; SIRT1: silent information regulator 1.

101], so this factor could significantly influence the obtained results. The age of the patients is also known to have an impact on the oxidant-antioxidant balance of the organism [102, 103]. The discussed studies were carried out on patients from the age of 40, whereas in the present study, the patients were older [102, 103]. It is worth mentioning that the activity of SOD-1 is dependent on the zinc level in the diet, whereas GPx is an enzyme dependent on selenium. Deficiencies resulting from an unbalanced diet may reduce the activity of SOD-1 and/or GPx. The lower activity of GPx in OCG compared to YCG may be the evidence of selenium deficiency in the diet of the elderly patients with LOCP cancer. Hydrogen peroxide (H_2O_2) is a substrate for both CAT and GPxs [104, 105]. In the present study, lower CAT and higher GPx activities were observed in the cancer patients compared to the healthy control group. The lower activity of CAT might be compensated by the increase in GPx activity. Thus, the glutathione-related antioxidant defense seems to be predominant in the patients. In summary, the results of the present study point to the increased ROS generation and reduction of antioxidant defense mechanisms, which are characteristic of neoplastic diseases [18]. Increased levels of lipid peroxidation and MDA could be a consequence of the disturbance of oxidant-antioxidant homeostasis and might be involved in the carcinogenesis.

Research by Liu et al. [91] indicates the important role of melatonin as a ROS scavenger in oral cancer. The research was conducted on human umbilical vein endothelial cells (HUVECs) and six human oral cancer cell lines, including SCC25, SCC9, Tca8113, Cal27, FaDu, and human normal oral keratinocytes (hNOKs). The addition of melatonin (1 mM) to the culture medium significantly reduced the level of ROS in the Cal27 and FaDu cells. Concurrently, melatonin reduced the proliferation and induced the apoptosis of oral cancer cells. Observed inactivation of ROS-reliant Akt signaling significantly decreased the mobility of cancer cells. Inhibition of angiogenesis and reduction in tumor mass were also found. Yang et al. [92] analyzed oral squamous cell carcinoma (OSCC) tissue arrays. The reduction of lysine-specific demethylase (LSD1) expression under the influence of melatonin (0.1 g/mL) was described. Lower LSD-1 expression significantly reduced tumor cell proliferation. Human nasopharyngeal carcinoma (HONE-1), NPC-39, and NPC-BM cell line incubated in a solution containing melatonin (50 ng/mL) were investigated by Ho et al. [93]. Presence of melatonin inhibited TPA-induced cell motility by regulating the matrix metalloproteinase-9 (MMP-9) expression in nasopharyngeal neoplasm cells. Many scientists point to the protective role of melatonin in oral and nasopharyngeal cavity diseases mainly by reducing oxidative stress [106–109]. However, in the present study, a statistically significant lower melatonin concentration in patients with lip, oral cavity, or pharynx cancer compared to the healthy group was only observed in OCG. During the aging, the synthesis and secretion of melatonin are reduced [110]. It could indicate that melatonin deficiency is not particularly involved in the pathogenesis of LOCP [106–109].

Vitamin D deficiency was found to correlate with mortality in the course of neoplastic diseases [44–46]. Calcitriol modulates immune response of the tumor microenvironment through the inactivation of the NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway [46]. The reference value for vitamin D in blood plasma is 75-125 nmol/L [111]. In our study, we observed that in the group of cancer patients, the vitamin D concentration was below the normal level. It is in accordance with the results of another research. Vitamin D deficiency was found to correlate with mortality in the course of neoplastic diseases [44–46]. The role of vitamin D in oral squamous cell carcinoma was investigated by Verma et al. [94]. Female C57BL/6 mice exposed to 4-nitroquinoline-1-oxide (4NQO) carcinogen were used. The animals were supplemented with vitamin D in the dose of 25-10,000 IU. The inhibition of tumor growth was observed. Beneficial effect in lowering oral mucositis in patients with head and neck cancer was also described by Bakr et al. [95]. The study involved 45 patients treated with radiotherapy divided into three groups. Two groups received topical oral vitamin D gel. Before the intervention, the levels of vitamin D in the blood serum of the patients were found to be deficient. The applied treatment not only reduced oral mucositis but also increased the level of vitamin D in the blood serum of patients. Also, Anand et al. [96] described vitamin D deficiency in oral cavity cancer patients. Moreover, the VDR overexpression in the course of oral cavity neoplasms was indicated in that study. Additionally, vitamin D appeared to play a special role in maintaining oral cavity health [112]. The surprising result of the present study is the negative correlation between CAT activity and vitamin D in the cancer group, suggesting that some regulatory mechanisms might be involved in the course of the disease. Undoubtedly, the role of vitamin D and its deficiency in LOCP cancer requires further research [112].

A significantly higher concentration of osteopontin was observed in our study in the LOCP patients. Also, Jeyasivanesan et al. [97] observed elevated osteopontin levels in patients with oral squamous cell carcinoma. Significant expression of osteopontin in salivary gland tumors was demonstrated in the study by Darling et al. [98]. Muramatsu et al. [99] analysed the effect of osteopontin levels on the invasiveness of oral cavity neoplasms. The study, performed on human oral squamous cell carcinoma cell lines, namely, HSC2, HSC3, HSC4, SAS, KB, and BSC-OF, revealed that high levels of osteopontin may increase the probability of metastasis. However, the mechanisms that link osteopontin and oral carcinomas have been not fully described yet. Presumably, osteopontin binds to integrin A4β1 and CD44 receptors, activating phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin (PI3K/AKT/mTOR1) pathway. In next step, mTOR1 regulates estrogen-related receptor alpha (ERRα), which binds to DNA and active transcription of osteopontin [113]. Overexpression of osteopontin was associated with increased angiogenesis, cancer cell proliferation, mobility, survival, invasion, and metastasis [113]. The positive correlation between osteopontin and lipid peroxidation, found in the present study, points to the association of the protein with deleterious oxidative processes in plasma membranes. Undoubtedly, these relations should be under further investigation.

The present study has some limitations. The small number of participants is a limiting factor. However, to the best of the authors' knowledge, no study has been conducted with the participation of patients with lip, oral cavity, and pharyngeal cancer of the European population, in which the mechanisms of antioxidant defense and the role of melatonin, vitamin D, and osteopontin were simultaneously analyzed.

5. Conclusions

The obtained results indicate a disruption of oxidant-antioxidant homeostasis in the lip, oral cavity, and pharyngeal cancer patients. Impaired antioxidant enzymatic defense and increased lipid peroxidation, correlated with high levels of osteopontin, were determined in this type of cancer. According to the results of the conducted study, melatonin seems not to be involved in the pathogenesis of the analyzed group of neoplasms. However, vitamin D deficiency in the LOCP patients was found. The role of elevated osteopontin in the pathogenesis of lip, oral cavity, and pharyngeal carcinoma requires further research. The simultaneous testing of vitamin D and osteopontin levels seems to be particularly noteworthy.

Data Availability

Data are available on request due to privacy/ethical restrictions.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

J.N., K.S.-G., and J.C. are responsible for the conceptualization; J.N., J.C., M.M., and. K.S.-G. for the methodology; J.N. and K.S.-G. for the formal analysis; J.N. K.S.-G., J.C., M.M., and M.P. for the investigation; J.N. for the data curation; J.N. for the writing—original draft preparation; J.N. and K.S.-G. for the writing—review and editing; J.N. for the visualization; A.W. and B.M. for the supervision; J.N. and K.S.-G. for the project administration; and A.W for the funding acquisition. All authors have read and agreed to the published version of the manuscript.

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14.1.3. Publikacja III

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Concentration of Selected Adipokines and Factors Regulating Carbohydrate Metabolism in Patients with Head and Neck Cancer in Respect to Their Body Mass Index

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Abstract: Head and neck cancers (HNCs) are a group of tumors not common in European populations. So far, not much is known about the role of obesity, adipokines, glucose metabolism, and inflammation in the pathogenesis of HNC. The aim of the study was to determine the concentrations of ghrelin, omentin-1, adipsin, adiponectin, leptin, resistin, visfatin, glucagon, insulin, C-peptide, glucagon-like peptide-1 (GLP-1), plasminogen activator inhibitor-1 (PAI-1), and gastric inhibitory peptide (GIP) in the blood serum of HNC patients depending on their body mass index (BMI). The study included 46 patients divided into two groups according to their BMI values: the normal BMI group (nBMI) included 23 patients with BMI < 25 kg/m^2 and the increased BMI group (iBMI) included patients with BMI $\geq$ 25 kg/m^2. A control group (CG) included 23 healthy people (BMI < 25 kg/m^2). Statistically significant differences in the levels of adipsin, ghrelin, glucagon, PAI-1, and visfatin were shown between nBMI and CG. In the case of nBMI and iBMI, statistically significant differences were observed in the concentrations of adiponectin, C-peptide, ghrelin, GLP-1, insulin, leptin, omentin-1, PAI-1, resistin, and visfatin. The obtained results indicate a disruption of endocrine function of adipose tissue and impaired glucose metabolism in HNC. Obesity, which is not a typical risk factor for HNC, may aggravate the negative metabolic changes associated with this type of neoplasm. Ghrelin, visfatin, PAI-1, adipsin, and glucagon might be related to head and neck carcinogenesis. They seem to be promising directions for further research.

Keywords: adipokines; biomarkers; body mass index; carbohydrate metabolism; cytokines; head and neck cancer; obesity

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1. Introduction

Head and neck cancers (HNCs) are a significant clinical and social problem. It is estimated that over 500,000 patients are diagnosed with HNC each year worldwide [1]. In 2020, there were 151,000 new cases of HNCs in Europe [2]. HNCs are a rare group of neoplasms characterized by an unfavorable prognosis, despite the significant development of diagnostic and therapeutic methods [3]. HNCs affect the organs of the head and neck, such as the lip, oral cavity, tongue, gum, pharynx, tonsil, larynx, paranasal sinuses, and salivary glands [4]. The neoplasms in this group are quite homogeneous in terms of morphology. The vast majority of HNCs include squamous cell carcinomas of varying degrees of differentiation, originating from the epithelium of the mucous membranes [5].

So far, several risk factors favoring the development of HNC have been identified. Irritation of the mucous membranes of the oral cavity and throat with cigarette smoke or alcohol, as well as chronic, mechanical damage to the tissues through ill-fitting dentures or broken teeth, may lead to carcinogenesis [6]. *Human papillomavirus* (HPV) and Epstein-Barr virus (EBV) infections may also be a cause of HNC [5,7,8]. The South Asian population is particularly vulnerable to HNCs, which may be related to socio-economic conditions [9]. The symptoms of HNC vary depending on the initial location of the cancer. Typical symptoms include pain and ulceration of the affected tissue, which over time may lead to breathing, swallowing, and speech difficulties [10]. Some patients with HNC have enlarged lymph nodes in the neck [4]. Surgery, chemotherapy, and radiotherapy are the most common forms of treatment for HNC patients. These methods are usually used together in combination therapy [11].

Obesity is a chronic disease of complex etiology associated with abnormal or excessive accumulation of adipose tissue, which poses a health risk [12]. It is estimated that 2.1 billion people in the world may be overweight or obese [13]. One of the methods of diagnosing obesity is by measuring the patient's body mass and height to calculate the body mass index (BMI) [13]. In people with a BMI $\geq$ 25 kg/m^2, a greater amount of adipose tissue may be accumulated [14]. Adipose tissue not only stores excess energy but also produces various types of substances [15]. Adipokines, hormones secreted by white adipose tissue, regulate inflammatory and metabolic processes as well as influence cell growth and proliferation [16]. Adipokines make obesity inextricably linked to low-grade chronic inflammation [17]. Inflammatory processes increase the risk of developing cancer [18]. According to a meta-analysis by Dobbins et al. [19], obesity is associated with a higher risk of colon, renal, gallbladder, pancreatic, leukemia, and breast cancer. In the course of cancer, metabolic pathways of carbohydrates and lipids are disturbed [20]. Although obesity is not a typical risk factor for HNC, the coexistence of obesity and HNC may have a significant impact on the course and prognosis of the disease [21]. The Cho et al. [22] study showed that being underweight (BMI < 18.5 kg/m^2) leads to a higher mortality in HNC patients, while obesity is associated with a better prognosis and patient survival. White adipose tissue inflammation in obesity may lead to insulin resistance and lower disease-free survival rates in HNC patients [21]. The level of adipose tissue appears to influence the course and prognosis of HNC. Thus, the ambiguous role of obesity and its complications as predisposing factors for HNC requires further research.

So far, only a few studies concentrated on the role of adipokines and glucose metabolism in patients with HNC have been conducted. Therefore, the aim of the presented study was an attempt to find potential links between obesity and HNC and to identify analytes related to obesity and glucose metabolism that may be associated with HNC carcinogenesis. For this purpose, it was planned to determine the concentrations of ghrelin, omentin-1, adipsin, adiponectin, leptin, resistin, visfatin, glucagon, insulin, C-peptide, glucagon-like peptide-1 (GLP-1), plasminogen activator inhibitor-1 (PAI-1), and gastric inhibitory peptide (GIP) in the course of HNC. Including HNC patients with both BMI values in the reference range and with elevated BMI values in the study could allow for a better understanding of the role of selected adipokines and factors regulating glucose metabolism in the course of HNC.

2. Results

The results of the anthropometric analyzes and clinical characteristics of the study participants are presented in Table 1. The group of patients with HNC was divided into two subgroups depending on BMI. The study included 23 HNC patients with normal body mass index values (nBMI) and 23 HNC patients with increased BMI (iBMI). The third group participating in the study included 23 healthy volunteers who qualified as a control group. There was a statistically significant difference in the body mass and BMI between the nBMI and iBMI groups. No significant differences were found between the nBMI and control groups in anthropometric and clinical characteristics.

Table 1. Anthropometric and clinical characteristics of patients with head and neck cancer (HNC groups) and healthy volunteers (control group). Each value represents the mean ± S.E.M.

Parameter	HNC				Control		p nBMI vs. iBMI	p nBMI vs. Control
	nBMI		iBMI					
	Mean	SEM	Mean	SEM	Mean	SEM		
n (female/male)	23 (10/13)	-	23 (9/14)	-	23 (11/12)	-	0.770785	0.773350
Age [yrs]	64.130	1.464	64.696	2.073	62.217	1.478	0.824801	0.362876
Body mass [kg]	61.957	1.787	90.957	2.556	62.652	1.524	<0.000001	0.768436
Height [cm]	169.130	1.464	170.957	1.172	168.304	1.202	0.335512	0.664882
BMI [kg/m^2]	21.601	0.445	31.089	0.755	22.066	0.358	<0.000001	0.420516
Ex-smoker or current smoker (y/n)	11/12	-	12/11	-	5/18	-	0.774186	0.065521

Abbreviations used: BMI: body mass index; HNC: head and neck cancer; iBMI: increased BMI group; nBMI: normal BMI group; SEM: standard error of mean. $p < 0.05$ was considered as statistically significant.

In the nBMI patients, statistically higher levels of adiponectin, omentin-1, and ghrelin were observed compared to the participants from the iBMI group. Individuals from the iBMI group presented significantly higher concentrations of insulin, leptin, C-peptide, GLP-1, PAI-1, resistin, and visfatin than the patients from the nBMI group. No significant differences were found between the nBMI and iBMI groups in the case of adipsin, GIP, and glucagon. The results of biochemical analyzes performed in HNC patients from the nBMI and iBMI groups are presented in Tables 2 and 3.

Table 2. Biochemical parameters with a statistically normal distribution analyzed in head and neck cancer (HNC) patients with respect to their body mass index (BMI).

Parameter	HNC				p Value
	nBMI		iBMI		
	Mean	SEM	Mean	SEM	
Adiponectin [µg/mL]	58.947	3.581	36.182	2.429	0.000004
Leptin [pg/mL]	1941.866	129.804	4030.817	273.135	<0.000001
Insulin [pg/mL]	334.688	25.264	684.051	42.573	<0.000001

Abbreviations used: iBMI: increased BMI group; nBMI: normal BMI group; SEM: standard error of mean. $p < 0.05$ was considered as statistically significant.

Table 3. Biochemical parameters with a statistically non-parametric distribution analyzed in head and neck cancer (HNC) patients with respect to their body mass index (BMI).

Parameter	HNC				p Value
	nBMI		iBMI		
	Median	IQR (Q1; Q3)	Median	IQR (Q1; Q3)	
Ghrelin [pg/mL]	282.341	229.697; 377.075	169.900	141.490; 196.700	0.000036
Omentin-1 [ng/mL]	701.330	660.220; 779.030	456.000	399.300; 688.960	0.000048
Adipsin [ng/mL]	677.935	475.431; 916.300	686.080	634.510; 815.832	0.775185
Resistin [pg/mL]	6846.331	5265.468; 7981.305	9089.872	7939.536; 12,098.560	0.000033
Visfatin [pg/mL]	1394.150	1171.907; 1647.002	1939.650	1692.205; 2298.355	0.000007
Glucagon [pg/mL]	1557.810	1452.890; 1639.300	1643.660	1398.320; 1891.490	0.783612

Table 3. *Cont.*

Parameter	HNC				*p* Value
	nBMI		iBMI		
	Median	IQR (Q1; Q3)	Median	IQR (Q1; Q3)	
C-peptide [pg/mL]	611.900	512.850; 706.740	700.550	603.880; 834.100	0.019874
GLP-1 [pg/mL]	257.500	213.360; 277.600	297.200	277.200; 308.020	0.011522
PAI-1 [pg/mL]	3544.930	3122.099; 3956.693	4476.310	3924.160; 4979.300	0.004597
GIP [pg/mL]	315.300	290.650; 400.900	333.400	299.800; 388.600	0.660384

Abbreviations used: GIP: glucose-dependent insulinotropic polypeptide; GLP-1: glucagon-like peptide-1; iBMI: increased BMI group; IQR: interquartile range; nBMI: normal BMI group; PAI-1: plasminogen activator inhibitor-1. $p < 0.05$ was considered as statistically significant.

In the nBMI group, statistically significant higher levels of adipsin, visfatin, glucagon, and PAI-1 were observed compared to the control group. Moreover, a statistically lower level of ghrelin was noted in the nBMI group compared to the control group. The analysis of the results showed no differences in the levels of omentin-1, GIP, adiponectin, C-peptide, GLP-1, insulin, leptin, and resistin between the nBMI and control groups. Tables 4 and 5 present the results of a laboratory analysis for the nBMI patients and the control group.

Table 4. Biochemical parameters with a statistically normal distribution analyzed in head and neck cancer (HNC) patients with normal body mass index values (nBMI) and a control group.

Parameter	HNC nBMI		Control Group		*p* Value
	Mean	SEM	Mean	SEM	
Omentin-1 [ng/mL]	727.701	18.184	721.247	20.433	0.814575
Adipsin [ng/mL]	680.076	49.457	443.729	20.163	0.000063
Visfatin [pg/mL]	1412.926	69.342	1241.184	29.803	0.027802
GIP [pg/mL]	339.807	22.837	290.509	12.393	0.064363

Abbreviations used: GIP: glucose-dependent insulinotropic polypeptide; SEM: standard error of mean. $p < 0.05$ was considered as statistically significant.

Table 5. Biochemical parameters with a statistically non-parametric distribution analyzed in head and neck cancer (HNC) patients with normal body mass index values (nBMI) and a control group.

Parameter	HNC nBMI		Control		*p* Value
	Median	IQR (Q1; Q3)	Median	IQR (Q1; Q3)	
Ghrelin [pg/mL]	282.341	229.697; 377.075	343.510	295.610; 434.450	0.034942
Adiponectin [µg/mL]	53.984	43.884; 75.405	45.673	39.945; 64.519	0.118805
Leptin [pg/mL]	1899.600	1416.650; 2393.790	1715.204	1576.584; 2998.286	0.253290
Resistin [pg/mL]	6846.331	5265.468; 7981.305	6781.360	5736.944; 7695.312	0.741750
Glucagon [pg/mL]	1557.810	1452.890; 1639.300	1429.350	929.720; 1595.870	0.008941
Insulin [pg/mL]	297.780	232.930; 420.230	284.970	212.290; 500.420	0.792065
C-peptide [pg/mL]	611.900	512.850; 706.740	684.038	431.604; 852.606	0.613355
GLP-1 [pg/mL]	257.500	213.360; 277.600	245.682	155.502; 291.314	0.333723
PAI-1 [pg/mL]	3544.930	3122.099; 3956.693	2926.950	2462.028; 3436.926	0.000661

Abbreviations used: GLP-1: glucagon-like peptide-1; IQR: interquartile range; PAI-1: plasminogen activator inhibitor-1. $p < 0.05$ was considered as statistically significant.

The correlation between the concentrations of the analyzed biomarkers was also evaluated. In the nBMI group, statistically significant negative correlations were observed

between ghrelin and GIP (r = −0.6284; $p = 0.001$), GIP and PAI-1 (r = −0.6662; $p = 0.001$), and omentin-1 and resistin (r = −0.4233; $p = 0.044$), whereas a significant positive correlation was found between ghrelin and PAI-1 (r = 0.6964; $p < 0.001$) as well as between insulin and leptin (r = 0.6359; $p = 0.001$). Figure 1 presents the significant correlations between selected parameters in the nBMI group. In the iBMI group, a negative correlation between age and resistin (r = −0.4479, $p = 0.032$) and a positive correlation between PAI-1 and visfatin (r = 0.8253; $p < 0.001$), adipsin and C-peptide (r = 0.7054; $p < 0.001$), as well as between insulin and C-peptide (r = 0.6462; $p = 0.001$) were noted. The statistically significant correlations in the iBMI group are presented in Figure 2.

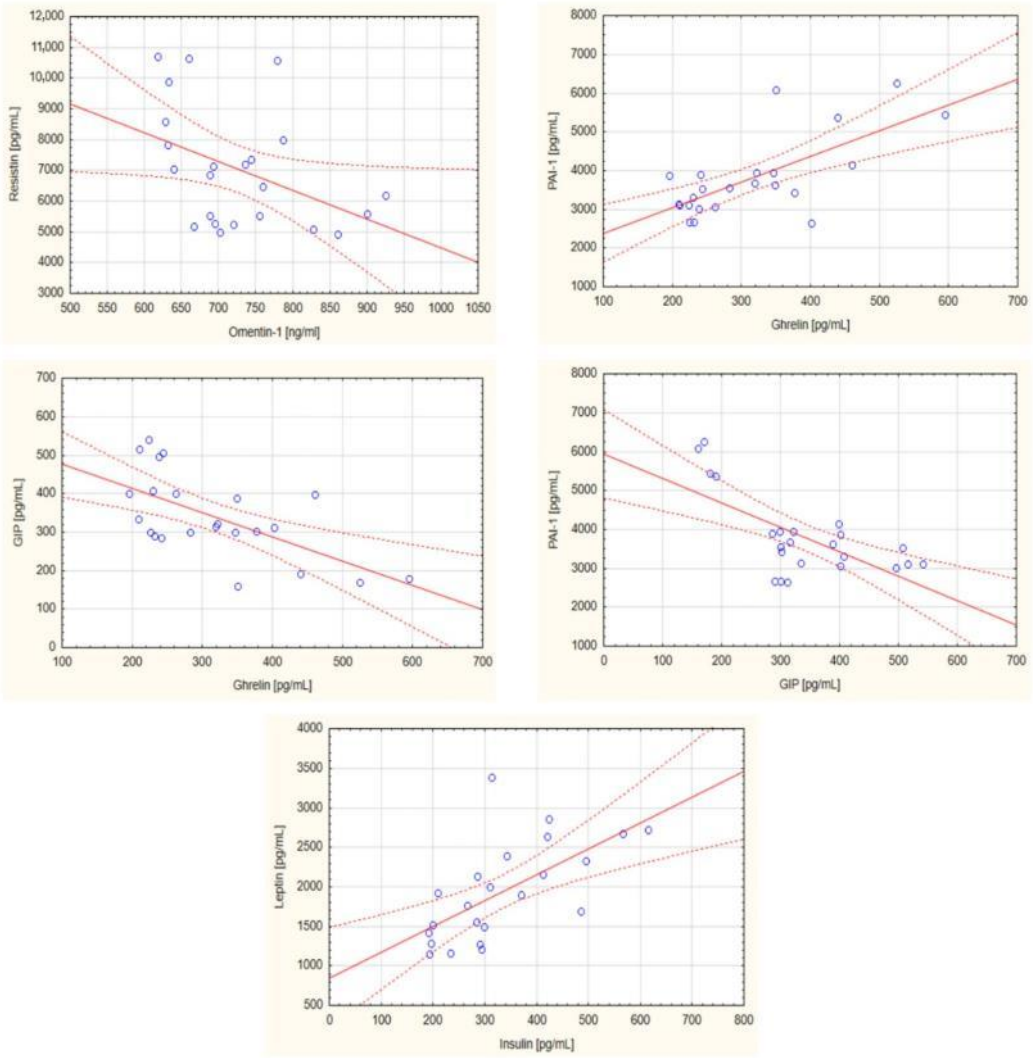

Figure 1. Statistically significant correlations between selected biochemical parameters in the normal body mass index group of head and neck cancer patients. Abbreviations used: GIP: glucose-dependent insulinotropic polypeptide; PAI-1: plasminogen activator inhibitor-1. The regression line is marked with a solid line, while the confidence intervals of 0.95 are marked with a dashed line. $p < 0.05$ was considered as statistically significant.

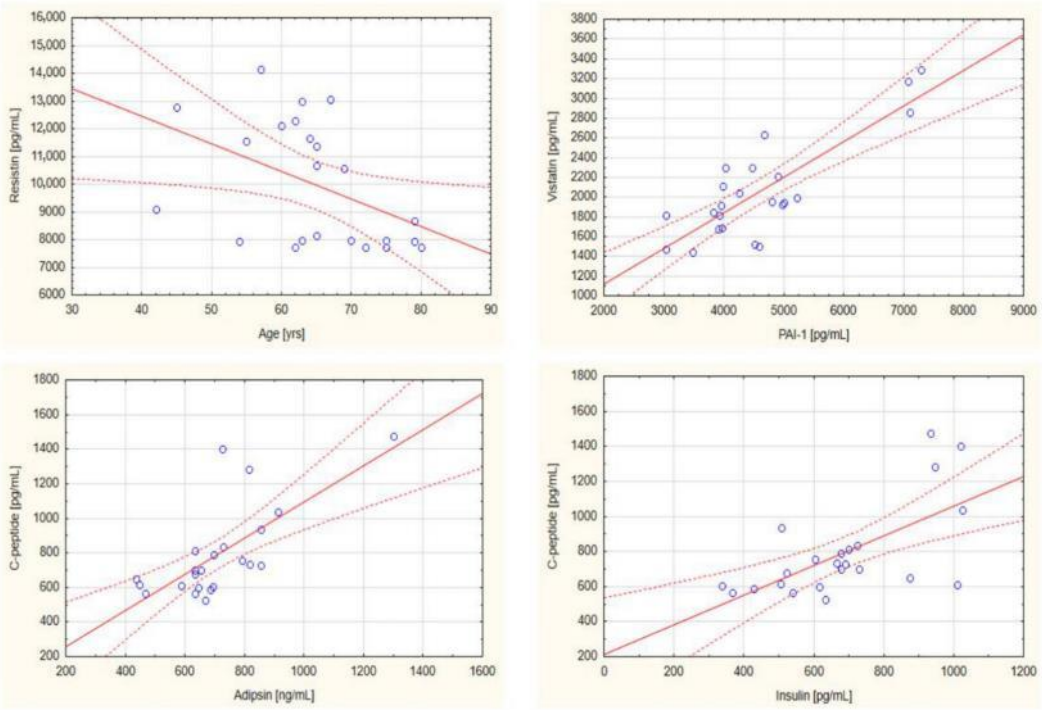

Figure 2. Statistically significant correlations between selected biochemical parameters in the increased body mass index group of head and neck cancer patients. Abbreviations used: PAI-1: plasminogen activator inhibitor-1. The regression line is marked with a solid line, while the confidence intervals of 0.95 are marked with a dashed line. $p < 0.05$ was considered as statistically significant.

Analogous statistical analysis was performed separately for the groups of women (nBMI vs. iBMI and nBMI vs. control group) and men (nBMI vs. iBMI and nBMI vs. control group). However, no statistically significant gender-specific differences or correlations were found. This could be due to the small number of subjects in the groups, which were additionally divided by gender. Nevertheless, although men are more likely to develop and die from HNC due to their smoking and hard alcohol consumption habits, gender is not a typical risk factor for HNC, which may have been the reason why we did not observe gender-specific differences in the studied parameters.

3. Discussion

In the course of both obesity and carcinogenesis, changes in the metabolic profile of adipose tissue have been observed [23–25]. This can lead to increased synthesis and secretion of many bioactive compounds, such as hormones, adipokines, inflammatory cytokines, and growth factors [26–28]. To date, more than 600 adipokines have been discovered. Most of the described adipokines play a key role in maintaining carbohydrate-lipid homeostasis [29]. These factors secreted by white adipose tissue contribute to the initiation and progression of several types of cancer by stimulating the metabolic reprogramming of cells [30]. Adipokines affect tumor metabolism and lead to cancer cell growth, proliferation, migration, invasion, epithelial-mesenchymal transition, angiogenesis, metastasis, and the development of multidrug resistance [31–34]. Low-grade chronic inflammation associated with obesity shapes the tumor microenvironment, affecting cell plasticity through epithelial-mesenchymal transition, dedifferentiation, immune cell polarization, reactive oxygen species, cytokines, and epigenetic mechanisms [35,36]. Liver, bladder, lung, colorectal, and gastric cancers are strongly associated with chronic inflammation [37,38].

Numerous studies have focused on the role of inflammation in carcinogenesis and the pharmacological reduction of inflammation as a potential anti-cancer therapy [35,39,40]. Cancer cells, due to their rapid proliferation, are characterized by a significant demand for glucose [20,41]. Increased glucose metabolism in neoplastic cells depends, inter alia, on their localization, increased expression of glucose transporting proteins from the glucose transporter (GLUT) family, enzymes such as phosphoglucomutase or hexokinase, the degree of cell proliferation, and the tumor vascularity [42]. HNCs have not been the subject of numerous studies, especially in the aspect of adipokine homeostasis and factors regulating carbohydrate-lipid metabolism. Therefore, the presented study might be a valuable contribution to the current state of knowledge in this area.

Ghrelin, also called "hunger hormone", is a short polypeptide hormone synthesized by enteroendocrine cells of the gastrointestinal tract [43]. Ghrelin is a hormone that regulates food intake and is secreted during fasting [43]. In people with obesity, a decrease in ghrelin levels is often observed [43]. In the course of HNC, we observed that ghrelin levels were lower in the iBMI patients compared to the nBMI patients. In the healthy individuals, we observed a higher level of ghrelin than in the nBMI patients. These results may suggest improper secretion of ghrelin in HNC patients and/or a relationship between this hormone and HNC carcinogenesis. It should be emphasized that patients with HNC have problems with food intake, which may lead to food restriction and starvation. According to Stempniewicz et al. [44], ghrelin presents anti-inflammatory, antioxidative, and antiapoptotic effects in oral mucositis. Hiura et al. [45] observed that during anti-cancer cisplatin-based chemotherapy, ghrelin levels were reduced in patients with advanced esophageal cancer.

Omentin-1, also known as intelectin 1, is a 34-kDa adipokine secreted primarily by visceral white adipose tissue [46]. It belongs to the group of adipokines with anti-inflammatory and antioxidant properties [46]. Recent studies have indicated that omentin-1 promotes insulin-dependent glucose transport in adipocytes [47]. The level of omentin-1 negatively correlates with BMI, especially in the course of type 2 diabetes mellitus [48]. In the presented study, the level of omentin-1 was significantly higher in the nBMI group compared to the iBMI group. No difference in serum omentin-1 concentration was observed between the HNC patients with normal BMI and the healthy controls. The obtained results could be explained by the disturbed hormonal activity of increased adipose tissue in HNC patients with increased BMI rather than by the influence of the neoplasm. Disturbed secretion of omentin-1 has been found in some studies concerning other cancer types. Shen et al. [49] described a study on a group of 41 patients with renal cell carcinoma. The authors indicated a significantly lower level of omentin-1 in the cancer patients compared to the control group. Both groups were characterized by a mean BMI in the reference range. This study did not include obese patients; however, researchers indicated a negative correlation between BMI and omentin-1 concentration. The role of selected adipokines in the course of postmenopausal breast cancer was described by Christodoulatos et al. [50]. The study group consisted of 103 females with a mean BMI of 27.7 ± 4.14 kg/m^2. The mean level of omentin-1 in the serum was significantly lower in the case of cancer patients compared to the control group. The authors indicated that the omentin-1 level was inversely correlated to the metabolic and inflammatory biomarkers, the cancer stage, and the number of infiltrated lymph nodes. Researchers suggested that the concentration of circulating omentin-1 may be a diagnostic marker for breast cancer. Currently, the mechanisms linking omentin-1 to tumor development remain unknown, but studies have indicated that omentin-1 affects the course of cancers with different localizations [51].

Adipsin, also called complement factor D, is a serine protease secreted mainly by adipocytes. It functions as an activator of the alternative pathway of the complement system [52]. Increased BMI and a greater volume of adipose tissue are associated with a high level of adipsin [53]. In the presented study, there were no differences in adipsin levels between the HNC groups. A statistically significant lower adipsin concentration was observed in the healthy people in the control group compared to the nBMI group. These

results suggest that adipsin might be a factor associated with carcinogenesis. This is in accordance with the results of other studies. In the study of Nezhad et al. [54], the cell lines UT-SCC-12A, UT-SCC-91, UT-SCC-105, UT-SCC-111, and UT-SCC-118 were used as models of cutaneous squamous cell carcinoma. It was observed that adipsin increased the proliferation of each cell line cell through the regulation of the extracellular signal-regulated kinases 1/2 (ERK1/2) signaling pathway. Mizuno et al. [55] indicated that adipsin and its downstream effector, hepatocyte growth factor, are active players in adipocyte-cancer cell interactions.

Adiponectin is a polypeptide hormone produced and secreted by adipocytes [56]. In the course of obesity and hypotrophy of adipose tissue, a decrease in the level of adiponectin is observed [57]. Adiponectin affects a number of metabolic processes, especially the metabolism of glucose and fatty acids in the liver and muscles, indirectly affecting the sensitivity of tissues to insulin and reducing inflammation [21,57]. Decreased adiponectin levels may lead to carcinogenesis [56]. Our results indicate that the obese HNC patients presented lower adiponectin levels compared to the nBMI group. No difference in adiponectin concentration was observed between the HNC patients with a normal BMI and the healthy individuals. Howard et al. [58] indicated that low adiponectin receptor 1 expression may be a predictor of improved overall survival in patients with oesophageal cancer.

Leptin is a well-known adipokine described as a pro-inflammatory biomolecule synthesized and secreted by white adipose tissue. It acts by activating transmembrane receptors (Ob-Rs) [59]. A positive correlation between the concentration of leptin in the blood plasma and the patient's BMI or the volume of adipose tissues has been found in numerous studies [59]. In our study, we observed higher leptin levels in the iBMI patients than in the nBMI group, as well as no differences between the nBMI group and the control group. In the article by Ozsoy et al. [60], male patients with HNC were tested for leptin levels. A group of pre-treatment ($n = 40$) patients with a BMI of 24.96 ± 4.02 kg/m^2 was compared with a control group with a BMI of 28.85 ± 3.52 kg/m^2. The results of the study indicated that the concentrations of leptin in the pre-treatment and control groups were similar. A statistically significant lower level of leptin was shown only in the group of patients with HNC post-treatment ($n = 40$; BMI 23.08 ± 3.92 kg/m^2) compared to the control group. Leptin can bind to Ob-R on breast cancer cells and enhance several tumor cell responses in tumor tissue by inappropriately activating multiple signaling pathways, such as MAPK and ERK1/2 activation, signal transducer and activator of transcription 3 (STAT3), and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Act) [61].

Resistin is a pro-inflammatory adipokine [62]. This polypeptide triggers cellular insulin resistance, stimulates the endothelium to accumulate lipids, and exhibits immunomodulatory properties [62]. Obesity and metabolic syndrome are associated with an elevated level of resistin [62]. In neoplastic diseases, resistin may enhance neoangiogenesis and metastasis by modulating vascular endothelial growth factor (VEGF) secretion [62]. In our study, the HNC patients with an increased BMI presented higher levels of resistin compared to the nBMI group. No differences were observed between the nBMI patients and the healthy control group. Nakajima et al. [63] studied resistin levels in patients with squamous cell carcinoma of the esophagus. The patients with a more advanced stage of the disease showed higher concentrations of resistin. Thus, it was suggested that resistin could be a potential biomarker for squamous cell carcinoma of the esophagus.

Visfatin, also known as nicotinamide phosphoribosyltransferase, is an enzyme belonging to the group of adipokines [64]. Visfatin is a rate-limiting enzyme in the biosynthesis of nicotinamide adenine dinucleotide (NAD^+) [65]. This adipokine is a pro-inflammatory protein, and its level increases in obesity, insulin resistance, and cardiometabolic diseases [66]. Recent studies have indicated that visfatin is involved in adipose tissue fibrosis [66]. In the course of breast cancer, the level of serum visfatin is elevated, and as a result, the promotion of the G1-to-S phase transition of the cell cycle is observed [65]. Additionally, visfatin leads to the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells/neurogenic locus notch homolog protein 1 (NF-κB/Notch1), Act/extracellular

signal-regulated kinases 1/2 (ERK1/2), and silent mating type information regulation 2 homolog 1 (SIRT1)/acetylated p53 signaling pathways [65]. In our study, the HNC patients had higher levels of visfatin than the healthy individuals. Moreover, the nBMI group presented a lower visfatin concentration than the iBMI group. Undoubtedly, these results point to the influence of visfatin in HNC carcinogenesis. Abdulsalam et al. [67] reported that visfatin levels were significantly higher in colon cancerous tissue compared to the paired adjacent non-cancerous tissue.

Glucagon is a hormone consisting of 29 amino acids, produced by the alpha cells of the islets of the pancreas [68]. Obesity can lead to impaired postprandial glucagon secretion [69]. What is more important is that glucagon not only regulates glucose balance but also energy homeostasis [69]. In this study, no differences in glucagon levels were observed between the nBMI and iBMI groups. On the other hand, a significantly lower concentration of glucagon in the blood serum of healthy people was observed compared to the nBMI group. These results may indicate a more intensive glucose metabolism in patients with HNC. According to Yagi et al. [70], glucagon may promote colon cancer cell growth by regulating the 5′ adenosine monophosphate-activated protein kinase (AMPK) and mitogen-activated protein kinase (MAPK) pathways.

Insulin is an anabolic peptide hormone with glucagon-antagonistic activity. Higher insulin levels are usually associated with obesity [71]. This phenomenon is related to the role of insulin, namely the intensification of lipid deposition in adipose tissue [71]. Obesity may lead to insulin resistance and even to the development of type 2 diabetes mellitus [71]. High insulin levels in hyperinsulinemia activate insulin/IGF signaling pathways followed by the activation of (PI3K)/Akt/mammalian rapamycin (mTOR) and MAPK signaling pathways, thus promoting cancer cell growth, survival, and mobility [72,73]. In our study, we observed significantly higher insulin levels in the iBMI patients compared to the nBMI patients. No difference in the serum insulin concentration was observed between the HNC patients with a normal BMI and the healthy control group. Vilaseca et al. [74] indicated that in patients with head and neck squamous cell carcinomas, glycemic metabolism is altered, which results in dysregulation of the insulin-glucagon system.

C-peptide is a metabolically inactive short peptide produced in the beta cells of the pancreas during the conversion of proinsulin to insulin [75]. In our study, the HNC patients with a normal body mass presented lower concentrations of C-peptide in their blood serum compared to the iBMI group. No difference in the serum C-peptide level was observed between the nBMI patients and the healthy controls. The meta-analysis by Guo et al. [76] indicated that the C-peptide concentration was not associated with an increased risk of prostate cancer. On the other hand, the studies of Arcidiacono et al. [77] indicated that the level of C-peptide may correlate with the development of Barrett's esophageal carcinogenesis. The role of C-peptide in the course of neoplastic diseases remains ambiguous. According to Thota et al. [78], obesity remains a factor influencing the increase in serum C-peptide concentration.

GLP-1 is a hormone secreted by the enteroendocrine cells of the gastric mucosa in response to food intake [79]. The effect of GLP-1 on tissues has been found to be impaired in obesity, which might be related to reduced GLP-1 secretion and/or reduced insulinotropic potency [80]. In our study, we observed that the average GLP-1 level was higher in the iBMI group than in the nBMI group. There were no differences between the nBMI group and the control group. Thus, the difference observed between the groups of HNC patients could be associated with the improper regulation of GLP-1 secretion in obesity. As in the case of GIP, the role of GLP-1 in carcinogenesis is not fully understood. A few studies have indicated that GLP-1 may be associated with pancreatic cancer [81].

PAI-1, or serpin E1, is a single-chain glycoprotein that belongs to the family of serine protease inhibitors [82]. PAI-1 has been considered to have pro-cancer and pro-inflammatory properties [83,84]. In the presented study, the level of PAI-1 was lower in the healthy controls than in the HNC patients with normal BMI. In addition, the iBMI group presented a higher concentration of PAI-1 than the nBMI group. These results

could strongly suggest the involvement of PAI-1 in HNC carcinogenesis. In the study by Pavón et al. [85], a high expression of PAI-1 in HNC patients was shown to increase the risk of metastatic recurrences after therapy due to an increase in tumor cell migration and resistance to cisplatin. The authors also indicated that a higher expression of PAI-1 was associated with a poor prognosis. PAI-1 might lead to PI3K/Act pathway activation. PAI-1 is involved not only in tumor metastasis but also in angiogenesis and has been found to accelerate the growth of tumor cells [83].

GIP is a short peptide produced by the mucosa of the small intestine, and its main action is to stimulate glucose-dependent insulin secretion by pancreatic β cells [86]. In the course of obesity, GIP synthesis and secretion are dysregulated [81]. In our study, we did not observe differences in the GIP levels in the patients participating in the experiment. This may be due to the very short biological half-life of GIP [81]. However, it has been found that incretins, which include GIP, might be related to pancreatic cancer [81].

The differences in omentin-1, adiponectin, leptin, resistin, insulin, C-peptide, and GLP-1 levels observed between the nBMI and iBMI groups in this study may be related to the differences in BMI and the volume of white adipose tissue. According to the results of the presented study, the influence of the above-mentioned parameters on HNC carcinogenesis seems questionable. However, statistical analysis revealed significant differences in adipsin and glucagon levels between the nBMI group and the healthy control. Thus, these parameters may be associated with HNC carcinogenesis. In turn, average serum concentrations of visfatin and PAI-1 were the highest in the iBMI group and the lowest in the control group. The opposite relationship was observed in the case of ghrelin. The contribution of those biomarkers to the course of HNC and obesity remains ambiguous. Ghrelin, visfatin, and PAI-1 may participate in HNC carcinogenesis and accompany obesity. Figure 3 presents potential interactions between obesity, adipokines, and HNCs.

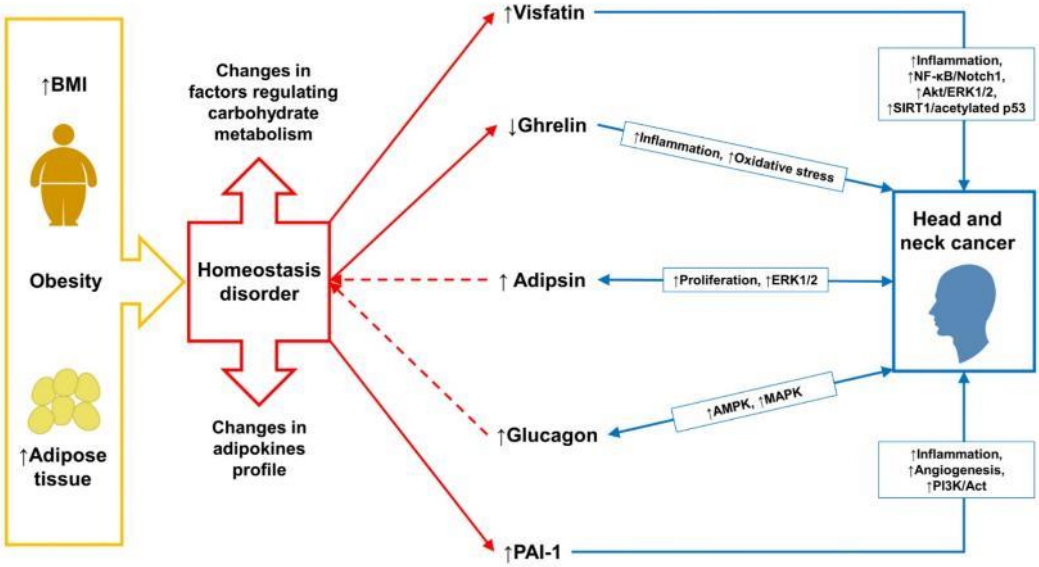

Figure 3. Possible interactions between impaired adipose tissue function in obesity and head and neck cancer. Abbreviations used: Act/ERK1/2: protein kinase B/extracellular signal-regulated kinases 1/2; AMPK: 5′ adenosine monophosphate-activated protein kinase; BMI: body mass index; MAPK: mitogen-activated protein kinase; NF-κB/Notch1: nuclear factor kappa-light-chain-enhancer of activated B cells/neurogenic locus notch homolog protein 1; PAI-1: plasminogen activator inhibitor-1; PI3K/Act: phosphatidylinositol 3-kinase/protein kinase B: SIRT1: silent mating type information regulation 2 homolog 1; dotted arrows—putative interactions.

The presented study has some limitations, including the small number of participants. However, to the best of the authors' knowledge, no study has been conducted with the participation of patients with HNC from the European population in which a wide range of parameters related to the endocrine role of adipose tissue, homeostasis of carbohydrate metabolism, and inflammation were simultaneously analyzed.

4. Materials and Methods

4.1. Study Subjects

This study involved 46 patients diagnosed with HNC. The condition for the inclusion of the patient in the study included the diagnosis of malignant neoplasms of the lip, oral cavity, or pharynx (according to the International Classification of Diseases—11th Revision (ICD-11): 2B60–2B69, 2B6A–2B6D), malignant neoplasms of the larynx (according to ICD-11: 2C23), or *carcinoma* in situ of the lip, oral cavity, or pharynx (according to ICD-11: 2E60.0) [87]. Exclusion criteria included the presence of acute and chronic diseases (infectious, autoimmune, genetic, and inflammatory) other than HNC and obesity. The assessment was made on the basis of a medical interview with the patient and an analysis of the patient's medical records. The study participants were divided into two groups of 23 patients each based on their BMI. Patients with a BMI < 25 kg/m^2 were assigned to the nBMI group. The iBMI group consisted of HNC patients with a BMI $\geq$ 25 kg/m^2. The classification of patients into the BMI-dependent groups was based on the WHO recommendations [88]. All patients were treated at the Prof. Franciszek Łukaszczyk Memorial Hospital's Oncology Center in Bydgoszcz, Poland. Participants were included in the study at the time of referral for planning radiotherapy using positron emission tomography–computed tomography (PET/CT). Patients were subjected to a histopathological examination. The histopathological analysis indicated that the study group included patients with G1 squamous cell carcinoma, G2 squamous cell carcinoma, nonkeratinizing G2 squamous cell carcinoma, or G2 keratinizing squamous cell carcinoma.

The control group consisted of 23 healthy people with anthropometric parameters similar to those of the nBMI patient group. The criteria for exclusion from the control group included chronic or acute diseases, such as cancer, diabetes, obesity, autoimmune disorders, and cardiometabolic disorders.

A questionnaire survey (Supplementary Questionnaire S1) was administered to study participants. The questions included in the questionnaire concerned addictions and other factors predisposing to the occurrence of HNC. The consent to participate in this research was voluntary and had no effect on the course of treatment. The study was accepted by the Bioethics Committee of the Nicolaus Copernicus University in Toruń, functioning at the Collegium Medicum in Bydgoszcz, Poland (consent no. KB 221/2018).

4.2. Study Design

Blood samples were collected in the morning after overnight fasting, between 7:00 a.m. and 9:00 a.m., from the median cubital vein by qualified medical personnel from the Department of Nuclear Medicine, Oncology Centre at Prof. Franciszek Łukaszczyk Memorial Hospital in Bydgoszcz, Poland. Each blood sample was collected into a 6 mL polypropylene tube with a clot activator and a gel separator. The samples were immediately transported under a reduced temperature condition to the laboratory of the Department of Medical Biology and Biochemistry, Faculty of Medicine, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland. Centrifugation (6000$\times$ g for 10 min at 4 °C) was performed to separate the blood serum from the blood clot. After centrifugation, the blood serum was aliquoted into Eppendorf tubes. Samples were stored at a temperature of −80 °C for further biochemical analysis.

4.3. Biochemical Analysis

Serum concentrations of omentin-1, adipsin, adiponectin, C-peptide, ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1, resistin, and visfatin were determined with the use

of commercially available research kits. The following kits were used accordingly: an enzyme-linked immunosorbent assay kit for human omentin-1 (BioVendor–Laboratorni medicina a.s., Brno, Czech Republic), Bio-Plex Pro™ human diabetes adipsin and adiponectin immunoassays (Bio-Rad Laboratories Inc., Hercules, CA, USA), Bio-Plex Pro™ human diabetes 10-plex immunoassay for C-peptide, ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1, resistin, and visfatin (Bio-Rad Laboratories Inc., Hercules, CA, USA). All analyses were performed in accordance with the manufacturer's instructions. The enzyme immune assay kits used in the study contained the reagents necessary for the analysis, such as standard concentration analytes and blank and control samples. The optical density for the omentin-1 research kit was tested with the BMG Labtech CLARIOstar multimode microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). Research kits manufactured by Bio-Rad Laboratories Inc. use fluorescence measurement to determine the level of individual analytes. Fluorescence was measured on a Bio-Plex® 200 system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The obtained results were expressed as pg/mL, ng/mL, or µg/mL.

4.4. Statistical Analysis

The study belongs to the category of observational case-control studies. Statistical analysis was performed with the use of Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). The Shapiro-Wilk test to assess the hypothesis of normal distribution, Levene's test to calculate the homogeneity of variances, and Pearson's correlation coefficient to evaluate the relationship between the measured parameters were used in this study. For analyzes that met the conditions of normal distribution, a Student's *t*-test for independent samples was performed, and the results were presented as a mean ± standard error of the mean (SEM). If the results did not meet the criterion of normal distribution, a non-parametric Mann–Whitney U test was used and the data was presented as a median and interquartile range (IQR–Q1; Q3).

5. Conclusions

The obtained results indicate a disruption of the endocrine function of adipose tissue and impaired glucose metabolism in the course of HNC. It has been shown that these changes are significantly intensified in obese HNC patients. In conclusion, neoplastic disease disturbs the homeostasis of glucose metabolism and increases the level of pro-inflammatory adipokines. Obesity, which is not a typical risk factor for HNC, may aggravate the negative metabolic changes associated with carcinogenesis. Among the analyzed parameters, ghrelin, visfatin, and PAI-1 seem to be particularly involved in the pathomechanisms of HNC development and/or progression. The levels of these analytes have been significantly altered in the HNC patients compared to healthy people. Interestingly, further changes have been observed in the obese HNC patients. Other markers of interest include adipsin and glucagon, which increased in the HNC patients compared to the controls. The parameters listed above seem to be related to head and neck carcinogenesis. Undoubtedly, further studies in this area are required; however, the presented results indicate new, potentially promising research directions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24043283/s1, Supplementary Questionnaire S1.

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Data Availability Statement: Data are available on request due to privacy/ethical restrictions.

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14.2. Oświadczenia autorów publikacji

14.2.1. Publikacja I

Nuszkiewicz, J.; Woźniak, A.; Szewczyk–Golec, K. *Ionizing Radiation as a Source of Oxidative Stress—The Protective Role of Melatonin and Vitamin D*. Int. J. Mol. Sci. 2020, 21, 5804, doi:10.3390/ijms21165804.

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Oświadczam, że mój wkład w powstanie niniejszej publikacji polegał na opracowaniu koncepcji artykułu, poszukiwaniu i analizie piśmiennictwa, przygotowaniu manuskryptu i przygotowaniu rycin oraz tabel.

Ponadto oświadczam, że udostępnienie publikacji nie będzie naruszało moich praw autorskich.

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Ponadto oświadczam, że udostępnienie publikacji nie będzie naruszało moich praw autorskich.

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14.2.2. Publikacja II

Nuszkiewicz, J.; Czuczejko, J.; Maruszak, M.; Pawłowska, M.; Woźniak, A.; Małkowski, B.; Szewczyk–Golec, K. *Parameters of Oxidative Stress, Vitamin D, Osteopontin, and Melatonin in Patients with Lip, Oral Cavity, and Pharyngeal Cancer*. Oxid. Med. Cell. Longev. 2021, 2021, 1–13, doi:10.1155/2021/2364931.

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Oświadczam, że mój wkład w powstanie niniejszej publikacji polegał na opracowaniu koncepcji artykułu, opracowaniu metodologii badania, przygotowaniu manuskryptu, przeprowadzeniu badań laboratoryjnych, akwizycji i analizie danych, przygotowaniu rycin.

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Oświadczam, że mój wkład w powstanie niniejszej publikacji polegał na nadzorowaniu realizacji badania.

Ponadto oświadczam, że udostępnienie publikacji nie będzie naruszało moich praw autorskich.

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Oświadczam, że mój wkład w powstanie niniejszej publikacji polegał na nadzorowaniu realizacji badania.

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Podpis

14.2.3. Publikacja III

Nuszkiewicz, J.; Czuczejko, J.; Dróżdż, W.; Woźniak, A.; Małkowski, B.; Szewczyk–Golec, K. *Concentration of Selected Adipokines and Factors Regulating Carbohydrate Metabolism in Patients with Head and Neck Cancer in Respect to Their Body Mass Index*. Int. J. Mol. Sci. 2023, 24, 3283, doi:10.3390/ijms24043283.

UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Lekarski
Collegium Medicum w Bydgoszczy

Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
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mgr Jarosław Nuszkiewicz
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OŚWIADCZENIE

Dotyczące publikacji: Nuszkiewicz, J.; Czuczejko, J.; Dróżdż, W.; Woźniak, A.; Małkowski, B.; Szewczyk-Golec, K. *Concentration of Selected Adipokines and Factors Regulating Carbohydrate Metabolism in Patients with Head and Neck Cancer in Respect to Their Body Mass Index*. Int. J. Mol. Sci. 2023, 24, 3283, DOI: 10.3390/ijms24043283.

Oświadczam, że mój wkład w powstanie niniejszej publikacji polegał na opracowaniu koncepcji badania, opracowaniu metodologii badania, przygotowaniu manuskryptu, przeprowadzeniu badań laboratoryjnych, akwizycji i analizie danych, przygotowaniu rycin.

Ponadto oświadczam, że udostępnienie publikacji nie będzie naruszało moich praw autorskich.

27.03.2023 r.
Data

Jarosław Nuszkiewicz
Podpis

dr n. med. Jolanta Czuczejko
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OŚWIADCZENIE

Dotyczące publikacji: Nuszkiewicz, J.; Czuczejko, J.; Dróżdż, W.; Woźniak, A.; Małkowski, B.; Szewczyk-Golec, K. *Concentration of Selected Adipokines and Factors Regulating Carbohydrate Metabolism in Patients with Head and Neck Cancer in Respect to Their Body Mass Index*. Int. J. Mol. Sci. 2023, 24, 3283, DOI: 10.3390/ijms24043283.

Oświadczam, że mój wkład w powstanie niniejszej publikacji polegał na uczestnictwie w przeprowadzeniu oznaczeń laboratoryjnych, zbieraniu materiału klinicznego.

Ponadto oświadczam, że udostępnienie publikacji nie będzie naruszało moich praw autorskich.

28.03.2023
Data

Jolanta Czuczejko
Podpis

dr hab. n. med. Wiktor Dróżdż, prof. UMK
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OŚWIADCZENIE

Dotyczące publikacji: Nuszkiewicz, J.; Czuczejko, J.; Dróżdż, W.; Woźniak, A.; Małkowski, B.; Szewczyk-Golec, K. Concentration of Selected Adipokines and Factors Regulating Carbohydrate Metabolism in Patients with Head and Neck Cancer in Respect to Their Body Mass Index. Int. J. Mol. Sci. 2023, 24, 3283, DOI: 10.3390/ijms24043283.

Oświadczam, że mój wkład w powstanie niniejszej publikacji polegał na nadzorowaniu realizacji badania.

Ponadto oświadczam, że udostępnienie publikacji nie będzie naruszało moich praw autorskich.

31.03.2023
Data

Wiktor Dróżdż
Podpis

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OŚWIADCZENIE

Dotyczące publikacji: Nuszkiewicz, J.; Czuczejko, J.; Dróżdż, W.; Woźniak, A.; Małkowski, B.; Szewczyk-Golec, K. *Concentration of Selected Adipokines and Factors Regulating Carbohydrate Metabolism in Patients with Head and Neck Cancer in Respect to Their Body Mass Index*. Int. J. Mol. Sci. 2023, 24, 3283, DOI: 10.3390/ijms24043283.

Oświadczam, że mój wkład w powstanie niniejszej publikacji polegał na nadzorowaniu realizacji badania.

Ponadto oświadczam, że udostępnienie publikacji nie będzie naruszało moich praw autorskich.

31.03.2023r.
Data

A. Woźniak
Podpis

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Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy
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OŚWIADCZENIE

Dotyczące publikacji: Nuszkiewicz, J.; Czuczejko, J.; Dróżdż, W.; Woźniak, A.; Małkowski, B.; Szewczyk-Golec, K. *Concentration of Selected Adipokines and Factors Regulating Carbohydrate Metabolism in Patients with Head and Neck Cancer in Respect to Their Body Mass Index*. Int. J. Mol. Sci. 2023, 24, 3283, DOI: 10.3390/ijms24043283.

Oświadczam, że mój wkład w powstanie niniejszej publikacji polegał na nadzorowaniu realizacji badania.

Ponadto oświadczam, że udostępnienie publikacji nie będzie naruszało moich praw autorskich.

2023.03.29
Data

Podpis

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OŚWIADCZENIE

Dotyczące publikacji: Nuszkiewicz, J.; Czuczejko, J.; Dróżdż, W.; Woźniak, A.; Małkowski, B.; Szewczyk-Golec, K. *Concentration of Selected Adipokines and Factors Regulating Carbohydrate Metabolism in Patients with Head and Neck Cancer in Respect to Their Body Mass Index*. Int. J. Mol. Sci. 2023, 24, 3283, DOI: 10.3390/ijms24043283.

Oświadczam, że mój wkład w powstanie niniejszej publikacji polegał na udziale w opracowaniu koncepcji badania, opracowaniu metodologii badania, analizie formalnej manuskryptu i krytycznym zrecenzowaniu artykułu.

Ponadto oświadczam, że udostępnienie publikacji nie będzie naruszało moich praw autorskich.

30.03.2023
Data

Podpis

14.3. Zgoda Komisji Bioetycznej

Zgoda numer KB 221/2018 wydana przez Komisję Bioetyczną Uniwersytetu Mikołaja Kopernika w Toruniu przy Collegium Medicum im. Ludwika Rydygiera w Bydgoszczy.

Uniwersytet Mikołaja Kopernika w Toruniu

Collegium Medicum im L. Rydygiera w Bydgoszczy

KOMISJA BIOETYCZNA

Ul. M. Skłodowskiej-Curie 9, 85-094 Bydgoszcz, tel.(052) 585-35-63, fax.(052) 585-38-11

KB 221/2018 Bydgoszcz, 27.03.2018 r.

Działając na podstawie art.29 Ustawy z dnia 5 grudnia 1996 roku o zawodzie lekarza (Dz.U. z 1997 r. Nr 28 poz. 152 (wraz z późniejszymi zmianami), zarządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz.U.Nr 47 poz.480) oraz Zarządzeniem Nr 21 Rektora UMK z dnia 4 marca 2009 r. z późn. zm. w sprawie powołania oraz zasad działania Komisji Bioetycznej Uniwersytetu Mikołaja Kopernika w Toruniu przy Collegium Medicum im Ludwika Rydygiera w Bydgoszczy oraz zgodnie z zasadami zawartymi w ICH – GCP

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(skład podano w załączeniu), na posiedzeniu w dniu **27.03.2018 r.** przeanalizowała wniosek, który złożyła kierownik badania:

dr hab. n. med. Karolina Szewczyk-Golec
Katedra Biologii Medycznej
Collegium Medicum w Bydgoszczy

z zespołem w składzie:

- prof. dr hab. n. med. Alina Woźniak, dr hab. n. med. Karolina Szewczyk-Golec, dr n. med. Bogdan Małkowski, dr n. med. Magdalena Bańkowska-Woźniak, dr n. med. Maciej Harat, dr n. med. Jolanta Czuczejko, mgr Jarosław Nuszkiewicz, lek. Rita Łopatto, lek. Marta Maruszak, mgr Paweł Waśniowski,

w sprawie badania:

„Wpływ radioterapii nowotworów tylnej ściany jamy nosowo-gardłowej oraz środkowego układu nerwowego na stężenie melatoniny i witaminy D oraz funkcjonowanie układu antyoksydacyjnego."

Po zapoznaniu się ze złożonym wnioskiem i w wyniku przeprowadzonej dyskusji oraz głosowania Komisja podjęła

Uchwałę o pozytywnym zaopiniowaniu wniosku

w sprawie przeprowadzenia badań, w zakresie określonym we wniosku pod warunkiem:

- poinformowania uczestników badania o celu oraz zakresie badań i uzyskania od każdego z nich osobnej, pisemnej, świadomej zgody na udział w badaniu, zgodnie z obowiązującymi przepisami, datowanej najpóźniej na moment rozpoczęcia badania a nie wcześniej niż data uzyskania z Komisji Bioetycznej zgody na takie badanie;
- zachowania tajemnicy wszystkich danych, w tym danych osobowych pacjentów, umożliwiających ich identyfikację w ewentualnych publikacjach;
- zapewnienia, że osoby uczestniczące w eksperymencie badawczym nie są ubezwłasnowolnione, nie są żołnierzami służby zasadniczej, nie są osobami pozbawionymi wolności, nie pozostają w zależności służbowej, dydaktycznej lub innej z prowadzącym badanie;

- sugerujemy uzyskanie podpisu uczestnika badania pod informacją o badaniu, lub sporządzenie formularza informacji i świadomej zgody na udział w badaniu na jednej kartce.

Jednocześnie informujemy, iż „Zgoda na udział w badaniu" winna zawierać m.in.: imię i nazwisko badanej osoby; Nr historii choroby pacjenta (L.ks.gł. Oddziału/Poradni) oraz datę i podpis badanej osoby, a także klauzule, że uczestnik badania wyraża zgodę na przetwarzanie danych osobowych dotyczących realizacji tematu badawczego, z wyjątkiem publikacji danych osobowych.

Kierownik badania zobowiązany jest do przechowywania wszystkich dokumentów dotyczących badania przez okres dwudziestu lat.

Zgoda obowiązuje od daty posiedzenia (27.03.2018 r.) do końca 2020 r.

Wydana opinia dotyczy tylko rozpatrywanego wniosku z uwzględnieniem przedstawionego projektu; każda zmiana i modyfikacja wymaga uzyskania odrębnej opinii. Wnioskodawca zobowiązany jest do informowania o wszelkich poprawkach, które mogłyby mieć wpływ na opinię Komisji oraz poinformowania o zakończeniu badania.

Od niniejszej uchwały podmiot zamierzający przeprowadzić eksperyment medyczny, kierownik zakładu opieki zdrowotnej, w której eksperyment medyczny ma być przeprowadzony, mogą wnieść odwołanie do Odwoławczej Komisji Bioetycznej przy Ministrze Zdrowia, za pośrednictwem Komisji Bioetycznej przy Collegium Medicum im. L. Rydygiera w Bydgoszczy, w terminie 14 dni od daty otrzymania niniejszej Uchwały.

Prof. dr hab. med. Karol Śliwka

Przewodniczący Komisji Bioetycznej

Otrzymuje:
dr hab. n. med. Karolina Szewczyk-Golec
Katedra Biologii Medycznej
Collegium Medicum w Bydgoszczy

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 27.03.2018 r.

Lp.	Imię i nazwisko	Funkcja	Podpis
1.	Prof. dr hab. med. Karol Śliwka	Przewodniczący	
2.	Prof. dr hab. med. Mieczysława Czerwionka-Szaflarska		
3.	Prof. dr hab. med. Anna Balcar-Boroń		
4.	Prof. dr hab. med. Marek Grabiec		
5.	Prof. dr hab. med. Zbigniew Włodarczyk		
6.	Dr hab. n. med. Katarzyna Pawlak-Osińska, prof. UMK		
7.	Dr hab. n med. Maria Kłopocka		
8.	Ks. dr hab. Wojciech Szukalski, prof. UAM		
9.	Dr n. med. Radosława Staszak-Kowalska		
10.	Mgr farm. Aleksandra Adamczyk		
11.	Mgr prawa Patrycja Brzezicka		
12.	Mgr prawa Joanna Połetek-Żygas	2-ca przewodniczącego	
13.	Mgr Lidia Iwińska-Tarczykowska		

Uniwersytet Mikołaja Kopernika w Toruniu

Collegium Medicum im L. Rydygiera w Bydgoszczy

KOMISJA BIOETYCZNA

Ul. M. Skłodowskiej-Curie 9, 85-094 Bydgoszcz, tel.(052) 585-35-63, fax.(052) 585-38-11

KB 221/2018 Bydgoszcz, 29.09.2020 r.

Działając na podstawie art.29 Ustawy z dnia 5 grudnia 1996 roku o zawodzie lekarza (Dz. U. z 1997 r. Nr 28 poz. 152 (wraz z późniejszymi zmianami), zarządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz. U. Nr 47 poz.480) oraz Zarządzeniem Nr 21 Rektora UMK z dnia 4 marca 2009 r. z późn. zm. w sprawie powołania oraz zasad działania Komisji Bioetycznej Uniwersytetu Mikołaja Kopernika w Toruniu przy Collegium Medicum im Ludwika Rydygiera w Bydgoszczy oraz zgodnie z zasadami zawartymi w ICH – GCP

Komisja Bioetyczna przy UMK w Toruniu, Collegium Medicum w Bydgoszczy

(której skład podano w załączeniu) na posiedzeniu w dniu **29.09.2020 r.** przeanalizowała prośbę o wyrażenie zgody na:

- przedłużenie okresu badań do końca 2023 roku,

którą złożyła:

dr hab. n. med. Karolina Szewczyk-Golec, prof. UMK
Katedra Biologii i Biochemii Medycznej
Collegium Medicum w Bydgoszczy

w sprawie badania:

„Wpływ radioterapii nowotworów tylnej ściany jamy nosowo-gardłowej oraz ośrodkowego układu nerwowego na stężenie melatoniny i witaminy D oraz funkcjonowanie układu antyoksydacyjnego."

Po zapoznaniu się ze złożonym dokumentem i w wyniku przeprowadzonej dyskusji oraz głosowania jawnego Komisja przyjęła do wiadomości podane informacje i wyraża zgodę na powyższe pod warunkami określonymi w uchwale Komisji podjętej w dniu 27.03.2018 r.

Zgoda na kontynuowanie przedmiotowego badania obowiązuje do końca 2023 r.

Prof. dr hab. med. Karol Śliwka

Przewodniczący Komisji Bioetycznej

Otrzymuje:
dr hab. n. med. Karolina Szewczyk-Golec, prof. UMK
Katedra Biologii i Biochemii Medycznej
Collegium Medicum w Bydgoszczy

Lista obecności

na posiedzeniu Komisji Bioetycznej

w dniu 29.09.2020 r.

Lp.	Imię i nazwisko	Funkcja/ Specjalizacja	Podpis
1.	Prof. dr hab. med. Karol Śliwka	Przewodniczący *medycyna sądowa*	
2.	Mgr prawa Joanna Połetek-Żygas	Z – ca przewodniczącego *prawniczka*	
3.	Prof. dr hab. med. Mieczysława Czerwionka-Szaflarska	*pediatra, alergologia i gastroenterologia dziecięca*	
4.	Prof. dr hab. med. Anna Balcar-Boroń	*pediatria, nefrologia*	
5.	Prof. dr hab. med. Marek Grabiec	*położnictwo, ginekologia onkologiczna*	
6.	Prof. dr hab. med. Zbigniew Włodarczyk	*chirurgia ogólna, transplantologia kliniczna*	
7.	Dr hab. n. med. Katarzyna Pawlak-Osińska, prof. UMK	*organizacja ochrony zdrowia, otolaryngologia*	
8.	Prof. dr hab. n med. Maria Kłopocka	*choroby wewnętrzne, gastroenterologia*	
9.	Ks. dr hab. Wojciech Szukalski, prof. UAM	*duchowny*	
10.	Dr n. med. Radosława Staszak-Kowalska	*pediatria, choroby płuc*	
11.	Mgr prawa Patrycja Brzezicka	*prawniczka*	
12.	Mgr farm. Aleksandra Adamczyk	*farmaceutka*	
13.	Mgr Lidia Iwińska-Tarczykowska	*pielęgniarska*	

14.4. Wzór kwestionariusza dla pacjenta

NUMER PRÓBKI: ..... (wypełnia badający)

ANKIETA

Szanowna/y Pani/Panie, zwracamy się z uprzejmą prośbą o wypełnienie poniższej ankiety, która stanowi część badania „Wpływ radioterapii nowotworów tylnej ściany jamy nosowo-gardłowej oraz OUN na stężenie melatoniny i witaminy D oraz funkcjonowanie układu antyosydacyjnego". **Udział w badaniu jest dobrowolny i bezpłatny, a uzyskane wyniki zostaną wykorzystane jedynie do celów naukowych.** Na poniższe pytania należy odpowiedzieć poprzez zaznaczenie jednej opcji (chyba, że w pytaniu podano inaczej).

Dziękujemy za poświęcony czas.

1. Płeć:
   - ☐ Kobieta
   - ☐ Mężczyzna
2. Wiek: .....
3. Masa ciała: .....[kg]
4. Wzrost: .....[cm]
5. Aktywność zawodowa:
   - ☐ Uczeń
   - ☐ Student
   - ☐ Osoba pracująca fizycznie
   - ☐ Osoba pracująca umysłowo
   - ☐ Bezrobotny
   - ☐ Rencista
   - ☐ Emeryt

---

6. Czy stosuje Pani/Pan suplementy diety zawierające witaminę D lub tran?
   - ☐ Tak
   - ☐ Nie
7. Czy w ostatnim miesiącu stosował/a Pani/Pan suplementy diety zawierające witaminę D lub tran?
   - ☐ Tak
   - ☐ Nie
8. Jak często spożywa Pani/Pan ryby?
   - ☐ Codziennie
   - ☐ Kilka razy w tygodniu
   - ☐ Raz na tydzień
   - ☐ Raz na dwa tygodnie
   - ☐ Rzadziej
9. Czy w ostatnich dwóch tygodniach korzystał/a Pani/Pan z solarium?
   - ☐ Tak
   - ☐ Nie
10. Proszę określić, ile czasu średnio spędza Pani/Pan na świeżym powietrzu w ciągu dnia:.....[min]
11. Czy pali Pani/Pan papierosy?
    - ☐ Tak
    - ☐ Nie
12. Czy pije Pani/Pan alkohol?
    - ☐ Tak (proszę określić jak często):
      - o Kilka razy w tygodniu
      - o Raz na tydzień
      - o Raz na dwa tygodnie
      - o Rzadziej
    - ☐ Nie
13. Czy uprawia Pani/Pan jakiś sport/ćwiczenia fizyczne?
    - ☐ Tak (proszę określić jak często):
      - o Codziennie
      - o Kilka razy w tygodniu
      - o Raz na tydzień
      - o Rzadziej
    - ☐ Nie
14. Jak określa Pani/Pan swoją dietę?
    - ☐ Zrównoważona
    - ☐ Bogatobiałkowa
    - ☐ Bogatotłuszczowa
    - ☐ Wegetariańska
    - ☐ Inna:.....
15. Czy przyjmuje Pani/Pan suplementy zawierające witaminy?
    - ☐ Tak
    - ☐ Nie