Pomorski Uniwersytet Medyczny w Szczecinie

ROZPRAWA DOKTORSKA

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Zaburzona homeostaza androgenowa a funkcje i morfologia wątroby – zwierzęcy model doświadczalny oparty na męskim pokoleniu potomnym (F1) od samców (F0) z farmakologicznie wywołanym deficytem DHT

Impaired androgen homeostasis and liver function and morphology – an experimental animal model based on male offspring (F1) from males (F0) with pharmacologically induced DHT deficiency

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Szczecin 2022

Niniejszą pracę pragnę zadedykować moim Rodzicom,
dzięki którym miałam możliwość kształcić się i zdobywać cenną wiedzę,
oraz Mężowi który stale mnie mobilizował i wspierał.

Składam najserdeczniejsze podziękowania **Promotorowi niniejszej pracy**
Pani dr hab. n. med. Agnieszce Kolasie za poświęcony czas oraz cenne rady,
a także za wsparcie i wyrozumiałość.

Pragnę podziękować również wszystkim Pracownikom Katedry Histologii
i Embriologii PUM za życzliwość,
która spotykała mnie na każdym kroku i okazaną pomoc.

Spis treści

WYKAZ STOSOWANYCH SKRÓTÓW

ACACA	karboksylaza alfa acetylo-Co	acetyl-CoA carboxylase alpha
ALIOS	amerykański zespół otyłości wywołany stylem życia	American lifestyle-induced obesity syndrome
ApoB	apolipoproteina B	apolipoprotein B
ApoM	apolipoproteina M	apolipoprotein M
AR	receptor androgenowy	androgen receptor
AR$^{-/-}$	myszy z wyłączoną ekspresją receptora androgenowego	AR knockout mice
ARKO	nokaut receptora androgenowego	AR knockout
ArKO	nokaut aromatazy	aromatase knockout
BrdU	bromodeoksyurydyna	bromodeoxyuridine
CLD	przewlekła choroby wątroby	chronic liver disease
CRP	białko C- reaktywne	C-reactive protein
DHT	dihydrotestosteron	dihydrotestosterone
DEN	dietylonitrozoamina	dimethylnitrosamine
E2	estradiol	estradiol
EDCs	substancje zaburzające funkcjonowanie układu hormonalnego	endocrine disrupting chemicals
ER	receptor estrogenowy	estrogen receptor
ERα	receptor estrogenowy alfa	estrogen receptor alpha
ERβ	receptor estrogenowy beta	estrogen receptor beta
ERαKO	myszy w wyłączoną ekspresją ER alfa	estrogen receptor alpha knockout mice
ESRRG	błonowy receptor estrogenowy gamma	estrogen-related receptor gamma
FA	kwasy tłuszczowe	fatty acid
FasL	ligand FASN	FASN ligand
FASN	syntaza kwasów tłuszczowych	fatty acid synthase
F1:Control	samce szczurów urodzone przez samice zapłodnione przez nietraktowane kontrolne samce szczurów	male rats born from females fertilized by untreated control male rats
F1:Fin	samce szczurów urodzone przez samice zapłodnione przez samce szczurów, którym podawano finasteryd	male rats born from females fertilized by finasteride-treated male rats
GK	glukokinaza	glucokinase

GLUT	transporter glukozy	glucose transporter
GLUT2	transporter glukozy 2	glucose transporter 2
Gpr30	błonowy receptor estrogenowy sprzężony z białkiem G	G protein-coupled estrogen receptor
HMG-CoA	reduktaza 3-hydroksy-3-metyloglutarylo-CoA	3-hydroxy-3-methylglutaryl-CoA reductase
H-AR$^{-/-}$	myszy pozbawione receptora androgenowego wybiórczo w wątrobie	hepatic androgen receptor knockout mice
HBV	wirus zapalenia wątroby typu B	hepatitis B virus
HCV	wirus zapalenia wątroby typu C	hepatitis C virus
HCC	rak wątrobowokomórkowy	hepatocellular carcinoma
HDL-c	lipoproteiny o dużej gęstości	high density protein
H-E	hematoksylina-eozyna	hematoxylin-eosin
HFD	dieta wysokotłuszczowa	high fat diet
HFHC	dieta wysokotłuszczowa i wysokocholesterolowa	high-fat and high-cholesterol diet
IHC	reakcja immunohistochemiczna	immunohistochemistry
IL	interleukina	interleukin
IL-6	interleukina 6	interleukin 6
IL-12A	interleukina 12A	interleukin 12A
INF	interferon	interferone
InR	receptor insulinowy	insulin receptor
IR	insulinooporność	insulin resistance
KCNN4	kanał potasowy aktywowany jonami wapnia	potassium calcium-activated channel
LDL-c	lipoproteiny o małej gęstości	low density protein
LKO	myszy z ERα-nokautem w komórkach wątroby	liver ERα-knockout
MetS	zespół metaboliczny	metabolic syndrome
NAFLD	niealkoholowa stłuszczeniowa choroba wątroby	nonalcoholic fatty liver disease
NASH	Niealkoholowe stłuszczeniowe zapalenie wątroby	nonalcoholic steatohepatitis
NF-κB	jądrowy czynnik transkrypcyjny NF kappa B	nuclear factor-kappa B
NK	„naturalni zabójcy”	„natural killer”

NLRP3	białko zawierające domenę pirynową 3	pyrin domain-containing protein 3
PAS	kwas nadjodowy-Schiffa	periodic acid-Schiff
PBC	pierwotne zapalenie dróg żółciowych	primary biliary cholangitis
PC	karboksylaza pirogronianowa	pyruvate carboxylase
PCNA	jądrowy antygen komórek proliferujących	proliferating cell nuclear antigen
PCOS	zespół policystycznych jajników	polycystic ovary syndrome
PEPCK	karboksykinaza fosfoenolopirogronianu	phosphoenolpyruvate carboxykinase
PI3K	kinaza fosfoinozytydu-3	phosphoinositide-3 kinase
PND	dzień po porodzie	postnatal day
PPARG	proliferator peroksysomów gamma	peroxisome proliferator-activated receptor gamma
PTP - 1B	fosfataza białkowo-tyrozynowea 1B	protein tyrosine phosphatase 1B
qRT-PCR	ilościowa reakcja łańcuchowej polimerazy z odwrotną transkrypcją	quantitative reverse transcription polymerase chain reaction
SGLT	transporter glukozy sprzężony z Na^+	sodium-coupled glucose transport
SHR	receptor hormonów płciowych	sex hormone receptor
SREBP - 1c	białko wiążące sekwencję odpowiedzi na sterole	sterol regulatory element binding protein
T	testosteron	testosterone
TG	trójglicerydy	triglyceride
TGF-β1	transformujący czynnik wzrostu beta 1	transforming growth factor beta 1
T2D	cukrzyca typu 2	type 2 diabetes
WB	western-blot	western-blot

1. NOTKA INFORMACYJNA

Niniejszą rozprawę doktorską pt. „*Zaburzona homeostaza androgenowa a funkcje i morfologia wątroby – zwierzęcy model doświadczalny oparty na męskim pokoleniu potomnym (F1) od samców (F0) z farmakologicznie wywołanym deficytem DHT*” stanowi cykl dwóch artykułów, jednego przeglądowego i jednego oryginalnego, w których jestem pierwszym autorem. Artykuły opublikowano w międzynarodowych czasopismach naukowych znajdujących się na liście *Journal Cytation Reports* (Thomson Reuters). Łączna wartość współczynnika oddziaływania (IF, ang. *Impact Factor*)
dla cyklu wymienionych prac (wg. Thomson Reuters Journal Citation Reports) wynosi 9,598 IF oraz 280 punktów Ministerstwa Edukacji i Nauki.

Rozprawę doktorską stanowią następujące publikacje:
1). Paulina Kur, Agnieszka Kolasa-Wołosiuk, Kamila Misiakiewicz-Has, Barbara Wiszniewska. *Sex Hormone-Dependent Physiology and Diseases of Liver*. International Journal of Environment Research and Public Health. 2020, 17, 2620; doi:10.3390/ijerph17082620. IF: 3,390. MEiN:140.
2). Paulina Kur, Agnieszka Kolasa-Wołosiuk, Marta Grabowska, Andrzej Kram, Maciej Tarnowski, Irena Baranowska-Bosiacka, Sylwia Rzeszotek, Małgorzata Piasecka, Barbara Wiszniewska. *The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, IR, and AR Expression in the Liver*. International Journal of Molecular Sciences. 2021, 22, 1242. https://doi.org/10.3390/ijms22031242. IF: 6,208. MEiN: 140.

Zgodnie z Rozporządzeniem Ministra Nauki i Szkolnictwa Wyższego z dnia 30 października 2015 roku (§5.2) do pracy zostały dołączone oświadczenia kandydata oraz pozostałych współautorów publikacji określające wkład w powstawanie prac stanowiących rozprawę doktorską.

Dodatkowo rozprawa doktorska została uzupełniona, o nieopublikowane wyniki badań, a przedstawione w niniejszej dysertacji.

Pracę wykonano w ramach badań statutowych Katedry i Zakładu Histologii i Embriologii, Pomorskiego Uniwersytetu Medycznego: WMS-167-02/S/16/2020-2022.

2. WPROWADZENIE

Zrównoważone funkcjonowanie układu hormonalnego jest niezbędne dla zachowania ciągłości gatunku poprzez kontrolowanie właściwego wzrostu i rozwoju zarodkowego oraz płodowego, a następnie postnatalnego poprzez prawidłowe przeprowadzanie procesów fizjologicznych. W ostatnich dekadach jednym z obszarów zainteresowań badaczy różnych dyscyplin jest wpływ czynników środowiskowych na zdrowie człowieka. Analizowanymi w tym aspekcie czynnikami są tzw. związki chemiczne zaburzające gospodarkę hormonalną (EDCs, ang. *endocrine disrupting chemicals),* które poprzez interferencję z układem hormonalnym wykazują niekorzystny wpływ na fizjologię organizmu [1]. Wśród znanych, antropogenicznych EDCs znajdują się substancje syntetyczne stosowane jako preparaty ochrony roślin, plastyfikatory stosowane w przemyśle, smary i rozpuszczalniki przemysłowe, niektóre środki higieny osobistej oraz produkty farmaceutyczne (np. niektóre leki). Zaburzenie homeostazy hormonalnej stało się poważnym problemem zdrowia publicznego, bowiem coraz częściej w etiologię wielu schorzeń wpisuje się właśnie rozchwianie fizjologii gruczołów endokrynnych [2].

Dane literaturowe dotyczące wpływu środowiskowych EDCs w większości przypadków opisują zaburzenia u osób/osobników bezpośrednio narażonych na ich działanie lub omawiają zaburzenia u potomstwa urodzonego przez matki narażone na te szkodliwe czynniki. Niemniej jednak, w zgodzie z badaniami *Trasler'a i Doerksen'a* [3] należy częściej podkreślać fakt, że nie tylko narażenie matki, ale także ojca na szkodliwe chemikalia może pociągać za sobą zmiany u potomstwa.

Finasteryd jest substancją czynną leków (Proscar®, Propecia®) stosowanych w terapii przerostu/raka prostaty, bądź leczenia młodych, przedwcześnie łysiejących mężczyzn [4, 5]. Finasteryd jest inhibitorem 5α-reduktazy typu 2, która katalizuje reakcję przekształcenia testosteronu (T, ang. *testosterone*) do dihydrotestosteronu (DHT, ang. *dihydrotestosterone*), zmienia zatem homeostazę androgenową organizmu [6]. Skutki uboczne działania finasterydu tzw. *finasteride side effects* głównie omawiane są w kontekście płodności męskiej [7, 8, 9, 10, 11, 12, 13, 14] oraz zaburzeń psychicznych [15], ostatnio jednak coraz częściej omawia się je również w kontekście międzypokoleniowym [16, 17, 6], czy epigenetycznym [18, 19].

Zważywszy na fakt, że finasteryd interferuje z układem endokrynnym (destabilizuje homeostazę androgenową) [6], może wpływać na fizjologię innych narządów/gruczołów ciała, których komórki wykazują ekspresję receptora dla androgenów. Do takich gruczołów zaliczana jest wątroba [20].

2.1. Uzasadnienie wyboru tematu

Wątroba jest jednym z najważniejszych gruczołów naszego organizmu. Komórki wątroby, hepatocyty odpowiadają za syntezę, magazynowanie (w formie glikogenu) i uwalnianie glukozy, przekształcanie węglowodanów i białek w tłuszcze, dokonują syntezy lipoprotein, fosfolipidów i cholesterolu. Komórki te partycypują również w procesach rozkładu lipidów do kwasów tłuszczowych, wytwarzają niemalże wszystkie białka osocza krwi (albuminy, globuliny, białka krzepnięcia, białka transportujące hormony steroidowe). Wątroba odpowiada za magazynowanie witamin (m.in. A, D, B_{12}) i żelaza oraz przetwarzanie hemu w bilirubinę, substratu do syntezy kwasów żółciowych. Oprócz funkcji detoksykacyjnej (neutralizacja toksyn, alkoholu, leków; przekształcanie amoniaku w mocznik) oraz sprzęgania i degradacji hormonów steroidowych, wątroba pełni także funkcje immunologiczne, nie tylko ze względu na dużą zawartość makrofagów (komórek Browicza-Kupffera), ale również ze względu na syntezę cytokin, interleukin (IL, ang. *interleukins*), interferonu (INF; ang. *interferon*), białka C reaktywnego (CRP; ang. *c-reactive protein*) i innych związków [21].

Te rozległe funkcje wątroby podlegają złożonym procesom kontrolnym. Coraz częściej dostrzega się tu rolę hormonów płciowych, a nawet mówi się o zależnych od płci chorobach wątroby i ich rokowaniu [22, 23]. Udowodniono na przykład, że wątroba samców szczurów ma wyższy poziom mikrosomalnych enzymów procesu β-oksydacji oraz 7-krotnie wyższą aktywność 2-hydrolazy estrogenowej w porównaniu do samic [24]. Mówi się także o tzw. "męskim wzorcu czynności wątroby", bowiem oprócz udowodnionej roli receptora androgenowego (AR, ang. *androgen receptor*) w rozwoju i progresji chorób wątroby takich, jak: rak wątrobowokomórkowy (HCC, ang. *hepatocellular carcinoma*), niealkoholowa stłuszczeniowa choroba wątroby (NAFLD, ang. *nonalcoholic fatty liver disease*), androgeny indukują ekspresję tzw. *male-specific genes* w wątrobie [25]. Z drugiej strony, jeżeli chodzi o dymorfizm płciowy, w wątrobie samic notuje się wyższą aktywność 5α-reduktazy [26].

Samce myszy pozbawione receptora androgenowego (ARKO, ang. *androgen receptor knock out*) w późniejszym wieku rozwijają otyłość [27, 28]; natomiast w innym modelu zwierzęcym, a mianowicie w badaniu prowadzonym na myszach pozbawionych receptora androgenowego (AR) wybiórczo w wątrobie (tzw. H-AR$^{-/-}$, ang. *hepatic AR knock-out mice*) wykazano, że tylko samce, a nie samice karmione wysokotłuszczową dietą (HFD, ang. *high-fat diet*) rozwinęły stłuszczenie wątroby i insulinooporność
(IR, ang. *insulin resistance*), a samce będące na zdrowej diecie wykazywały umiarkowane stłuszczenie wątroby w starszym wieku. W populacji ludzkiej, wykazano iż obniżenie ekspresji AR koreluje z ciężkością alkoholowego uszkodzenia wątroby [29]. Ponadto, w trakcie nasilania się HCC [30], marskości, uszkodzeń czy regeneracji wątroby [31, 32] wzrasta ekspresja TGF-β1 (ang. *transforming growth factor beta 1*), czynnika którego synteza regulowana jest przez kompleks androgen-AR, bowiem w genie kodującym ten czynnik obecne są tzw. *androgen response sequence* [33].

2.2. Hormonozależny metabolizm lipidów w wątrobie

Niskie stężenie testosteronu koreluje ze zwiększonym stłuszczeniem wątroby u mężczyzn [34, 35]. Suplementacja testosteronem mężczyzn z cukrzycą typu 2 (T2D, ang. *type 2 diabetes*), otyłością trzewną czy deficytem androgenów skutkowała redukcją tkanki tłuszczowej w trzewiach [36, 37] oraz zmniejszyła objawy związane z niealkoholowym stłuszczeniem wątroby i zespołem metabolicznym (MetS, ang. *metabolic syndrome*) [38]. U podstaw molekularnych tych procesów może stać supresyjna rola testosteronu wobec ekspresji enzymów regulujących syntezę kwasów tłuszczowych, i tę protekcyjną rolę T wobec stłuszczenia wątroby dowiedziono na myszach z deficytem androgenów, suplementowanych testosteronem i karmionych dietą wysoko tłuszczową. Ekspresja kluczowych enzymów szlaku syntezy kwasów tłuszczowych: karboksylazy alfa acetylo-CoA (ACACA, ang. *acetyl-CoA carboxylase alpha)* i syntazy kwasów tłuszczowych (FASN, ang. *fatty acid synthase*) była niższa u myszy otrzymujących T, w porównaniu do zwierząt otrzymujących placebo [39]. Autorzy tej publikacji sugerują również wpływ testosteronu na odkładanie się lipidów w wątrobie, które jest niezależne od klasycznego, jądrowego, wewnątrzkomórkowego receptora androgenowego.

Suplementacja szczurów dihydrotestosteronem, aktywniejszym metabolitem testosteronu pociągnęła za sobą obniżenie poziomów cholesterolu, w tym frakcji lipoprotein o małej gęstości (LDL-c, ang. *low-density lipoprotein cholesterol*) i apolipoproteiny B (ApoB, ang. *apolipoprotein B*) oraz podwyższenie fosforylacji reduktazy 3-hydroksy-3-metyloglutarylo-CoA (HMG-CoA, ang. *3-hydroxy-3-methylglutaryl-CoA reductase*) [40]. Zarówno w badaniach *in vivo* na myszach oraz *in vitro* na ludzkich komórkach HepG2 wykazano hamujący wpływ DHT na apolipoproteinę M (ApoM, ang. *alpolipoprotein M*) na poziomie mRNA i białka poprzez proteinową kinazę C, czyli niezależnie od aktywności klasycznego, wewnątrzkomórkowego AR [41]. Suplementacja DHT myszy po orchidektomii skutkowała otyłością związaną z obniżeniem nakładu energetycznego i β-oksydacji lipidów w wątrobie; DHT podwyższył poziom HDL-c (ang. *high-density lipoprotein cholesterol*), triglicerydów i znacząco zredukował ekspresję genu kodującego 7α-hydroksylazę, co pociągnęło za sobą obniżenie produkcji żółci [42].

2.3. Hormonozależny metabolizm glukozy w wątrobie

Metabolizm lipidów i węglowodanów jest ściśle ze sobą powiązany i wzajemnie zależny poprzez substraty, metabolity, enzymy oraz mechanizmy regulacyjne. Przykładowo, lipogeneza *de novo* wolnych kwasów tłuszczowych z acetylo-CoA jest regulowana przez insulinę, która aktywuje białko wiążące sekwencję odpowiedzi na sterole (SREBP-1c, ang. *sterol regulatory element-binding protein*), kontrolującego transkrypcję enzymów lipogennych takich, jak syntaza kwasów tłuszczowych [21]. Dlatego też np. w NAFLD oprócz ogólnej otyłości stwierdza się również insulinooporność [43]. Postuluje się również, że kluczowym czynnikiem patogenezy IR jest akumulacja triglicerydów w hepatocytach [44] wraz ze zwiększoną produkcją endogennej glukozy u osób zarówno szczupłych, jak i otyłych [45, 46].

Już wczesne badania na hipogonadycznych myszach z brakiem receptora androgenowego udokumentowały ich oporność na insulinę [47]. Jednak szczegóły dotyczące mechanizmów leżących u podstaw tego, w jaki sposób sygnalizacja androgenowa poprzez AR reguluje wrażliwość na insulinę w poszczególnych narządach ciągle pozostaje niejasna. Wg. *Lin i wsp*. [47] insulinooporność samców myszy H-AR$^{-/-}$ wynikała ze spadku aktywności kinazy fosfoinozytydu-3 (PI3K, ang. *phosphoinositide-*

3 kinases) i podwyższenia ekspresji karboksykinazy fosfoenolopirogronianu (PEPCK, ang. *phosphoenolpyruvate carboxykinase*) w korelacji ze wzrostem ekspresji fosfatazy białkowo-tyrozynowej 1B (PTP-1B, ang. *protein-tyrosine phosphatase 1B*).

Pod względem klinicznym, zwiększoną IR i upośledzoną tolerancję glukozy obserwuje się u mężczyzn z niedoborem testosteronu [48]. Suplementacja testosteronem mężczyzn z cukrzycą typu 2, otyłością trzewną, czy deficytem androgenów obniża oporność na insulinę, glikemię i hipercholesterolemię [36, 37]. Jednocześnie istnieją doniesienia, że u zdrowych mężczyzn, którym farmakologicznie zahamowano aktywność obydwu izoform 5α-reduktazy (dutasterydem typu 1, finasterydem typu 2) udokumentowano wzrost insulinooporności hepatocytów tylko w przypadku dutasterydu [49]. Żaden z użytych w doświadczeniu inhibitorów konwersji T do DHT nie miał wpływu na poziom glukozy i insuliny we krwi pacjentów; ale w innej analizie wykazano, że szybkość syntezy endogennej glukozy mocno wzrosła po zastosowaniu dutasterydu, zgodnie ze zwiększoną insulinoopornością wątroby [49]. *Livingstone i wsp.* dowiedli, iż finasteryd indukuje hiperinsulinemię i stłuszczenie wątroby u otyłych samców szczurów szczepu Zucker, zarówno u kontrolnych, jak i wykastrowanych [50]. W tym samym badaniu wykazano insulinooporność u szczurów z deficytem, bądź z zahamowaną aktywnością 5α-reduktazy typu 1 [50].

2.4. Regeneracja, degeneracja, karcynogeneza w odpowiedzi na hormony płciowe

Rolę receptora androgenowego udowodniono dla procesów karcynogenezy hepatocytów w przebiegu HCC wywołanej takimi czynnikami, jak wirusowe zapalenie wątroby typu B (HBV, ang. *hepatitis B virus*), wirusowe zapalenie wątroby typu C (HCV ang. *hepatitis C virus*) czy alkohol [51, 52, 53, 22, 54]. W badaniach *in vitro* ludzkich hepatocytów (linie: HepG2, SMMC-7721, L02) wykazano, że DHT nie tylko indukuje zatrzymanie cyklu komórkowego, ale także inicjuje apoptozę w komórkach wątroby linii SMMC-7721 i L02 poprzez kaskadę PKR/eIF2α/GADD153. Zatem szlak androgenowy odgrywa rolę w fizjologii komórek wątroby i regulowaniu ich apoptozy, której deregulacja może być zaangażowana w patogenezę chorób wątroby [55]. Badania wykazują, że również sygnalizacja estrogenowa kontroluje proliferację hepatocytów.

Co ważne, sygnalizacja estradiolem (E2, ang. *estradiol*), za pośrednictwem receptora estrogenowego alfa (ERα, ang. *estrogen receptor alpha*), ma kluczowe znaczenie dla skutecznej regeneracji wątroby – wzrost poziomu E2 jest jednym z sygnałów inicjujących odnowę wątroby, zatem terapia z wykorzystaniem estradiolu może być korzystna dla stymulacji regeneracji wątroby u ludzi [56].

3. GŁÓWNE ZAŁOŻENIA I CELE PRACY DOKTORSKIEJ ORAZ SPODZIEWANE KORZYŚCI BADANIA

Wyżej przedstawione dane literaturowe, dotyczą zaburzeń metabolicznych w wątrobie, wynikających z:

(i) nieprawidłowości w sygnalizacji (up-/down-regulacja AR),

(ii) zaburzenia homeostazy androgenowej (deficyt/suplementacja T/DHT),

(iii) zahamowania aktywności jednej z izoform 5α-reduktazy, i dotyczą populacji męskiej pokolenia bezpośrednio dotkniętego wspominanymi nieprawidłowościami (i-iii). Mało natomiast wiadomo na temat skutków ubocznych w męskim pokoleniu potomnym, będących konsekwencją przyjmowania przez pokolenie ojcowskie leków wpisujących się w definicję *endocrine disrupting chemicals.*

Dlatego też, celem pracy badawczej była ocena tkanek pokolenia potomnego (F1:Fin) uzyskanego od samic, krytych szczurami z wywołanym farmakologicznie (finasterydem) deficytem DHT (F0:Fin) w odniesieniu do grupy kontrolnej (F1:Control) w tym:

1). analiza markerów metabolizmu cukrowcowo-lipidowego:

- akumulacja glikogenu w hepatocytach (reakcja PAS),
- ekspresja na poziomie mRNA: karboksylazy pirogronianowej (PC, ang. *pyruvate carboxylase*), glukokinazy (GK, ang. *glucokinase*), karboksylazy acetylo-CoA alfa (ACACA), syntazy kwasów tłuszczowych (FASN), receptora gamma aktywowanego przez proliferator peroksysomów (PPARG, ang. *peroxisome proliferator-activated receptor-gamma*) w wątrobie,
- ekspresja na poziomie mRNA: receptora insulinowego (InR, ang. *insulin receptor*) oraz transportera glukozy 2 (GLUT2, ang. *glucose transporter 2*) w wątrobie,
- poziom glukozy, insuliny i triglicerydów we krwi

2). analiza wrażliwość komórek wątroby na hormony płciowe:

- androgeny (ekspresja AR na poziomie mRNA – qRT-PCR i białka – IHC),
- estrogeny (ekspresja ERα – IHC)

3). analiza zdolności proliferacyjnej hepatocytów

- immuoekspresja PCNA (ang. *proliferating cell nuclear antigen*).

Spodziewanym rezultatem badania jest ustalenie, czy przyjmowanie finasterydu przez młodych mężczyzn w wieku reprodukcyjnym może negatywnie wpływać na funkcje wątroby ich męskiego potomstwa. Istotność tego zagadnienia wynika z globalnego wzrostu otyłości, zapadalności na cukrzycę i niealkoholowe stłuszczenie wątroby, które są procesami hormonozależnymi.

4. MODEL BADAWCZY

4.1. Zwierzęta

Materiał do badań stanowiły zebrane wątroby (utrwalone i zbloczkowane w parafinie oraz zamrożone w -70ºC), które były wypreparowane od 7-, 14-, 21-, 28- i 90-dniowych samców (F1:Fin) urodzonych z samic, krytych przez samce otrzymujące finasteryd (F0:Fin; Proscar®, MSD, Cramlington, UK) dłużej, niż czas trwania jednej spermatogenezy (od 56 dnia podawania leku do momentu zakończenia pozyskania potomstwa, czyli przez ok. 6 miesięcy w dawce 5mg/dzień). Męskie pokolenie potomne (F1:Control) od nietraktowanych finasterydem samców (F0:Control) stanowiło grupę kontrolną.

Eksperyment (pozyskanie pokolenia potomnego samców szczurów w różnym postnatalnym wieku rozwojowym) przeprowadzono w pełnej zgodności z polskim prawem oraz za zgodą Lokalnej Komisji ds. Doświadczeń na Zwierzętach w Szczecinie (Uchwała nr 23/2010 z dnia 21 lipca 2010 r.).

Praca badawcza obejmowała 5 grup wiekowych szczurów płci męskiej, rasy Wistar. W sumie w badaniu przeanalizowano wątroby od 50 osobników. Poniżej przedstawiono oznaczenia poszczególnych grup zwierząt oraz liczebność osobników w grupie (n):

F1:Control 7 (n=5),	F1:Fin 7 (n=5),
F1:Control 14 (n=5),	F1:Fin 14 (n=5),
F1:Control 21 (n=5),	F1:Fin 21 (n=5),
F1:Control 28 (n=5),	F1:Fin 28 (n=5),
F1:Control 90 (n=5)	F1:Fin 90 (n=5)

4.2. Pozyskane tkanki

Pozyskane tkanki posłużyły do przygotowania skrawków parafinowych i zostały poddane:

- ✓ rutynowemu barwieniu hematoksyliną-eozyną (H-E, ang. *hematoxylin and eosin*) celem oceny morfologicznej,
- ✓ barwieniu metodą PAS (ang. *periodic acid Schiff*) celem detekcji glikogenu w hepatocytach
- ✓ reakcji immunohistochemicznej (IHC, ang. *immunohistochemistry*) z wykorzystaniem specyficznych przeciwciał pierwszorzędowych celem oceny ekspresji: receptora androgenowego, receptora estrogenowego alfa, receptora insulinowego, transportera glukozy 2 oraz PCNA, aby sprawdzić wrażliwość komórek na androgeny, estrogeny, insulinę oraz transport glukozy do komórki, jak również aktywność proliferacyjną hepatocytów.

Zamrożone próbki wątrób posłużyły do przygotowania homogenatów tkankowych i izolacji RNA do analizy molekularnej (qRT-PCR, ang. *quantitative reverse transcription polymerase chain reaction*) celem oceny ekspresji czynników zaangażowanych w procesy zależne od insuliny (ekspresja InR) i glukozy (ekspresja GLUT2) oraz enzymów związanych z metabolizmem lipidów: karboksylazy pirogronianowej, glukokinazy, karboksylazy alfa acetylo-CoA, syntazy kwasów tłuszczowych, receptora gamma aktywowanego przez proliferatory peroksysomów, jak również związanych z wrażliwością komórki na androgeny (ekspresja AR).

Zamrożona surowica krwi posłużyła do pomiaru stężenia glukozy, trójglicerydów (wykonano w Laboratorium Centralnym SPSK2, PUM) oraz insuliny (wykonano w laboratorium weterynaryjnym).

5. METODY BADAŃ

5.1. Rutynowe barwienie histologiczne i histochemiczne

Barwienie hematoksyliną-eozyną zostało wykonane z przygotowanych na świeżo odczynników, natomiast do reakcji PAS użyto komercyjnego zestawu firmy Bio-Optica® (nr cat.: 04-130802, Mediolan, Włochy).

5.2. Reakcja immunohistochemiczna

Do reakcji IHC wykorzystano komercyjny zestaw Dako LSAB+System-HRP (DakoCytomation, K0679) natomiast przeciwciała pierwszorzędowe skierowane były przeciwko: AR (SantaCruz Biotechnology, sc-7305, rozcieńczenie 1:50), ERα (sc-7305, 1:250), InR (Abcam, ab 60946, 1:20), GLUT2 (Invitrogen, nr cat.: PA5-77459, 1:250), PCNA (ab 60946, 1:250).

Uzyskane wyniki z barwienia PAS oraz IHC (dla GLUT2, ERα, PCNA) zeskanowano (skaner ScanScope AT2, Leica) i poddano densytometrycznej, komputerowej analizie obrazu (ImageScope wersja 11.2.0.780, Aperio Technologies) z zastosowaniem algorytmu v9 (wersja 9.1; Aperio Technologies) celem ilościowej oceny poziomów powyższych markerów w grupie badanej względem grupy kontrolnej.

5.3. Badania ekspresji genów na poziomie mRNA techniką qRT-PCR

Ekspresja mRNA dla AR, InR, GLUT2, GK, PC, ACACA, FASN i PPARG została przeprowadzona metodą PCR w czasie rzeczywistym (qRT-PCR), przy użyciu sond typu TaqMan. Z homogenatów wątroby wyizolowano całkowity RNA za pomocą zestawu AllPrep DNA/RNA Mini Kit firmy Qiagen. Stężenie i czystość izolowanego RNA określono przy pomocy spektrofotometru Lambda Bio+, a następnie przeprowadzono odwrotną transkrypcję z użyciem zestawu FirstStrand cDNA synthesis kit. Ilościowe oznaczanie poziomu mRNA wykonano metodą PCR czasu rzeczywistego na urządzeniu ABI 7500 Fast, wykorzystując do tego odczynnik Power SYBR Green PCR Master Mix. Poziom ekspresji genu w próbce został porównany do kontroli endogennej – GAPDH (gen referencyjny).

5.4. Metody statystyczne

Wyniki uzyskane z qRT-PCR oraz oznaczeń stężenia glukozy, insuliny, trójglicerydów i androgenów (T, DHT; [6]) w surowicy zostały przeanalizowane za pomocą programu Statistica 6.1 (StatSoft, Kraków, Polska). Dla każdego z parametrów obliczono średnie i odchylenia standardowe (X±SD). Rozkład normalny wyników dla poszczególnych zmiennych wykazano za pomocą testu Shapiro-Wilka. Ponieważ większość rozkładów odbiegała od rozkładu normalnego, do dalszej analizy wykorzystano testy nieparametryczne. Aby ocenić różnice między grupami, przeprowadzono: nieparametryczny test-U Manna-Whitney'a i test Kruskal'a-Wallis'a z testem wielokrotnych porównań Dunn'a post hoc ($p<0,05$). Dodatkowo, poziomy T i DHT w surowicy zostały skorelowane z poziomami mRNA dla GLUT2, InR i AR według współczynnika korelacji rang Spearman'a (Rs). Ten sam test zastosowano do skorelowania stężenia glukozy w surowicy z wiekiem zwierząt oraz poziomem transkryptu dla GLUT2, a także skorelowania poziomów immunoekspresji ERα z PCNA.

6. SYNTETYCZNE STRESZCZENIA PUBLIKACJI STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

6.1. Sex Hormone-Dependent Physiology and Diseases of Liver

➢ ***Zależny od hormonów płciowych metabolizm glukozy w zdrowej wątrobie, oporność na insulinę (IR) i cukrzycę typu drugiego (T2D)***

Transportery komórkowe zaangażowane w transport glukozy – ich ekspresja w IR, T2D i MetS: Wątroba jest jednym z narządów zaangażowanych w metabolizm glukozy ze względu na zdolność do jej produkcji (glukogeneza), i magazynowania w postaci glikogenu (glikogeneza), dzięki czemu w razie potrzeby może być rozkładana w szlaku glikolitycznym (glikogenoliza) lub przekształcana w kwasy tłuszczowe na drodze lipogennej (lipogeneza). Transport glukozy jest kierowany przez transportery glukozy sprzężone z Na^+ (SGLT, ang. *sodium-glucose transporter*) lub nie sprzężone z sodem transportery glukozy (GLUT, ang. *glucose transporter*). Wykazano, że u wykastrowanych szczurów podwyższony poziom glukozy we krwi oraz wyższy poziom GLUT2 (na poziomie mRNA i białka) uległ normalizacji po podaniu egzogennego testosteronu, dlatego można przypuszczać, że testosteron bezpośrednio wpływa na transkrypcję i translację genu GLUT2. W zwierzęcych modelach doświadczalnych wykazano, że niedobór testosteronu zwiększał wątrobową syntezą glukozy (hiperglikemia) i powodował wystąpienie objawów podobnych do T2D lub MetS. Z kolei u kobiet zaobserwowano, iż wysoki poziom testosteronu wiązał się z podwyższonym ryzykiem wystąpienia cukrzycy. Jednocześnie wykazano, że estrogeny mają niewielki wpływ na ekspresję GLUT2 i receptora insuliny w wątrobach samców szczurów.

Związek między androgenami/receptorem androgenowym/5alfa-reduktazą a wątrobową homeostazą glukozy: Obniżona ekspresja AR u mężczyzn sprzyjać może insulinooporności, prowadząc dalej do rozwoju T2D. Potwierdzają to badania przeprowadzone *in vivo* na samcach myszy z wyłączoną ekspresją AR ($AR^{-/-}$), u których wraz z wiekiem stopniowo spadała wrażliwości na insulinę oraz osłabiała się tolerancja glukozy. Badania sugerują również, że brak aktywności 5α-reduktazy, enzymu przekształcającego T w DHT, indukuje IR.

Związek między estrogenami/receptorami estrogenowymi/aromatazą a wątrobową homeostazą glukozy: Pacjenci płci męskiej z nieaktywnym ERα oraz z deficytem aromatazy, wykazywali obniżony metabolizm glukozy, insulinooporność i hiperinsulinemię, co wiązało się z rozwojem T2D oraz MetS. Egzogenne podawanie estrogenu przywracało prawidłowy metabolizm glukozy jedynie u mężczyzn z deficytem aromatazy. Badania prowadzone na samicach szczurów donoszą, że wycięcie jajników nie wpływa na poziom magazynowania glikogenu w wątrobie ani na ekspresję genu PEPCK, głównego enzymu szlaku glukoneogenezy, jednak po podaniu egzogennego estradiolu doszło do wzrost akumulacji glikogenu i zmniejszenia ekspresji PEPCK.

Z kolei inne badania wskazują, że po usunięciu jajników u otyłych samic szczurów, ekspresja genów lipogennych (SREBP-1c; FASN) i adipogennych (PPARG) w wątrobie znacznie wzrosła, a podanie egzogennego E2 lub agonisty ERα zmniejszyło ekspresję SREBP-1c, FASN i PPARG w hepatocytach.

Tabela 1. Podsumowanie danych dotyczących zaburzeń równowagi hormonalnej oraz ekspresji receptorów hormonów płciowych w odniesieniu do metabolizmu węglowodanów w wątrobie, jako przyczyna wielu fizjologicznych dysfunkcji, zespołów czy chorób (z podziałem na płeć i uwzględnieniem czynników środowiskowych np. dieta).

Zaburzony metabolizm węglowodanów u mężczyzn	
↓ Testosteron	T2D, MetS
AR (brak)	IR, T2D
AR knockout	↓ Metabolizm glukozy, IR, hyperinsulinemia
AR knockout + HFD	↓ Wrażliwość na insulinę
5α-red1 knockout + ALIOS	Hyperinsulinemia
5α-red1 knockout + HFD	Hyperinsulinemia
↓ ERα	↓ Metabolizm glukozy, IR, hyperinsulinemia, T2D, MetS
ERαKO	↓ Tolerancja glukozy, IR w wątrobie
↓ Testosteron	T2D, MetS
Zaburzony metabolizm węglowodanów u kobiet	
↓ Testosteron	↑ Glukoza
↓ Estrogen	↑ Cukrzyca

➢ ***Zależny od hormonów płciowych, metabolizm lipidów w zdrowej wątrobie, w niealkoholowej stłuszczeniowej chorobie wątroby i zespole metabolicznym***

Związek między estrogenami/receptorami estrogenowymi/aromatazą a wątrobową homeostazą lipidów: W przypadku wątrobowego metabolizmu lipidów należy podkreślić, że również estrogeny mają tu kluczowe znaczenie. U kobiet z rakiem piersi leczonych tamoksyfenem (selektywny modulator ER, o działaniu antyestrogennym) obserwuje się, jako powikłanie leczenia, stłuszczenie wątroby spowodowane upośledzeniem β-oksydacji kwasów tłuszczowych (FA, ang. *fatty acid*). Ochronna funkcja estrogenów jest głównie przypisywana sygnalizacji poprzez ERα ponieważ, jak wykazano w analizie mikromacierzy, samce i samice myszy z wyłączoną ekspresją ERα (ERαKO, ang. *estrogen receptor alpha knockout mice*) wykazują stłuszczenie wątroby z powodu nadekspresji genów lipogennych i obniżonej ekspresji genów zaangażowanych w przemiany lipidów. Estrogenny szlak regulacji funkcji wątroby również w dużym stopniu zależy od aktywności aromatazy, enzymu przekształcającego androgeny w estrogeny. U samców myszy z nokautem aromatazy (ArKO, ang. *aromatase knockout*), powstałe stłuszczenie wątroby można normalizować przez podawanie egzogennych estrogenów, przy czym efekt ten nie był obserwowany u samic.

Związek między niealkoholową stłuszczeniową chorobą wątroby (NAFLD)/niealkoholowym stłuszczeniowym zapaleniem wątroby (NASH) a hormonami płciowymi: Hipotezy dotyczące związku poziomu androgenów z wystąpieniem NAFLD nie są spójne. Może to wynikać ze stosowania różnych modeli zwierzęcych, metod leczenia pacjentów lub kombinacji różnych substytutów hormonów steroidowych. Coraz powszechniej uważa się jednak, że to stosunek T do DHT jest najważniejszy w rozwoju i progresji NAFLD, a nie ich ogólne/całkowite stężenia.

Tabela 2. Podsumowanie danych dotyczących zaburzeń równowagi hormonalnej oraz ekspresji receptorów hormonów płciowych, w odniesieniu do metabolizmu lipidów w wątrobie, jako przyczyna wielu fizjologicznych dysfunkcji, zespołów czy chorób (z podziałem na płeć i uwzględnieniem czynników środowiskowych np. dieta).

Zaburzony metabolizm lipidów u mężczyzn	
↓ Testosteron	↑albo ↓Stłuszczenie wątroby
5α-red1 knockout + ALIOS	Stłuszczenie wątroby
5α-red1 knockout + HFD	↑ TG, stłuszczenie wątroby
AR- knockout w wątrobie + HFD	Stłuszczenie wątroby, IR
↓ AR	↑ TG w wątrobie, stłuszczenie wątroby
ARKO	↑ TG w wątrobie, otyłość, IR
ArKO	Stłuszczenie wątroby, otyłość
↓ Aromataza	Zaburzenia metaboliczne
↓ ERα + HFD	↑ TG, ↑ diacyloglicerydy, IR
ERαKO	Stłuszczenie wątroby
LKO + ↓ Gpr30 + HFD	↑ TG, ↑ diacyloglicerydy
Zaburzony metabolizm lipidów u kobiet	
↓ Estrogen	↑ LDL-C, ↓ HDL-C, stłuszczenie wątroby
↓ Estrogen + HFD/HFHC	NASH
LKO + ↓ Gpr30 + HFD	↑TG, ↓HDL-C, ↑ diacyloglicerydy ↑ akumulacja lipidów w wątrobie, IR
ERαKO	Stłuszczenie wątroby
ArKO	Stłuszczenie wątroby, otyłość
Hyperandrogenizm + PCOS	Otyłość, IR, NAFLD

- ***Wirus zapalenia wątroby typu B i C a rak wątrobowokomórkowy***

Zakażenie HBV i jego późniejsze konsekwencje są różne u mężczyzn i kobiet. Wpływ różnic płciowych (takich jak: stężenia hormonów płciowych i ich wzajemnych proporcji) na wrodzoną odpowiedź immunologiczną w zakażeniu HBV jest w dużej mierze nieznany. Nieco więcej wiadomo na temat różnic między płciami w nabytej odpowiedzi immunologicznej w przebiegu zakażenia HBV. Udowodniono, że HCC po zakażeniu HBV rozwija się częściej u mężczyzn niż u kobiet. Może to oznaczać, że w patogenezie HCC ważną rolę odgrywają hormony płciowe.

Związek między estrogenami/ERs a ostrą niewydolnością wątroby, taką jak HCC indukowany HBV: Dymorfizm płciowy w chorobach wątroby związanych z wirusem zapalenia wątroby typu B może wynikać z bezpośredniego działania estrogenów poprzez ich receptory. Wykazano bowiem, że polimorfizm ERα prowadzi do nieprawidłowej odpowiedzi komórek odpornościowych na estrogen w ostrej niewydolności wątroby związanej z HBV. Przeciwwirusowa modulacja odpowiedzi immunologicznej przez hormony płciowe może również pomóc w wyjaśnieniu częstszego występowania raka wątrobowokomórkowego u mężczyzn. Wykazano bowiem, że HCC indukowany dietylonitrozoaminą (DEN, ang. *dimethylnitrosamine,* chemiczny karcynogen) miał cięższy przebieg u samców niż u samic myszy, ze względu na zwiększoną produkcję IL-6 przez komórki Kupffera w wątrobie samców. Badania te sugerują, że estrogeny hamują IL-6 poprzez zmniejszenie aktywacji jądrowego czynnika transkrypcyjnego NF kappa B (NF-κB, ang. *nuclear factor-kappa B*) poprzez Myd 88 - adaptor dla receptorów toll podobnych. Zatem, fizjologiczne dawki egzogennych estrogenów mogą hamować przerzuty HCC nie tylko poprzez zmniejszenie wydzielania IL-6, ale także poprzez obniżenie poziomu czynnika wzrostu hepatocytów.

Związek między androgenami/AR a ostrą niewydolnością wątroby, taką jak HCC indukowany HBV: Mechanizmy związane z receptorem androgenowym, które mogą pośredniczyć w ekspansji HCC, obejmują modulację odporności wrodzonej. Wykazano, że AR może tłumić ekspresję IL-12A na poziomie transkrypcji, poprzez bezpośrednie wiązanie się z regionem promotora genu kodującego tę interleukinę. Powoduje to obniżenie cytotoksyczności komórek NK (ang. *natural killer*) wobec komórek raka wątroby. Z drugiej strony, istnieją dowody na to, że aktywowany AR może hamować przerzuty HCC, indukując apoptozę transformowanych nowotworowo hepatocytów poprzez modulację fosforylacji kinazy p38, która, jak wykazano, jest zależna od mitogenów i odgrywa znaczącą rolę w patogenezie raka wątrobowokomórkowego.

Współzależność niekodujących mRNA w powstawaniu i progresji HCC: Progresja HCC jest związana z kilkoma długimi niekodującymi RNA (lncRNA), które mają klastry miR-374b/421 i miR-545/374a. Biorąc pod uwagę, że gen kodujący błonowy receptor estrogenowy gamma (ESRRG, ang. *estrogen related receptor gamma*) jest

potencjalnym genem docelowym dla miR-545, postawiono hipotezę, że może on być związany
z mniejszą częstością występowania HCC wywoływanego przez HCV u kobiet. Zauważono także kluczową rolę estradiolu w regulacji aktywacji ekspresji p53 i miR-23a, co może być podstawą dla zrozumienia różnic w przebiegu i patogenezie raka wątrobowokomórkowego u kobiet i mężczyzn.

Złośliwość HCC a hormony płciowe: Egzogenny testosteron wpływa na podwyższenie ekspresji regulatorów cyklu komórkowego, takich jak cyklina D1, E czy kinaza CDK2. Jednocześnie obniża on ekspresję białek p53 i p21, i poprawia żywotność hepatocytów. Odwrotny efekt obserwuje się w przypadku estradiolu, który hamuje markery cyklu komórkowego hepatocytów i zwiększa ekspresję p53, osłabiając żywotność hepatocytów i komórek raka wątrobowokomórkowego. Wydaje się również, że stosunek DHT do T jest istotnym wskaźnikiem, ponieważ jego podwyższenie można zaobserwować u pacjentów z HCC w przeciwieństwie
do pacjentów z marskością wątroby oraz u osób zdrowych.

Hipotezy dotyczące roli ERs w HCC: Wykazano, że obniżona ekspresja mRNA dla ERα w wyniku hamowania aktywności kanału jonowego (KCNN4, kanał potasowy aktywowany $Ca2^{+}$; ang. *potassium calcium-activated channel subfamily N member 4*) poprzez TRAM-34 (potencjalny immunosupresant) prowadzi do zmniejszenia aktywacji NF-kappaB, czynnika, zaangażowanego w rozwój HCC. Inne badania dowodzą również, że niskie dawki endogennego estradiolu wzmacniają nowotwór stymulują jego progresję, podczas gdy wysokie dawki egzogennego estradiolu hamują jego powstawanie.

Odpowiedź immunologiczna w niewydolności wątroby a hormony płciowe: Zaobserwowano korelację pomiędzy ERβ, a inflamosomen NLRP3 (ang. *nucleotide-binding oligomerization domain*). Ekspresja ERβ była znacząco obniżona w tkance HCC w porównaniu z prawidłową tkanką wątroby; ponadto ekspresja ERβ wykazywała istotną ujemną korelację z postępem choroby i dodatnią korelację z poziomem ekspresji składników inflamasomu NLRP3. Należy zaznaczyć, iż utrata inflamasomu NLRP3 w chorobowo zmienionej tkance HCC przyczynia się do progresji nowotworu.

Aktywność aromatazy/estrogenów/ERs (i ich wariantów) w HCC: W komórkach raka wątroby wykazano znaczne podwyższenie aktywności aromatazy, co prowadzi do wzrostu lokalnej konwersji estrogenów z androgenów. Natomiast w próbkach ludzkiej wątroby z marskością poziom aromatazy jest umiarkowany i niemal niewykrywalny
w nienowotworowej tkance wątroby ludzkiej. Być może zatem, poziom miejscowej aromatyzacji androgenów jest skorelowany z transformacją nowotworową wątroby.

- ***Inne stany patologiczne i HCC***

Marskość wątroby spowodowana zwłóknieniem wątroby w przewlekłej chorobie wątroby (CLD, ang. *chronic liver disease*) może prowadzić do rozwoju HCC. Dodatkowo zaobserwowano, że zwłóknienie wątroby będące konsekwencją CLD występuje częściej u mężczyzn i to już w młodym wieku. Natomiast u pacjentów z pierwotnym zapaleniem dróg żółciowych (PBC, ang. *primary biliary cirrhosis*) progresję tej choroby w kierunku HCC obserwuje się rzadko, jednak częściej ona występuje u mężczyzn i zależna jest od czasu rozpoznania oraz stopnia zaawansowania PBC.

- ***Stany patologiczne związane z drożnością dróg żółciowych***

Istnieją sugestie, że hormony, zwłaszcza płciowe, odgrywają kluczową rolę w modulowaniu wzrostu cholangiocytów w warunkach uszkodzenia wątroby. Badania kliniczne wykazały, że pacjenci z późnym stadium PBC mieli znacznie obniżoną ekspresję ER w cholangiocytach. Modulatory ER poprawiają parametry cholestazy w surowicy tych pacjentów. Niewiele jednak wiadomo na temat wpływu androgenów na nabłonek dróg żółciowych. Ostatnie badania wskazują na ekspresję AR w cholangiocytach oraz stymulujący efekt testosteronu na wzrost wydzielania żółci podczas cholestazy.

6.2. The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, InR, and AR Expression in the Liver

Wstęp. Wątroba jest jednym z narządów odpowiedzialnych za metabolizm glukozy. Wchłanianie i uwalnianie glukozy zależne jest od aktualnych potrzeb organizmu, i w hepatocytach odbywa się głównie poprzez GLUT2. Wątroba jest heterogenna pod względem zawartości glikogenu, zależnie od strefy zrazika klasycznego, bowiem komórki różnią się między sobą natlenieniem i aktywnością metaboliczną. Dodatkowo akumulacja glikogenu w wątrobie może być zależna od diety, wieku, i innych czynników np. hormonozależnych. Przykładowo, niski poziom T prowadzi do zmniejszenia tolerancji glukozy i insulinooporności, szczególnie u starszych mężczyzn. Niedobór testosteronu wiąże się również z upośledzoną glikemią na czczo. Brak T u wykastrowanych szczurów powoduje objawy podobne do T2D lub MetS takie jak: hiperglikemia w wyniku zahamowania sekrecji insuliny, zwiększony wychwyt glukozy, nadekspresja GLUT2 i podwyższona aktywność fosforylazy glikogenu w wątrobie. Natomiast wysoki poziom T u ludzi wiąże się z niskim ryzykiem wystąpienia cukrzycy u mężczyzn, ale wysokim u kobiet. Wykazano, że u samców szczurów Zucker z genetyczną otyłością (kastrowanych i niekastrowanych) podawanie finasterydu (inhibitor 5α-reduktazy) powoduje hiperinsulinemię, a mężczyźni z łysieniem androgenozależnym także stanowią grupę ryzyka rozwoju nietolerancji glukozy lub T2D. Zatem zaburzona homeostaza androgenowa u samców szczurów pokolenia potomnego, którym ojcom podawano finasteryd mogła prowadzić do zmian metabolizmu hepatocytów, ich wrażliwości na insulinę czy androgeny.

Cel. Celem badania było sprawdzenie czy podawanie finasterydu miało międzypokoleniowy wpływ na: (i) gromadzenie glikogenu w wątrobie skorelowane z poziomem mRNA dla GLUT2, (ii) związek między poziomami T i DHT w surowicy, a poziomem mRNA dla GLUT2, InR i AR w hepatocytach, (iii) poziom glukozy w surowicy w zależności od wieku zwierząt oraz poziomu transkryptu GLUT2 w hepatocytach potomstwa szczurów płci męskiej (F1:Control vs F1:Fin).

Metody. Badania przeprowadzono na szczurach szczepu Wistar zgodnie z polskim prawem i za zgodą Lokalnej Komisji Etycznej ds. Doświadczeń na Zwierzętach w Szczecinie (Uchwała nr 23/2010z dnia 21 lipca 2010 r.). Pokolenie rodzicielskie F0 (F0:Control, n=5; F0:Fin, n=5) dało męskie pokolenie F1 (F1:Control,

n=5; F1:Fin, n=5). Szczury ojcowskie w grupie F0:Fin otrzymywały finasteryd (Proscar, MSD, Cramlington, UK) w dziennych dawkach 5 mg/kg/mc., celem wywołania deficytu DHT. Przedmiotem badań były wątroby od 7-, 14-, 21-, 28- i 90-dniowych samców (F1:Fin) urodzonych z samic, krytych przez samce grupy F0:Fin. Natomiast męskie pokolenie potomne (F1:Control) od nietraktowanych finasterydem samców (F0:Control) stanowiło grupę kontrolną. Preparaty histologiczne wątrób wybarwiono metodą PAS (zasadą Schiff'a) w celu wizualizacji glikogenu oraz przeprowadzono reakcję IHC w celu wykrycia immunoekspresji GLUT2, InR i AR. Preparaty wybarwione metodą PAS oraz metodą IHC dla wykazania immunoekspresji GLUT2 w hepatocytach poddano ilościowej, komputerowej analizie obrazu. Homogenaty wątrób wykorzystano do analizy qRT-PCR celem oceny poziomów transkryptów GLUT2, InR, AR. Za pomocą współczynnika korelacji Spearman'a, (i) zawartość glikogenu w hepatocytach skorelowano z immunoekspresją GLUT2, (ii) poziomy T i DHT w surowicy skorelowano z poziomami transkryptów GLUT2, InR i AR, (iii) a stężenie glukozy w surowicy skorelowano z wiekiem zwierząt oraz z poziomem mRNA dla GLUT2.

Wyniki. W każdej grupie wiekowej szczurów F1:Fin, w hepatocytach zaobserwowano podwyższoną akumulację glikogenu w porównaniu do F1:Control, (i) ale nie korelowała ona ze zmianami immunoekspresji GLUT2 w hepatocytach. Dodatkowo u zwierząt F1:Fin, poziomy transkryptów dla GLUT2, InR i AR oraz ich immunoekspresja były istotnie statystycznie obniżone w porównaniu z grupą kontrolną. W grupie szczurów F1:Control współczynnik korelacji Spearman'a nie wykazał statystycznie istotnej korelacji między (ii) stężeniem T oraz DHT w surowicy a poziomami transkryptów GLUT2, InR i AR. W grupie F1:Fin statystycznie istotną, silnie, ujemną korelację wykazano tylko pomiędzy transkryptem AR a stężeniem obydwu androgenów we krwi. Samce szczurów z grupy F1:Fin charakteryzowały się podwyższonym poziomem glukozy w surowicy w porównaniu z F1:Control. Jednakże współczynnik korelacji Spearman'a w obu grupach zwierząt nie wykazał żadnej statystycznie istotnej korelacji (iii) między stężeniem glukozy w surowicy a wiekiem oraz poziomem mRNA dla GLUT2. Ponadto, u dojrzałych płciowo (90PND) szczurów F1:Fin obserwowano

stłuszczenie wątroby (w załączniku – Appendix A – na prośbę Recenzentów omówiono dodatkowo wykonane analizy dotyczące metabolizmu lipidów w wątrobie).

Wnioski. Reasumując, szczury grupy F1:Fin charakteryzują się wyższą akumulacją glikogenu wątrobowego w wyniku hiperglikemii, obniżoną ekspresją (na poziomie mRNA i białka) GLUT2, InR oraz AR w hepatocytach oraz stłuszczeniem wątroby. Można zatem wnioskować, że finasteryd ma działanie międzypokoleniowe i prawdopodobnie epigenetyczne skutki uboczne, które mogą prowadzić do niektórych zespołów metabolicznych, takich jak hiperglikemia, insulinooporność, cukrzyca typu 2 lub stłuszczenie wątroby u mężczyzn. Ponadto, finasteryd powinien stać się przedmiotem badań w stosunkowo nowej dziedzinie farmakologii farmakoepigenomice.

7. PUBLIKACJE STANOWIĄCE ROZPRAWĘ DOKTORSKĄ

7.1. Sex Hormone-Dependent Physiology and Diseases of Liver

International Journal of
Environmental Research and Public Health

MDPI

Review

Sex Hormone-Dependent Physiology and Diseases of Liver

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Received: 3 March 2020; Accepted: 7 April 2020; Published: 11 April 2020

Abstract: Sexual dimorphism is associated not only with somatic and behavioral differences between men and women, but also with physiological differences reflected in organ metabolism. Genes regulated by sex hormones differ in expression in various tissues, which is especially important in the case of liver metabolism, with the liver being a target organ for sex hormones as its cells express estrogen receptors (ERs: ERα, also known as ESR1 or NR3A; ERβ; GPER (G protein-coupled ER, also known as GPR 30)) and the androgen receptor (AR) in both men and women. Differences in sex hormone levels and sex hormone-specific gene expression are mentioned as some of the main variations in causes of the incidence of hepatic diseases; for example, hepatocellular carcinoma (HCC) is more common in men, while women have an increased risk of autoimmune liver disease and show more acute liver failure symptoms in alcoholic liver disease. In non-alcoholic fatty liver disease (NAFLD), the distinction is less pronounced, but increased incidences are suggested among men and postmenopausal women, probably due to an increased tendency towards visceral fat accumulation.

Keywords: gender-dependent liver failure; hepatic glucose metabolism; insulin resistance; type 2 diabetes; metabolic syndrome; hepatic lipid metabolism; non-alcoholic fatty liver disease; cirrhosis; hepatocellular carcinoma; transgenic animal models; clinical cases

1. Sex Hormone-Dependent Glucose Metabolism in a Healthy Liver, in Insulin Resistance (IR) and in Diabetes (T2D)

1.1. Cellular Transporters Involved in Glucose Transport: Their Expression in IR, T2D and MetS (metabolic syndrome)

The liver is one of the organs responsible for glucose metabolism due to its production of glucose (glucogenesis), which is stored as glucogen (glycogenogenesis) (Figure 1), and is degraded as needed via the glycolytic pathway (glycogenolysis) or converted to fatty acids by the lipogenic pathway (lipogenesis). Glucose transport is directed by the Na^+-coupled glucose transporters (SGLT) or glucose transporters (GLUT). Among the former, SGLT1 and SGLT2 serve as transporters, and SGLT3 is a glucose sensor. In humans, the GLUT family includes 14 isoforms which have diverse affinities and different expression profiles, thus enabling tissue adaptation of glucose uptake via *GLUT* gene expression [1]. Glucose absorption and release depends on the current needs of the body, and take place mainly through the activation of GLUT2, one of GLUT's isoforms. Bi-directional transport via GLUT2 is responsible for the glucose balance in the cell, and GLUT2 up-regulation plays a more important role in the export of glucose than in its import to the liver [1]. Nevertheless, to ensure proper expression of glucose-dependent genes in the liver, it is necessary to maintain a proper balance between intracellular and extracellular GLUT2-dependent glucose concentrations [2]. In the liver, the

GLUT2 level is increased by glucose, insulin and fatty acid synthase (FASN) stimulation, and studies on GLUT2 knockout mice confirm that glucose uptake by hepatocytes is a major source of glucose for lipogenesis [2].

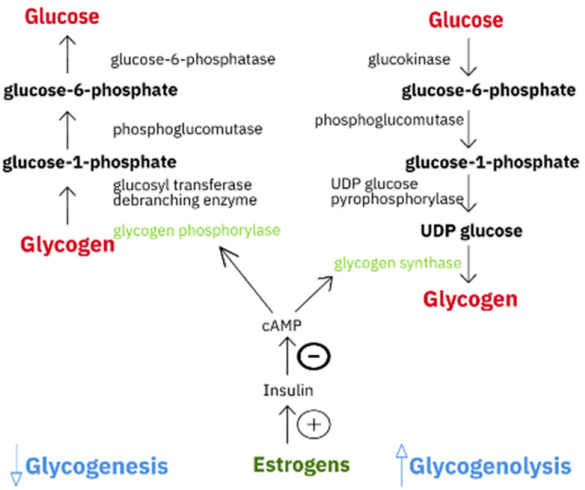

Figure 1. Graphical comparison of glycogenesis and glycogenolysis pathways.

Epidemiological studies show sex differences in type 2 diabetes (T2D) and indicate a higher prevalence in men than in women [3]. Men are also more likely to suffer from obesity and effects of sedentary lifestyle than women, probably due to differences in insulin sensitivity and regional fat storage [4], which may be due to disrupted sex hormone homeostasis. In a study on castrated rats, an increased level of glucose in blood and a higher level of GLUT2 mRNA and protein expression was found as a result of endogenous androgens deficiency [5]. Supplementation with testosterone (T) or testosterone with estradiol (E2) normalized the level of GLUT2 mRNA and protein expression in the liver of the rats, whereas supplementation with E2 alone had no effect [5]. In vitro data indicated that the addition of testosterone and 17β estradiol to the medium of non-malignant Chang liver cells significantly increased the insulin receptor mRNA expression and glucose oxidation and that these processes were not the effect of insulin action. So, compared to the individual effects of T and E2, their combination significantly increased the glucose oxidation which is similar to the effect of insulin [6]. In the aforementioned study by Muthusamy [5], testosterone deficient rats were characterised by increased hepatic glucose synthesis (hyperglycaemia) and symptoms similar to T2D or metabolic syndrome (MetS) [5]. Because the normal testosterone level improved the levels of GLUT2 mRNA and protein expression, it can be supposed that T directly influences the *GLUT2* gene transcription and translation [5]. Another report indicated that exogenous T encouraged synthesis of glycogen in both castrated and non-castrated rats [7]. At the same time, in humans, a high level of testosterone was related to a low risk of diabetes in men and a high risk in women [7]. An excess of androgens in women with polycystic ovary syndrome (PCOS) disrupts hepatic glucose metabolism as a result of a reduced glucose concentration in blood due to insulin action and glycogen synthesis; furthermore, PCOS predisposes women to insulin resistance (IR) [8,9]. However, estrogens have been shown to have little effect on GLUT2 and the insulin receptor in the livers of male rats, although this caused an increase in the insulin receptor levels in the human liver cell line (HepG2) [10] and non-malignant Chang liver cells [6]. Furthermore, testosterone supplementation resulted in the non-malignant Chang liver cells up-regulating the mRNA level for the insulin receptor and increasing insulin sensitivity [6].

1.2. Relationship between Androgens/Androgen Receptor/5α-Reductase and Hepatic Glucose Homeostasis

A lack of an androgen receptor (AR) in males promotes IR which could promote T2D development. For example, an in vivo study performed on AR knockout ($AR^{-/y}$) male mice showed a gradual decrease in sensitivity to insulin and attenuated glucose tolerance which increased with age. Aging $AR^{-/y}$ mice also exhibited accelerated hyperinsulinemia [4]. Another experiment on sex-dependent insulin resistance was carried out on hepatic AR knockout mice, where overweight male H-$AR^{-/y}$, but not female H-$AR^{-/-}$ mice, fed a high-fat diet (HFD) were characterized by reduced sensitivity to insulin as a result of increased expression of protein-tyrosine phosphatase 1B (PTP1B, negative regulator of the insulin signaling pathway). So, the hepatic androgen receptor, as a positive factor, could also play an important role in avoiding IR development [11]. The authors of the publication suggest that "strategies aimed at increasing AR activity specifically in the liver through tissue-selective AR modulators could therefore improve both hepatic insulin and leptin sensitivity and improve both lipid and glucose homeostasis". In another study 5α-reductase-knockout (5αR1$^{-/-}$, but not 5αR2$^{-/-}$) mice with an ALIOS diet (American lifestyle-induced obesity syndrome) had decreased hepatic mRNA expression of genes involved in insulin signaling [12]. However, overweight male Zucker rats (castrated and non-castrated) showed hyperinsulinemia induced by finasteride (which is an inhibitor of 5α-R2 and not 5α-R1) [13]. Male 5αR1-knockout mice on HFD showed a higher average weight gain and hyperinsulinemia then wild type (WT) animals [13]. This suggests that lack of activity of 5α-reductase, the enzyme that converts T to DHT, induces IR. Interestingly, in another study [14], expression of the ERα transcript in the liver was decreased by DHT treatment of orchidectomized (ORX) male mice, although there was no significant impact on ERβ or AR transcripts.

1.3. Relationship between Estrogens/Estrogen Receptors/Aromatase and Hepatic Glucose Homeostasis

The relationship between estrogen concentration and metabolic homeostasis has also been found in a study carried out on aromatase-deficient patients and aromatase-knockout animals (ArKO mice); these patients, such as a male patient with inactive ERα [15], displayed diminished glucose metabolism, insulin resistance and hyperinsulinemia [16]. Exogenous estrogen supplementation of the male patient with attenuated ERα did not recover homeostasis of glucose and did not restore insulin to normal level, whereas the aromatase-deficient patients revised their metabolic anomalies [15]. Estrogen supplementation increases synthesis and release of insulin [17,18], and may also change liver GLUT expression [1]. Therefore, these sex hormones are important in hepatic insulin clearance. Postmenopausal women, after an oral hormone replacement therapy (HRT) at low doses, did not show any changes to IR but exhibited a slight increase in hepatic insulin clearance [19]. Estrogens also reduce gluconeogenesis and increase liver glycogen synthesis and storage, and lower blood glucose levels [20]. As well as this, observations on ovariectomized (OVX) rodents support the view that estrogens decrease glucose levels [21,22]. This correlates with increased glucagon signalling due to an increased expression of the glucagon receptor (GLR), which stimulates glucose production by activating gluconeogenic enzymes in OVX rats [23]. As a glucose imbalance after an ovariectomy is not reversed by exogenous E2 [23], it can be assumed that this disturbed homeostasis was due to a lack of progesterone. While the classic, nuclear progesterone receptor (PR) has not been detected in the liver [24], progesterone—in addition to binding to membrane PR [25]—can also bind to the nuclear AR [26] and thus induce metabolic effects in human hepatocytes, similar to hepatic glycogenolysis and gluconeogenesis regulated by the epinephrine/β2-adrenergic receptor pathway as a result of estrogen action [27].

T2D and MetS development are known to be related to the polymorphism in the gene encoding ERα; for example, a study on men with identified ERα-deficiency showed an imbalance in glucose metabolism [28]. ERα knockout (ERαKO) mice also display attenuated glucose tolerance with IR in their livers, while ERβ knockout (ERβKO) mice have normal glucose tolerance, which suggests that ERα and not ERβ plays an important role in regulating glucose homeostasis in the liver [20,29]. The normalization of glucose homeostasis, insulin concentration and the reverse obesity in mice

with ERα deficiency and removed ovaries, suggests that ERβ may be a base of diabetogenic and adipogenic phenotype. In contrast, ERβKO mice had better insulin sensitivity and glucose tolerance without increased body fat storage. That is why ERα is indicated as an important factor in metabolic regulation [29]. Similarly, a declining glucose tolerance was also observed in GPER1 knockout mice (G protein-coupled estrogen receptor 1), although GLUT2 expression and glucokinase activity were not altered [30]. The mouse GLUT2 promoter includes both a PPAR-γ (peroxisome proliferator activator receptor-gamma) response element as well as a steroid hormone response element [31,32], and that is why ERs with PPAR-γ could together regulate the gene encoded glucose transporter type [33].

Ovariectomies of female Holstman rats did not affect liver glycogen levels or phosphoenolpyruvate carboxykinase (PEPCK, the main enzyme in the gluconeogenesis pathway) gene expression, but after E2 replacement the expression was altered (glycogen–increased, PEPCK–decreased) [34]. However, according to another study [35], following the ovariectomy of obese female rats the expression of lipogenic (sterol-regulatory element-binding protein 1c, SREBP-1c; FASN) and adipogenic (PPAR-γ) genes in the liver increased significantly, and administration of exogenous E2 or an ERα agonist (16a-LE2) reduced hepatic expression of SREBP-1c, FASN and PPAR-γ, while an ERβ agonist (8β-VE2) comparably increased PPARγ expression to the same level of mRNA as in non-treated ovariectomized animals [35]. Both agonist of ERs not only decreased lipogenesis but also lowered triglyceride (TG) accumulation in the liver. Therefore, the systemic insulin sensitivity was improved by the activation of ERα and also ERβ, most likely as a result of the anabolic activity of ERβ [35]. (Summary of the above data in Table 1.)

Table 1. Summary data on impact of the hormone imbalance, disturbance of the sex hormone receptors and the enzyme activity/expression on the hepatic metabolism of carbohydrate in relation to the gender as a cause of many physiological dysfunctions, syndromes or diseases.

Hormone Imbalance or Receptor/Enzyme Dysfunction	Results	References
Disturbed carbohydrate metabolism in male		
↓ Testosterone	Hyperglycemia, T2D, MetS	Muthusamy et al. [5]
AR (lack)	IR, T2D	Lin et al. [4]
AR knockout	↓ Glucose metabolism, IR, hyperinsulinemia	Lin et al. [4]
AR knockout + HFD	↓ Sensitivity to insulin	Lin et al. [11]
5α-red1 knockout + ALIOS	Hyperinsulinemia	Dowman et al. [12]
5α-red1 knockout + HFD	Hyperinsulinemia	Livingstone et al. [13]
↓ ERα	↓ Glucose metabolism, IR, hyperinsulinemia, T2D, MetS	Zirilli et al. [16], Yamada et al. [28]
ERαKO	↓ Glucose tolerance, hepatic IR	Bryzgalova et al. [20], Nilsson et al. [29]
↓ Testosterone	Hyperglycemia, T2D, MetS	Muthusamy et al. [5]
Disturbed carbohydrate metabolism in female		
↓ Testosterone	↑ Glucose	Kelly et al. [7]
↓ Estrogens	↑ Diabetes	Saengsirisuwan et al. [21], Feigh et al. [22]

ALIOS—American lifestyle-induced obesity syndrome, AR—androgen receptor; ERα—estrogen receptor alpha, ERαKO—ERα knockout, HFD—high-fat diet, IR—insulin resistance, MetS—metabolic syndrome, T2D—type 2 diabetes, 5α-red1—5α reductase type 1.

2. Sex Hormone-Dependent Lipid Metabolism in the Normal Liver, in Nonalcoholic Fatty Liver Disease (NAFLD), Obesity and in Metabolic Syndrome (MetS)

There is an increasing amount of evidence from animal cell culture and clinical studies that testosterone/androgens control the expression of important regulatory proteins involved not only in glycolysis and glycogen synthesis but also in lipid and cholesterol metabolism [7]. For example, dihydrotestosterone treatment of orchidectomized male mice caused obesity, decreased energy utilization and fat oxidation, ane increased HDL-C and TG levels correlating with lowered bile acid synthesis as a result of down-expression of 7α-hydroxylase gene [14]. In addition, in AR knockout male mice ($AR^{-/y}$) the exogenous DHT did not reverse the metabolic anomalies and IR [11].

2.1. Relationship between Estrogens/Estrogen Receptors/Aromatase and Hepatic Lipid Homeostasis

In the case of hepatic lipid metabolism, it should be emphasized that estrogens are also crucial. In both sexes, estrogen signalling via ER is important in the regulation of lipogenesis, as evidenced by experimental animal models and clinical studies. Women with breast cancer were treated with tamoxifen (selective modulator of ER, with anti-estrogenic effect) although hepatic steatosis caused by an impairment of fatty acids (FA) β-oxidation in estrogen deficient livers is a frequent complication associated with this therapy [1]. Genome-wide analysis demonstrates that transcriptional activity of ERα oscillates depending on the phase of the mouse estrous cycle and this oscillation is required for pulsating transcription of FA and cholesterol genes. This ER-dependent physiological programming changes during gestation and after termination of fallopian tube as a result of progressing age or surgically induced menopause, signifying that ER signalling is crucial for appropriate liver physiology in relation to the energetic necessities of reproductive age. Therefore, any changes in the amplitude and frequency of the cycle are related with the accumulation of fat in the liver [36]. Accordingly, the oscillation of ER expression has great importance for limiting fat deposition in the livers of women of reproductive age, and appropriate HRT in post-menopausal women or after surgical menopause, and has an important role for hepatic metabolism [36]. This protective function of estrogens is mainly attributed to ERα signalling [37] because, as was shown in microarray analysis, male and female ERαKO mice exhibit a fatty liver due to the up-expression of lipogenic genes and down-expression of genes involved in lipid intake [34]. Mice with liver ERα-knockout (LKO) [38] and Gpr30-deficient mice (GPR30; orphan G protein-coupled receptor 30) [39] fed a HFD had increased liver triglycerides and diacylglycerides, and female rather than male mice had significantly lower HDL-C level along with an increase in fat liver accumulation with insulin resistance. Thus, both ERα and GPER (G protein-coupled ER, also known as GPR 30) must be present in the liver cells to maintain lipid homeostasis.

The estrogenic pathway of regulation of the liver function also heavily depends on the activity of aromatase, an enzyme that converts androgens to estrogens. In aromatase knockout (ArKO) male mice, but not in ArKO female mice, the developed liver steatosis could be normalized by the administration of exogenous estrogens [40], and impaired hepatic FA β-oxidation was caused by disturbing the activity of peroxisomal very long-chain acyl-CoA synthetase (VLACS), fatty acyl-CoA oxidase (AOX) and medium-chain acyl-CoA dehydrogenase [41,42]. These impairments were inverted by exogenous E2 [41] or treatment with pitavastatin that is able to re-establish FA β-oxidation via the PPAR-α and abolish hepatic steatosis [42]. As such, estrogen therapy is used to aid recovery from metabolic anomalies in aromatase-deficient patients [15]. In castrated male rats, E2 supplementation also decreased FA production and lipid collection, and prevented nonalcoholic fatty liver disease [43]. Similar to the increase in hepatic triglycerides and diacylglyceride in liver ERα-knockout HFD fed mice, the alteration of insulin-stimulated ACC (acetyl-CoA carboxylase) phosphorylation and DGAT1/2 (diacylglycerol O-acyltransferase 1/2) protein levels were also observed due to decreased insulin sensitivity [38]. Males on a HFD showed that estrogen (via ERα) helps avoid not only hepatic but also whole body IR. Therefore, intensifying hepatic estrogen-ERα pathways could reduce the effect of obesity, diabetes and cardiovascular risk [38]. Another study on a mouse knockout model (ArKO) [44] showed obesity and liver steatosis due to an impaired FA β-oxidation and an increased FASN level in the liver

of both female and male mice. This is in line with the findings of Foryst-Ludwig et al. [45], according to whom ERα mainly mediates beneficial metabolic effects of estrogens such as anti-lipogenesis, improvement of insulin sensitivity and glucose tolerance, and reduction of body fat mass. In contrast, ERβ activation seems to be detrimental for the maintenance of regular glucose and lipid homeostasis. Hypoestrogenemia caused by ovarian senescence significantly increases the risk of steatohepatitis and liver fibrosis severity both in humans with NAFLD and in zebrafish with experimental steatosis [46]. Therefore, estrogen deficiency promotes the development of NASH (nonalcoholic steatohepatitis) and estrogen treatment improved NASH progression in bilateral ovariectomized mice fed a high-fat and high-cholesterol (HFHC) diet [47].

It was also shown that not only classic, nuclear ERα and ERβ act in liver metabolism. Some very interesting results are presented in a study carried out on a "membrane-only ERα" mouse model (MOER, ligand-binding domain of receptor is present within the plasma membrane) injected with propyl-pyrazole-triol or trisphenol (PPT), a selective agonist of ERα [48]. This experiment showed that the expression of many lipid synthesis-related genes was decreased in "MOER" mice but was not suppressed in ERKO mice, indicating that only membrane-localized ERα was necessary for the suppression of these genes (cholesterol, TG and FA content was decreased only in livers from MOER mice exposed to PPT, but not in the livers from the ERKO mice). Therefore, the inhibition of gene expression mediated by membrane-localized ERα caused the aforementioned metabolic phenotype that did not require nuclear ERα. Consequently, the membrane-localized ERα is responsible for protection against hyperlipidemia by reducing the expression of some genes involved in lipid synthesis in the liver [48]. Although ERα in the liver is considered anti-lipogenic, data from literature on the role of ERβ in the liver is not consistent. Mice with a lack of ERβ are heavier but their livers are lighter as a result of reduced hepatic TG storage accompanied by whole body higher insulin sensitivity [49], indicating that ERβ in the liver can perform lipogenic and diabetogenic functions, because—as was documented by Foryst-Ludwig et al. [49]—this receptor deactivates the adipocytic gene expression induced by PPAR-γ and finally leads to a reduction in adipogenesis. This is confirmed by the discoveries of some mutations in the ERβ gene of obese female adolescents or women with bulimia [50,51].

2.2. Correlation of Non-Alcoholic Fatty Liver Disease (NAFLD)/Non-Alcoholic Steatohepatitis (NASH) with Sex Steroids

Non-alcoholic fatty liver disease (NAFLD) includes the entire spectrum of steatohepatitis as a non-alcoholic steatohepatitis (NASH) with or without fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [52–55], related to systemic features [56,57] and excessive mortality from cardiovascular and liver diseases [58–61]. Histologically indistinguishable from alcoholic liver disease, the NAFLD [62] is closely related to insulin resistance [63] and metabolic syndrome [53,54].

There are studies which show that androgens protect against NAFLD [43], because low serum T levels and hepatic steatosis in men are closely related [64]. However, other reports show opposite results, with androgens promoting the development and progression of NAFLD [65,66]. In vitro data similarly suggests that exposure to excessive amounts of androgens (including corticosterone) can lead to lipogenesis [12]. These inconsistencies may result from the use of various animal models, genders, methods of treatment or combinations of various steroid hormone replacements. In addition, it is the T to DHT ratio that is most important for the development and progression of NAFLD rather than the concentrations of T or DHT [67]. In the human liver, both isoforms of 5α-reductase (5αR1, 5αR2) are present, and the level of the first isoform becomes higher with the growing severity of NAFLD symptoms. Mice with 5α-reductase knockout ($5\alpha R1^{-/-}$, $5\alpha R2^{-/-}$) do not convert testosterone into DHT. Implementing an ALIOS diet for these knockout mice induced a development of great hepatic steatosis only in $5\alpha R1^{-/-}$, but not $5\alpha R2^{-/-}$ [12]. This steatosis was driven largely by impaired corticosterone clearance rather than decreased DHT [12]. Similarly, male 5αR1-knockout mice on a HFD diet also demonstrated hepatic steatosis as a result of hepatic reduction in FA β-oxidation and increased TG accumulation [13]. The authors of the mentioned study also observed hepatic steatosis

in obese male Zucker rats, both intact and castrated, after treatment with finasteride (5α-reductase type 2 inhibitor) [13]. The hepatic steatosis was independent of DHT, but changes in 5αR1 activity with non-selective 5α-reductase inhibition in overweight men with prostate disease could indicate the beginning and progression of hepatic metabolic failure [13].

In another study [68], a very low T serum level in feminised (Tfm) male mice on a normal diet showed increased lipid accumulation although this was significantly less than cholesterol-fed Tfm mice. Tfm mice on a normal diet demonstrated increased gene expression of hormone sensitive lipase, stearoyl-CoA desaturase-1 (SCD1) and PPAR-γ, although acetyl-CoA carboxylase alpha (ACACA) and FASN were not altered. Yet testosterone supplementation caused a reduction in the lipid deposition in the liver of Tfm mice compared to placebo-treated Tfm as a result of a decrease in the expression of key regulatory enzymes of fatty acid synthesis [68]. Hepatic AR-knockout (H-AR$^{-/y}$) male (but not female) mice on a HFD diet also developed hepatic steatosis as a result of a rise in SREBP-1c and PPAR-γ [4,11]. Moreover, the insulin resistance of these male mice was associated with a decline of phosphoinositide-3 kinase (PI3K) action, increased phosphoenolpyruvate carboxykinase (PEPCK) expression, and correlated with increased protein-tyrosine phosphatase 1B expression (PTP1B). Loss of AR in aging H-*AR*$^{-/y}$ male mice caused a rise in hepatic TG volume, so that hepatic androgen receptors may be a key for avoiding liver steatosis development. Lin et al. [11] proposed the development of AR agonists to target hepatic AR and thus improved the effectiveness of therapies used in metabolic syndrome in male patients. Male mice with complete (not only hepatic) AR knockout (ARKO) developed increasing triglyceride deposition in liver, obesity, and severe IR [4]. As hepatic AR has a greater effect in men than in women [7,11], Kanaya et al. [69] performed an experiment to try to better understand how elevated androgen levels regulate food intake and obesity in females. Ovariectomized female mice treated with DHT (non-aromatazable androgen) exhibited increased food intake, significantly higher lipids storage in the liver, and other signs of biochemical dysfunction (increased fasting glucose, impaired glucose tolerance, resistance to leptin) [69].

The aforementioned reports indicate that androgens have a major influence on lipid metabolism in female livers. There are also many indications that hyperandrogenic women with PCOS may indirectly increase the risk of NAFLD by obesity, IR, and directly by the hepatotoxic effect (significantly increased level of alanine aminotransferase (ALT)) [70]. Compared to premenopausal women, men and postmenopausal women have higher LDL-C and lower HDL-C concentration in blood, so estrogens could play an important role in decreased hepatic fats storage [71]. This indicates that menopause is related to increased body weight and higher risk of metabolic diseases. In an OVX mice model of menopause [72], increased adiposity was prevented by estrogen replacement. In that study, treatment with E2 was associated with general reduction of adipose tissue mass (because of down-regulation of lipogenic genes under the control of SREBP-1c). In the liver, endogenous E2, similar to the adipose tissue, caused a decrease in the expression of lipogenic genes. It was shown by D'Eon et al. [72], that in the liver, estradiol participated in free fatty acids dividing during oxidation and prevented TG storage by up-regulation of *PPAR-δ* and by direct initiation of AMP-activated protein kinase (AMPK). Accordingly, genomic and non-genomic actions of E2 promote leanness in OVX mice independently of reduced energy intake [72].

There is ample evidence from screening studies that the prevalence of NAFLD is higher in males compared to females, regardless of age [73–78]. In a study examining the incidence of NAFLD in women, 7.5% of those going through menopause and 6.1% of postmenopausal women were found to have NAFLD, in comparison to 3.5% of premenopausal women [76]. The increased risk of NAFLD did not correlate with hormone replacement therapy. The incidence of NAFLD in women rose with age, but did not change with age in men [76]. Thus, this indirectly indicates that endogenous (contrary to exogenous) estrogens could play a protective function against the advancement of NAFLD in women. On the other hand, there is data indicating that hormone-replacement therapy may lessen the risk of diabetes, but its mechanisms are unclear [79]. In contrast, an Italian multicentre study on almost 5500 healthy hysterectomised women who received tamoxifen or placebo for five years showed that the

medicament increased the risk of NAFLD/NASH development only in overweight and obese women with features of MetS [80]. A study on women with T2D documented that low doses of hormone replacement therapy significantly reduced liver enzymes: AST, ALT, GGT (γ-glutamyltransferase), and ALP (alkaline phosphatase) in serum, potentially due to a reduced level of hepatic fat accumulation [81]. Authors of this publication indicate that the explanation for the HRT improvement of liver physiology could help in the search of the effective treatment of non-alcoholic fatty liver disease among women. (Summary of the above data in Table 2.)

Table 2. Summary data on impact of the hormone imbalance, disturbance of the sex hormone receptors and the enzyme activity/expression on the hepatic metabolism of lipids in relation to the gender as a cause of many physiological dysfunction, syndromes and diseases.

Hormone Imbalance or Receptor/Enzyme Dysfunction	Results	References
disturbed lipids metabolism in male		
↓ Testosterone	↑ Hepatic steatosis ↓ Hepatic steatosis	Vőlzke et al. [64] Jones et al. [65] Schwingel et al. [66]
5α-red1 knockout + ALIOS	Hepatic steatosis	Dowman et al. [12]
5α-red1 knockout + HFD	↑ TG, hepatic steatosis	Livingstone et al. [13]
Hepatic AR- knockout + HFD	Hepatic steatosis, IR	Lin et al. [4], Lin et al. [11]
↓ AR	↑ TG in liver, hepatic steatosis	Lin et al. [4]
ARKO	↑ TG in liver, obesity, IR	Lin et al. [4]
ArKO	Liver steatosis, obesity	Hewitt et al. [40], Fisher et al. [44]
↓ Aromatase	Metabolic anomalies	Maffei et al. [15]
↓ ERα + HFD	↑ TG, ↑ diacylglyceride, IR	Zhu et al. [38]
ERαKO	Fatty liver	Bryzgalova et al. [20]
LKO + ↓ Gpr30 + HFD	↑ TG, ↑ diacylglyceride	Zhu et al. [38], Meoli et al. [39]
disturbed lipids metabolism in female		
↓ Estrogen	↑ LDL-C, ↓ HDL-C, hepatic steatosis	Trapani et al. [71], Chen et al. [1]
↓ Estrogen + HFD/HFHC	NASH	Kamada et al. [47]
LKO + ↓ Gpr30 + HFD	↑ TG, ↑ diacylglyceride, ↓ HDL-C, ↑ fat liver accumulation, IR	Zhu et al. [38], Meoli et al. [39]
ERαKO	Fatty liver	Bryzgalova et al. [34]
ArKO	Liver steatosis, obesity	Fisher et al. [44]
Hyperandrogenism + PCOS	Obesity, IR, NAFLD	Bohdanowicz-Pawlak et al. [70]

ALIOS—American lifestyle-induced obesity syndrome, AR—androgen receptor, ARKO—AR knockout, ArKO—aromatase-knockout, ERα—estrogen receptor alpha, ERαKO—ERα knockout, Gpr30—orphan G protein-coupled receptor 30, HDL-C—high-density lipoprotein HFD—high-fat diet, HFHC—high-fat an high-cholesterol diet, IR—insulin resistance, LDL-C—low-density lipoprotein, LKO—liver ERα knockout, MetS—metabolic syndrome, NAFLD—non-alcoholic fatty liver disease, PCOS—polycystic ovary syndrome, T2D—type 2 diabetes, TG—triglycerides, 5α-red1—5α reductase type 1.

3. HBV/HCV and Hepatocellular Carcinoma (HCC, Hepatoma)

Hepatitis B (HBV) and hepatitis C (HCV) are two hepatotropic viruses belonging to the family of Hepadnaviridae and Flaviviridae (respectively), differing in genome, life cycle and molecular

prediction. HBV is a DNA virus that has an ability to integrate into the DNA of the host cell. In contrast, HCV is an RNA virus that replicates in cytoplasmic membranous host cell networks. The innate and adaptive immune responses are the main mechanism involved in determining persistent hepatitis infection, and the innate immune response is the first line of defence against viral infections [82].

The HBV contagion and subsequent consequences of infection are different in males and females [83–89]. The effects of sex differences, especially sex hormones, on the innate immune response to HBV are largely unknown, which is at least partly due to the lack of appropriate research models. Slightly more is known about gender differences in the adaptive immune response to HBV infection. For instance, after a prophylactic vaccination against HBV, women have a higher titre of anti-HBV antibodies than men [90]. Hepatocellular carcinoma (HCC) development, pathogenesis and disease progression-induced hepatitis B infection show gender-related differences [91]. HBV-related HCC occurs more often in men than in women [92]. Rates of liver cancer in men are typically 2 to 4 [93] or even 3 to 5 [94] times higher than in women. Gender-related variation in liver cancers is common in mammals, from rodents to humans, and was firstly described in mice in the late 1930s, with female mice being resistant to liver cancer [95]. The remarkable gender disparity suggests an important role of sex hormones in HCC pathogenesis [96]. It is probable that the specific immune response of the host is reflected in HBV replication and viral protein levels. Likewise, in a study conducted on HBV infected mice, males had up-expressed DNA and protein of HBV in comparison to females. The reduced functionality (not the number) of $CD8^+$ T lymphocytes was accompanied by increased numbers of regulatory T cells (T reg) in males which may explain why, among male HBV human patients, there are more infections and more failed cases of immunotherapy than in women [91].

3.1. Relationship between Estrogens/ERs and HBV-Related Acute Liver Failure Such as HCC

The sexual dimorphism of hepatitis B virus-related liver diseases may be related to estrogen and its receptors. One possible explanation is that the ERα polymorphism leads to a defective immune cell response to estrogen in HBV-related acute liver failure [97]. Antiviral modulation of immune responses by sex hormones can also help to explain the prevalence of HCC in men, as in the case of chemically induced HCC by diethylnitrosamine (DEN, a chemical carcinogen), which is more severe in males than in female mice, due to an increased production of IL-6 by Kupffer cells (in a manner dependent on the Toll-like receptor adaptor protein MyD88) in the male liver [98]. Interleukin 6 (IL-6) is a multifunctional cytokine that is largely responsible for the hepatic response to systemic infection or inflammation, often referred to as an 'acute phase response' [99]. Naugler et al. [98] demonstrated that estrogens inhibited IL-6 by reducing the activation by Myd 88-induced NF-κB. Physiological doses of estrogens can suppress metastasis of HCC not only by decreasing IL-6 expression but also by decreasing hepatocyte growth factor levels [100]. The hepatocarcinogenic effect of IL-6 in hepatocytes can be stopped by inhibiting transcription factor STAT3 and reducing the activity of mitogen-activated protein kinase JNK (c-Jun N-terminal kinases) [98]. The protection against the development of liver cancer in carcinogen-treated mice also depends on ERα-mediated estrogen signaling of forkhead box protein A (Foxa) factors such as Foxa1 and Foxa2 [101] pioneer transcription factors in the liver, crucial for steroid hormone signalling (estrogens and androgens) as essential controllers of variations of liver cancer in terms of gender [95]. The integrative genomic analysis showed that the risk of HCC in women might be associated with the SERPINA6-rs1998056 regulated by FOXA/ERα [102].

3.2. Relationship between Androgens/AR and HBV-Related Acute Liver Failure Such as HCC

Mechanisms through AR that can mediate the expansion of HCC also include the modulation of innate immunity. Shi et al. [103]. showed that AR could suppress IL-12A expression at the transcriptional level via direct binding to the IL-12A promoter region which results in repressing the efficacy of natural killer (NK; related innate immune surveillance) cell cytotoxicity against liver cancer cells. On the other hand, there is also evidence that activated AR can inhibit HCC metastasis

by inducing cellular apoptosis by modulation of p38 kinase phosphorylation [104], shown to be mitogenic-dependent and playing a significant role in HCC [105–107].

In addition to affecting the immune response, sex hormones can also directly affect the activity of the virus. In general, the HBV surface antigen (HBsAg) circulates at a higher level in the serum of male mice than in females [108], and its level decreases after castration, thus indicating that the expression of the viral antigen and viral replication are regulated by androgens [109]. The HBV genome integrated into the host cell DNA contains two androgen respond elements (ARE) in the enhancer region I. When the AR-androgen complex is internalized to hepatocytes, it binds to both the nuclear and viral ARE sequences, thereby activating the transcription of the HBV genome and the production of HBV X (HBx) protein [110]. This protein, in turn, facilitates dimerization of AR and enhances transcriptional activity of AR by activating Src kinase, thus creating a feedback loop that can promote hepatocarcinogenesis [92]. The AR further acts in conjunction with other molecules, such as cell cycle-related kinases (CCRKs), which in turn activate oncogenic β-catenin in hepatocytes. This mechanism indicates that androgens/AR signalling may promote the development of HBV-related hepatocellular carcinoma and explains the higher incidences of HCC as well as higher HBV titres in male serum than female [111]. Conversely, estrogen signalling probably inhibits hepatocarcinogenesis and protects against HBV-related HCC progression. The molecular mechanism of estrogen is mediated by the binding to the nuclear ERα which inhibits the enhancer I of HBV and transcription of integrated viral genomes [92,111].

3.3. Complicity of Noncoding mRNAs in the Onset and Progression of HCC

Progression of HCC is also related with several long noncoding RNAs such as lncRNAs, which have miR-374b/421 and miR-545/374a clusters. Considering that the estrogen-related receptor gamma (ESRRG) is a potential target gene of miR-545, it has been hypothesized that this mechanism may be associated with a lower incidence of HVC-induced HCC in women. As the miR-545 and miR-374a were up-expressed in male *versus* female HCC individuals in a study by Zhao et al. [112], the authors of the study concluded that the up-expressed miR-545/374a cluster could be related to a low chance of recovery, and suggested the employment of miR-545/374a levels in sera for HCC diagnostics. The role of E2 in regulating the activation of p53 and miR-23a expression could be crucial to understanding the sex differences observed in HCC [113]. In miRNA PCR array, Huang et al. [113] found more than a two-fold alteration in apoptotic miRNAs (25 was upregulated and 10 was downregulated) in E2-treated cells. Among these miRNAs, expression of miR-23a was related to p53 functional status in the male-derived liver cell-lines. Moreover, miR-23a expression correlated negatively with the expression of target gene X-linked inhibitor of apoptosis protein (XIAP), but positively with the caspase-3/7 activity. So, a decrease of XIAP may contribute to caspase-3 activity and cell apoptosis. The authors of the study emphasize the huge potential of miRNAs as biomarkers and therapeutic agents thanks to their ability to control gene expression. In research in which lentivirus-mediated ERα small interfering RNA (siRNA) was transfected into HCC cells (Hep3B), the downregulation of ERα expression caused the inhibition of cell proliferation, reduced cell invasion, slowed-down cell population at S phase, and increased the rate of apoptosis [114]. According to these authors, ERα may play a very important role in carcinogenesis of HCC and its knockdown may offer a new potential gene therapy approach for human liver cancer in the future. In addition, it has been proved that the promotor of pri-miR-216a has an androgen-responsive element [115]. The up-expression of miR-216a was mainly noticed in male patients, as a result of transcriptional activation of pri-miR-216a by the androgen signaling further reinforced by X protein (HBV protein) [115].

3.4. HCC Malignancy and Sex Hormones

Generally, a correlation between the axis of androgen/androgen receptor and HCC incidence have been confirmed, but the mechanism is still largely unknown. For example, it is proposed that androgen/AR complex after binding to promoter of Nanog (pluripotency factor) can promote HCC

stemness. It is worth emphasizing that, in HCC tissues, AR expression was abnormally high and showed a correlation with Nanog expression [116]. Another study revealed a "vicious circle" of androgenic signaling. This signaling increases the expression of CCRK (cycle-related kinase, a direct AR transcriptional target), which results in the activation of the Wnt/β-catenin/TCF (T cell factor) pathway that finally leads to up-expression of AR in HCC cells [117]. CCRK was overexpressed in approximately 70% of HCCs and was significantly correlated with tumor staging. Thus, the interaction of AR/CCRK stimulates cell cycle progression and induces tumor formation (promotion of hepatocarcinogenesis) [117]. It was noted that the expression of matrix metalloproteinase 9 (MMP9), an important marker of migration, adhesion, invasion and metastasis of liver cancer [118], was higher in HCC tumors in mice lacking specific AR in the liver (L-AR$^{-/y}$) compared to WT-animals. It was also found that AR suppresses cell migration via suppression of nuclear factor kappa B (NF-κB)-MMP9 pathway [104]. In their next paper, the authors showed that AR affects cell adhesion and cellular mobility through the AR-β1-integrin-PI3K/AKT signaling pathway in HCC [119]. The L-AR$^{-/y}$ mice with carcinogen-induced HCC developed more undifferentiated and larger size tumors at the metastatic stage and died earlier with increased lung metastasis [104]. These results indicated that hepatic AR may play dual opposite roles, to promote HCC initiation but suppress HCC metastasis.

DEN-injected female mice exhibited scarcer dysplastic foci and less acute early stage of HCC than males, with more differentiated tumors and fewer metastases [120]. Castration of these mice down-regulated cyclin E kinase and amplified hepatocyte apoptosis, and estradiol/progesterone enhanced those effects. In control female mice, cyclin E kinase activity was lower than in males, and testosterone administration of ovariectomized mice increased cyclin E and its kinase activity and accelerated hepatocarcinogenesis. Moreover, exogenous testosterone not only up-expressed cell cycle regulators (cyclin D1 and E, CDK2) but also down-expressed p53 and p21, which improved hepatocyte viability. Conversely, E2 inhibited hepatocyte cell cycle markers, increased p53 and reduced hepatocyte and HCC viability. This study showed that both sex hormones determined the male predominance to hepatocarcinogenesis: castration of male mice delayed the onset of HCC [120]. Moreover, the DHT to T ratio is also an essential indicator, because it is elevated in patients with HCC in contrast to patients with cirrhosis or healthy individuals [121]. Furthermore, the size and cell division activity of HCC significantly declines after blood DHT levels drop [122]. Therefore, in terms of tumorigenesis, DHT (a more active T metabolite and AR ligand) cannot be omitted. According to Yu et al. DHT reinforces hepatocellular carcinoma cell division depending on AR activation [123], and the decline in HCC malignancy after AR antagonism treatment is linked with a decrease in blood DHT [124]. This data confirms the observations of Dowman et al. [12], where more than half of the mice after one year of the ALIOS diet revealed hepatocellular lesions similar to those observed in HCC, compared to one-fifth of 5αR2$^{-/-}$ and zero of 5αR1$^{-/-}$ (isoform 5α-reductase knockout) mice. Because of this, it has been proposed that the 5αR1 deletion could have protective function against the NAFLD-associated HCC expansion, and this enzyme isoform may become a therapeutic target [12].

3.5. Hypothesis about the Role of ERs in HCC

Although hepatocellular carcinoma is known to be accompanied by decreased expression of ERs, their role in HCC is not fully understood [125]. There are some studies on the effects of estrogen/ERs signaling on various tumor suppressors, but their results are inconclusive. The development and invasion/progression of HCC and other cancers are associated with metastasis-associated protein 1 (MTA1) gene expression [126–128]. Additionally, the results of research carried out by Deng et al. [129] show that ERα up-regulation inhibits the division and spread of HCC. On the other hand, the MTA1 overexpression lowers ERα-controlled inhibition of HCC cells' division and metastasis. These results indicate a co-regulation of ERα and MTA1 in the response to HCC, providing a basis for understanding the gender-related difference in HCC progression. Overexpression of ERα has also been shown to mediate apoptosis in ERα-negative Hep3B cells via the binding of ERα to specificity protein 1 (SP1). Then this complex (ERα-SP1) binds to the TNFα gene promoter, inducing the expression of

active caspase 3 in a ligand-dependent manner [125]. It was also shown that decreased expression of ERα mRNA due to inhibition of ion channel (KCNN4; Ca^{2+}-activated K^+ channel) by TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-pyrazole) led to a decrease in activation of NF-kappaB, the factor known to be involved in the development of HCC [130]. Therefore, TRAM-34 is proposed as a new therapeutic target for the treatment of HCC.

In addition, E2 may also inhibit the progression of HCC, since E2-suppressed cell cycle markers, increased p53-regulated p21, Bcl-X_L and Bax expression, consequently reducing the viability of HCC cells [120]. Interestingly, estradiol was shown to have a dual effect: in hepatocytes, increasing estradiol concentrations promoted cell survival, while the opposite effect was observed in HCC cells. A primary culture of hepatocytes and HCC cells clearly responded differently to estradiol stimulation with respect to cell death [120]. These dual effects of estradiol have been described before: low doses of endogenous estradiol are tumor-enhancing, while high doses of exogenously delivered estradiol inhibit tumor formation [131,132]. This is probably why some of the results of research on the effects of estrogen on HCC are contradictory, in addition to the existence of various estrogen receptor variants [133]. One of ER's alpha receptors is vER (variant estrogen receptor) which does not have the hormone-binding domain but has a normal DNA-binding domain, responsible for the transcription of estrogen-dependent genes [134]. In chronic hepatitis, vER transcripts, in contrast to wtER (wild-type ER), are present more frequently in men and in HBsAg-positive subjects than in individuals with antibodies to HCV. In HCC male patients the vER transcript is overexpressed or is the only one expressed form [133]. The much more frequent presence of vER in men, mainly those with HBsAg, both in the early stages of the disease and chronic hepatitis, indicates that this variant of estrogen receptor promotes the uncontrolled proliferation and development of hyperplasia, and may be a mechanism of neoplastic alteration in men [133]. The hepatocellular carcinoma cells that express vER are highly malignant [134,135], because of the raised proliferation rate and because they are insensitive to tamoxifen (antiestrogen). Fortunately, megestrol (a drug that blocks wtER and vER) does have some influence on the success of therapy in HCC with the expression of vER [134].

3.6. Immune Response in Liver Failure and Sex Hormones

Yet another role of estrogen receptors in HCC progression was shown by Wei et al. [136] who presented a novel link between estrogen receptor β and the NLRP3 inflammasome (an intracellular multiprotein complex involved in the innate immune response to pathogens) in hepatocarcinogenesis. They demonstrated that expression of ERβ was significantly downregulated in HCC tissue compared with normal liver tissue; moreover, ERβ expression had a significant negative correlation with disease progression and a positive correlation with the expression level of NLRP3 inflammasome components. It is known that loss of NLRP3 inflammasome in HCC tissue contributes to tumor progression. Treatment with 17β estradiol significantly inhibited the malignant behavior of HCC cells through E2/ERβ/MAPK pathway-mediated upregulation of the NLRP3 inflammasome [136]. E2 could achieve the same effect (suppression of tumor growth) via regulating the polarization (producing distinct functional phenotypes as a reaction to specific microenvironmental stimuli/signals) of macrophages [137]. During this process, 17β-estradiol suppressed macrophage activation and HCC development alternatively by inhibiting the interaction between ERβ and ATPase coupling factor 6 (ATP5J, an ATPase component), and then blocking the JAK1-STAT6 signaling pathway [137]. These results could contribute to the implementation of a new HCC therapeutic strategy based on the discovered aforementioned mechanism.

3.7. Activity of Aromatase/Estrogens/ERs (and Variants) in HCC

A study by Carruba et al. [138,139] carried out on nontumoral, cirrhotic, and malignant human liver tissue samples (in vivo) and in HepG2, HuH7, and HA22T cells (in vitro) revealed for the first time that the level of the aromatase enzyme is significantly increased in liver cancer cells (malignant human liver tissue and HepG2 hepatoma cells), which leads to an increase in the local conversion of

estrogens from androgens. Aromatase expression is moderate (or intermediate) in cirrhotic human liver samples (or HuH7 cells) and undetectable (or very low) in nontumoral human liver tissue (or HA22T cells) [138–140]. The level of local androgen aromatization is correlated with the degree of malignancy of the liver tissue/cell line. Therefore, locally elevated estrogen formation affects the development and progression of cancer tissues and cells (HCC, HepG2) by activating the rapid signalling pathway mediated via amphiregulin (AREG; a member of the EGF family), a ligand of EGF-R (epidermal growth factor receptor) [138,140]. Moreover, elevated expression of AREG corresponds with ubiquitous expression of hERalpha46 (human variant of ERα) [138,139] and occasional expression of the hERβ2/Cx (human variant of ERβ) [139]. Either none or a low expression of wild-type ERα and ERβ is observed in liver cancer cells and malignant tissues, and the pattern of wtERα is inversely related to aromatase expression [140]. Therefore, the elevated estrogen production induced to a higher aromatase activity could induce liver tumor cell growth through a variant ERα-mediated mechanism. Furthermore, the modification in activity of aromatase-estrogen-amphiregulin-EGF-R axis in issue injury or inflammation could result in growth of tumours such as liver, breast or prostate and progress of chronic diseases such as diabetes, obesity, Alzheimer's and heart disease [140]. Other studies also confirm that the change in ERα status (from wild type ERα66 to the ERα36 splice variant, but not to the ERα46 splice variant) influences HCC development [141]. Probably, due to the existence of numerous ER splicing variants with diverse action, many HCC patients have not responded properly to anti-estrogen treatment. This was possibly caused by hERα66 which inhibits the activation of hERβ in an estrogen-dependent and independent manner [142].

Both prognostic factors and survival rate after therapeutic HCC resection differed between sexes, with female patients having a better overall survival rate than male patients (women had a less invasive tumor phenotype), but this survival benefit was only observed in cases of tumor-node-metastasis stage I diseases compared with males at the same stage; although female patients had a greater prevalence of increased serum alpha-fetoprotein (AFP), AFP and tumor number had prognostic significance only for males; vascular invasion and serum g-glutamyl transpeptidase (GGT) levels were independent risk factors for early recurrence in female patients, whereas AFP and GGT level were independent risk factors for late recurrence [143]. These authors suggest that because estrogens may have a protective effect against early-, but not late-stage, HCC, more aggressive treatment should be attempted for female patients with recurrent HCC [143]. The effective treatments for hepatocellular carcinoma are hepatectomy and liver transplantation, although the risk of recurrence is still high, particularly in patients with a large pool of circulating cancer cells (CTCs) positive for cancer stem cell/progenitor cell mercers. In this area, the results of a study performed on two AR knockout mouse models with spontaneous HCC, which showed a negative relation between HCC recurrence/progression after hepatectomy expression of AR in CTCs, are very interesting and promising. AR-regulated suppression of HCC is a solid sign that this receptor could act as a gatekeeper of HCC recrudescence after surgery [144].

4. Other Pathological Conditions and HCC

It was also shown that cirrhosis, as a result of liver fibrosis in chronic liver disease (CLD), could lead to neoplasia in hepatocellular carcinoma [145]. A cohort study of over 12,000 patients showed that males with CLD were younger (52.9 vs. 58.7 yrs.) and additionally more frequently suffered from alcoholic liver disease (11.4% vs. 6.9%) than women with CLD [146]. Researchers of this analysis have highlighted significant gender differences in terms of the etiologic factors and the onset of chronic liver disease. On this basis, it can be concluded that fibrosis as a consequence of CLD may also be gender-dependent. According to Saginelli et al. [146] factors such as NF-κB, STAT3 and JNK could be linkers with the onset of HCC in patients with cirrhosis. In an inflammatory mouse model ($mdr2^{-/-}$ mice with cholangitis, chronic liver inflammation and finally HCC), the TNF-NF-κB axis had a pro-carcinogenic effect on the liver. It was demonstrated that inhibition of NF-κB by doses of anti-TNF-α stopped HCC progression [147]. In lymphotoxin (LT) transgenic mice models, the

overexpression of LT is related with chronic inflammation and infiltration into the liver by T, B and dendritic cells, with cytokine (IL-1β, IFN-γ, IL-6) over production reaching the highest concentration in HCC. These mice also had elevated production of chemokines (CXCL1, CCL7, CXCL10) as a result of NF-κB activation [148]. Activation of NF-κB is a frequent and early event of human HCC [149,150]. As it had been described earlier [98,100], by reducing Myd 88-induced NF-κB or STAT3/JNK kinase-pathway, estrogens may inhibit IL-6 or hepatocyte growth factor, and then this activity can suppress the progression of liver fibrosis and chronic liver disease. It has also been noted that there are noncirrhotic patients with HCC that have a better overall survival and disease-free survival than cirrhotic patients with HCC [145].

A not often noted and not well known progression is the development of hepatocellular carcinoma in patients with primary biliary cirrhosis (PBC). An early study carried out on almost 400 patients with PBC has shown that only 14 patients developed HCC; and the appearance rate was higher in patients with advanced-stage PBC, with age at the time of diagnosis and male gender more associated with the development of HCC [151]. In the decade following, there was the point of view that the disease overwhelmingly affected females. In epidemiological studies, only 7–11% of documented PBC patients were males, but with a higher risk of life-threatening complications such as gastrointestinal bleeding and hepatoma [152]. In PBC, females demonstrated enhanced antibody production and cell-mediated responses, in addition to an increased CD4 T cell number, probably because, normally T decreases IgG and IgM production by plasmocytes in healthy males and females, or because of co-expression of ER and AR on B cells, whereas CD8 T cells, monocytes, neutrophils and NK cells express only ER [152].

Obesity related to leptin secretion is also a significant predictor of HCC in humans. In the context of sex steroid dimorphism, it is not known whether estrogens antagonize the action of leptin in women. HCC line HepG2 cells cultured with leptin and E2, PPT, DPN (bis-hydroxy-phenyl-propionitrile, a ERβ selective agonist) or G-1 (GPER selective agonist) were studied. The results of the experiment showed that E2/ERs upset the oncogenic function of leptin in the HepG2 cells via preventing their division and promoting their death; and these events were linked with regression of changes in SOCS3/STAT3 induced by leptin, up-regulation of p38/MAPK as a result of ERβ action, and up-regulation of ERK due to the action of ERα and GPER. Additionally, it was shown that agonists of ERα, ERβ and GPER induced cell apoptosis in the HepG2 line [153]. This further data demonstrates the protective role of estrogens in the expansion of HCC, and that estrogen receptors could be a target in the prevention/treatment of leptin-induced HCC.

5. Pathological Condition Associated with the Biliary Tree

The biliary tree (network of intra- and extra-hepatic bile ducts) is lined with a specific type of epithelial cell known as cholangiocyte. They are a heterogeneous (biochemically and functionally) highly dynamic population of cells that modify (via transcytotic transport of various ions like Cl^-, HCO_3^-, Ca^{2+}, Na^+, K^+, solutes, water and also glucose) hepatocyte-derived bile under the direction of hormones, cytokines, growth factors and neurotransmitters [154]. Other functions of cholangiocytes are proliferation, injury repair, fibrosis, angiogenesis and regulation of blood flow [155]. It was documented that cholangiocytes can undergo damage or pathological proliferation during chronic cholestatic liver diseases (cholangiopathies), primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), polycystic liver disease (PCLD) and cholangiocarcinoma (CCA) [155]. During biliary fibrosis, proliferating bile duct epithelial cells, along with hepatic stellate (Ito) cells, are the dominant source of connective tissue growth factor (CTGF); additionally, in this pathological condition, the elevated mRNA level of TGF-β1 that is produced not only by Ito cells but also by activated cholangiocytes seemed to be the main source of this profibrogenic factor [156]. Within the hepatic parenchyma are also oval cells (stem cells). These cells are heterogeneous and bipotent in terms of their developmental maturity or their commitment to either the hepatocytic or biliary lineage. Studies in rodents demonstrated that oval cells not only are associated with an increased risk of HCC in chronic liver disease, but also can proliferate and form ductule-like structures during carcinogenesis and biliary

obstruction, and have been also indicated to have the potential involvement of bile epithelium in fibrosis associated with other chronic liver diseases [157].

Suggestions exist that hormones, especially the sex hormones, play a key role in the modulation of cholangiocyte growth in a damaged liver [155]. For example, bile duct ligation (BDL) caused an increase in ER-β expression in cholangiocytes in comparison to control animals [158]. Clinical studies have shown that patients with late-stage PBC had markedly reduced ER expression in cholangiocytes. ER modulators improve the serum parameters of cholestasis in PBC patients [159]. An in vitro study documented that estrogens, by Src-Shc-ERK1/2 signalling mechanisms, modulate cholangiocyte proliferation and secretion [160,161]. This was confirmed in an experiment on rats with bile duct ligation after tamoxifen or ICI 182,780 9 (anti-estrogen) treatment; the BDL rats had significantly lower weight of intrahepatic bile ducts (IBDM) compared to the control as a result of impaired proliferation and increased apoptosis [155]. Another experimental cholestasis study showed that ovariectomised (OVX) female rats after BDL had significantly reduced bile duct mass associated with a decreased expression of ERβ. Exogenous E2 caused a normalization of bile duct mass, ERβ expression and cholangiocyte proliferation in comparison to untreated BDL rats [162]. This is why it is highly likely that estrogens might delay the progress of cholangiopathies into ductopaenia [163].

Generally, there is little data that described the influence of androgen on biliary epithelium. For example, Yang et al. [164] showed the expression of AR in cholangiocytes and that testosterone stimulated biliary growth and secretion during cholestasis. The cAMP level in cholangiocytes from BDL rats was higher than cAMP levels from normal cholangiocytes [165,166]. Castration of the BDL rats inhibited the stimulatory effects of secretin on cAMP levels in cholangiocytes, and bile and bicarbonate secretion in bile fistula rats; and exogenous testosterone restored the functional secretory activity (secretin stimuli bile and bicarbonate secretion) of cholangiocytes in the castrated BDL rats [164]. Reduced serum testosterone levels as a result of castration or anti-testosterone treatment led to a decrease of IBDM in normal and BDL rats in comparison to the non-castrated rats; and then endogenous testosterone partly compensated for the castration-induced loss of IBDM. Moreover, in the bile duct of BDL castrated rats and BDL rats treated by anti-testosterone, there was an increase in apoptosis compared with BDL rats [164].

On the basis of the above mentioned studies, it was proposed that not only estrogens [155] but also testosterone is important in sustaining biliary proliferation and ductal secretory activity in pathological conditions like functional damage of the biliary epithelium [164].

Author Contributions: Conceptualization: A.K.-W., P.K.; data curation: A.K.-W., P.K.; formal analysis: K.M.-H.; investigation: A.K.-W., P.K.; resources: PUM (see funding); writing—original draft: P.K.; writing—review & editing: A.K.-W., B.W. All authors have read and agreed to the published version of the manuscript.

Funding: The publishing and English editing of the publication was financed from funds for research and development (statutory activity of the Pomeranian Medical University, WLA-167-02/S/16/2020).

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

Abbreviations

ACACA	acetyl-CoA carboxylase alpha
ACC	acetyl-CoA carboxylase
AFP	alpha-fetoprotein
ALIOS	American lifestyle-induced obesity syndrome
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMPK	AMP-activated protein kinase
AOX	fatty acyl-CoA oxidase
AR	androgen receptor
$AR^{-/y}$	AR knockout male mice
ARE	androgen response element
AREG	amphiregulin

ARKO	AR konockout
ArKO	aromatase-knockout
AST	aspartate aminotransferase
ATP5J	ATPase coupling factor 6
BDL	bile duct ligation
CCA	cholangiocarcinoma
CCRKs	cell cycle-related kinases
CLD	chronic liver disease
CTCs	circulating tumor cells
CTGF	connective tissue growth factor
DEN	diethylnitrosamine
DGAT1/2	diacylglycerol O-acyltransferase 1/2
DHT	dihydrotestosterone
DPN	bis-hydroxy-phenyl-propionitryle
E2	estradiol
EGF-R	epidermal growth factor receptor
ER	estrogen receptor
ERα/ERβ	estrogen receptor alpha/estrogen receptor beta
ERαKO/ERβKO	ERα knockout/ERβ knockout
ESRRG	estrogen-related receptor gamma
FA	fatty acid
FASN	fatty acid synthase
Foxa	forkhead box protein A
GGT	γ-glutamyltransferase
GLUT	glucose transporter
GLR	glucagon receptor
GPER	G protein-coupled estrogen receptor, known as Gpr30
Gpr30	orphan G protein-coupled receptor 30
H-AR$^{-/y}$	hepatic AR knockout male mice
H-AR$^{-/-}$	hepatic AR knockout female mice
HBsAg	HBV surface antigen
HBV	hepatitis B
HBx	HBV X protein
HCC	hepatocellular carcinoma
HCV	hepatitis C
HDL-C	high-density lipoprotein
HepG2	human liver cell line
HFD	high-fat diet
HFHC	high-fat and high-cholesterol diet
HRT	hormone replacement therapy
IBDM	intrahepatic bile duct mass
IFN-γ	interferon γ
IL-1β	interleukin 1β
IL-6	interleukin 6
IL-12A	interleukin 12A
JNK	c-Jun N-terminal kinase
KCNN4	Ca^{2+}-activated K^+ channel
L-AR$^{-/y}$	liver AR knockout male mice
LDL-C	low-density lipoprotein
LKO	liver ERα-knockout
lncRNA	long non-coding RNAs
LT	lymphotoxin
MetS	metabolic syndrome
miR	microRNA
MMP9	matrix metalloproteinase 9
MOER	ERα at the plasma membrane

MTA1	metastasis-associated protein 1
MyD88	myeloid differentiation primary response 88
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NF-Kb	nuclear factor kappa B
NK	natural killer
ORX	orchidectomized
OVX	ovariectomized
PBC	primary biliary cirrhosis
PCLD	polycystic liver disease
PCOS	polycystic ovary syndrome
PEPCK	phosphoenolpyruvate carboxykinase
PI3K	phosphoinositide 3-kinase
PPAR-α	proliferator-activated receptor-alpha
PPAR-δ	peroxisome proliferation activator receptor-delta
PPAR-γ	peroxisome proliferator activator receptor-gamma
PPT	propyl-pyrazole-triol or trisphenol
PR	progesterone receptor
PSC	primary sclerosing cholangitis
PTP1B	protein-tyrosine phosphatase 1B
SCD1	stearoyl-CoA desaturase-1
SGLT	Na^+-coupled glucose transporters
siRNA	small interfering RNA
SREBP-1c	sterol-regulatory element-binding protein 1c
STAT3	signal transducer and activator of transcription 3
T	testosterone
T2D	type 2 diabetes
TCF	T cell factor
Tfm	testicular feminized male mice
TG	triglycerides
TGF-β1	transforming growth factor beta 1
TNFα	tumor necrosis factor alpha
vER	variant estrogen receptor
VLACS	very long fatty acyl-CoA synthetase
WT	wild type
wtER	wild type estrogen receptor
XIAP	X-linked inhibitor of apoptosis protein
5αR	5α-reductase
5αR1/5αR2	5α-reductase type1/5α-reductase type 2
$5\alpha R1^{-/-}/5\alpha R2^{-/-}$	5α-reductase type1/5α-reductase type 2 knockout

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7.2. The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, InR, and AR Expression in the Liver

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The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, IR, and AR Expression in the Liver

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Abstract: Background: A growing body of data indicates that the physiology of the liver is sex-hormone dependent, with some types of liver failure occurring more frequently in males, and some in females. In males, in physiological conditions, testosterone acts via androgen receptors (AR) to increase insulin receptor (IR) expression and glycogen synthesis, and to decrease glucose uptake controlled by liver-specific glucose transporter 2 (GLUT-2). Our previous study indicated that this mechanism may be impaired by finasteride, a popular drug used in urology and dermatology, inhibiting 5α-reductase 2, which converts testosterone (T) into dihydrotestosterone (DHT). Our research has also shown that the offspring of rats exposed to finasteride have an altered T–DHT ratio and show changes in their testes and epididymides. Therefore, the goal of this study was to assess whether the administration of finasteride had an trans-generational effect on (i) GLUT-2 dependent accumulation of glycogen in the liver, (ii) IR and AR expression in the hepatocytes of male rat offspring, (iii) a relation between serum T and DHT levels and the expression of GLUT2, IR, and AR mRNAs, (iv) a serum glucose level and it correlation with GLUT-2 mRNA. Methods: The study was conducted on the liver (an androgen-dependent organ) from 7, 14, 21, 28, and 90-day old Wistar male rats (F1:Fin) born by females fertilized by finasteride-treated rats. The control group was the offspring (F1:Control) of untreated Wistar parents. In the histological sections of liver the Periodic Acid Schiff (PAS) staining (to visualize glycogen) and IHC (to detect GLUT-2, IR, and AR) were performed. The liver homogenates were used in qRT-PCR to assess GLUT2, IR, and AR mRNA expression. The percentage of PAS-positive glycogen areas were correlated with the immunoexpression of GLUT-2, serum levels of T and DHT were correlated with GLUT-2, IR, and AR transcript levels, and serum glucose concentration was correlated with the age of animals and with the GLUT-2 mRNA by Spearman's rank correlation coefficients. Results: In each age group of F1:Fin rats, the accumulation of glycogen was elevated but did not correlate with changes in GLUT-2 expression. The levels of GLUT-2, IR, and AR transcripts and their immunoreactivity statistically significantly decreased in F1:Fin animals. In F1:Fin rats the serum levels of T and DHT negatively correlated with androgen receptor mRNA. The animals from F1:Fin group have statistically elevated level of glucose. Additionally, in adult F1:Fin rats, steatosis was observed in the liver (see Appendix A). Conclusions: It seems that treating male adult rats with finasteride causes changes in the carbohydrate metabolism in the liver of their offspring. This can lead to improper hepatic energy homeostasis or even hyperglycaemia, insulin resistance, as well as some symptoms of metabolic syndrome and liver steatosis.

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Citation: Kur, P.; Kolasa-Wołosiuk, A.; Grabowska, M.; Kram, A.; Tarnowski, M.; Baranowska-Bosiacka, I.; Rzeszotek, S.; Piasecka, M.; Wiszniewska, B. The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, IR, and AR Expression in the Liver. *Int. J. Mol. Sci.* **2021**, *22*, 1242. https://doi.org/10.3390/ijms22031242

Academic Editor: Małgorzata Kotula-Balak
Received: 7 January 2021
Accepted: 25 January 2021
Published: 27 January 2021

Keywords: glycogen storage; GLUT-2; AR; IR; finasteride; DHT deficiency; serum androgens and glucose concentration; zones of hepatic lobules

1. Introduction

The liver is one of the organs responsible for glucose metabolism. It is involved in the production of glucose and its storage as glycogen—a branched polymer of glucose produced mostly in the liver, in skeletal muscles and, to a smaller degree, in the brain. Glucose can be converted into glycogen and vice versa other via synthesis or degradation involving various steps in the glycogen metabolism pathways [1]. Glucose is transported in various ways including by glucose transporters (GLUT). In humans there are 14 isoforms (GLUT-1–GLUT-14) with diverse binding affinities and different expression profiles, that's why are responsible for differences in glucose uptake by various human tissues [2]. Glucose absorption and release is dependent on the current needs of the body, and in hepatocytes, takes place mainly through the activation of GLUT-2 [3].

The liver is heterogenic in terms of the hepatocyte glycogen content, depending on the location of hepatocytes in the zones of the hepatic lobules. The zones differ in oxygenation, metabolic activity, and the phenotype of cells [4,5]. In zone 1 (also known as the periportal zone), glycogen forms large discrete aggregates, while glycogen in zone 3 (also known as the perivenous zone) is dispersed homogenously within the cytoplasm [6]. Glucose uptake and glycolysis occur intensively in zone 3, and the intensity of these processes decreases towards zone 1. In contrast, glucose delivery and gluconeogenesis (the generation of glucose from certain non-carbohydrate carbon substrates) are most intense in zone 1 and decrease in intensity towards zone 3 [6].

Glycogen accumulation in the liver can be diet-dependent [7–9] and age-dependent [9], e.g., younger mice store slightly more glycogen than older ones [10]. Moreover, an in vitro study showed that the primary culture of hepatocytes derived from old rats (around 24 months) exhibited a higher potential for glucose production when compared with the hepatocytes derived from younger rats (around 4-months old) [11]. Many data link increased hepatic glucose production to type 2 diabetes (T2D) [4].

Carbohydrate metabolism is also affected by levels of sex hormones [12–14]. Low testosterone (T) levels lead to reduced glucose tolerance and insulin activity (insulin resistance), particularly in elderly men [15,16]—effects which are correlated with type 2 diabetes. Testosterone therapy has been shown to significantly improve glucose tolerance and T2D in men [17]. Moreover, independent of obesity and metabolic syndromes (MetS) in men, testosterone deficiency is also associated with impaired fasting glucose and glucose intolerance [18]. Generally, testosterone is considered a key factor in gender related metabolic syndrome [19]. The lack of T in castrated rats causes symptoms similar to T2D or MetS, e.g., increased hepatic glucose synthesis (hyperglycaemia) as a consequence of inhibited insulin secretion, Akt phosphorylation, glucose uptake, glycogen synthase activity, GLUT-2 over-expression and glycogen phosphorylase activity in the liver. Supplementing castrated rats with testosterone or T with estradiol (E2) normalized the level of GLUT-2 mRNA and protein expression in the liver, whereas supplementation with E2 alone had no effect. Since normalization the testosterone level improved GLUT-2 expression, it can be presumed that T directly influences the *GLUT-2* transcription and translation [20]. Kelly et al. [21] also confirmed that exogenous T can stimulate synthesis of glycogen in both castrated and non-castrated rats. In humans, a high level of testosterone is related to a low risk of diabetes in men but a high risk in women [22,23].

The irreversible reduction in T into dihydrotestosterone (DHT) is carried out by 5α-reductase (EC1.3.99.5) [24]. 5α-reductase isozymes 1 and 2 are well studied [25]. It was reported that 5α-reductase-knockout mice (5αR1$^{-/-}$, but not 5αR2$^{-/-}$) on an American lifestyle-induced obesity syndrome (ALIOS) diet had a decreased hepatic mRNA expression in the genes involved with insulin signaling and developed hepatic steatosis [26]. The

imbalance between T and DHT could be also caused by the inhibition of 5α-reductase type 2 by medicaments such as finasteride used by men suffering from benign prostatic hyperplasia (BPH), prostate cancer, and androgenic alopecia (AGA) [27,28]. Men with premature balding represent a risk group for the development of impaired glucose tolerance or T2D [29]. It was also shown that in male Zucker rats with genetic obesity (castrated and non-castrated), the finasteride treatment results in hyperinsulinemia [30]. Male 5αR1-knockout mice on a high-fat diet (HFD) showed a higher average weight gain and hyperinsulinemia comparing to wild animals. This may suggest a lack of activity in 5α-reductase and induces insulin resistance [30].

However, androgen concentration is not the only factor which influences glucose homeostasis. A key role is also played by the androgen receptor (AR)—a target for T, DHT, and other androgens. Many studies indicate that AR deletion contributes to the development of late visceral obesity with leptin resistance, insulin resistance and increased lipogenesis in adipose tissue and the liver [14]. A lack of androgen receptors in males promotes insulin resistance that could promote T2D development. An experiment carried out on hepatic AR-knockout mice fed a HFD showed that male H-AR$^{-/y}$ (not female H-AR$^{-/-}$) were overweight and were characterized by reduced sensitivity to insulin as a result of an increased expression of the protein-tyrosine phosphatase 1B (PTP1B), negative regulator of the insulin signaling pathway. So, the hepatic androgen receptor (as a positive factor), could also play an important role in avoiding insulin resistance development [31].

In our previous study on the same animal model, we showed that the offspring (F1:Fin) of rats exposed to finasteride had altered levels of serum androgens (T, DHT) and adverse changes in the morphology and physiology of testes and epididymides [32,33]. Given this trans-generational effect of finasteride on the male reproductive system and the aforementioned information on the role of androgens and AR in glucose metabolism, the aim of this study was to assess whether the androgen (T, DHT) imbalance in the F1:Fin generation of rats from males receiving finasteride can affect the accumulation of glycogen in the liver (androgen-dependent organ), serum glucose concentration and the hepatic mRNAs and proteins, GLUT2, IR, and AR expression.

2. Results

2.1. Percentage of PAS-Positive Glycogen Area in the Liver

2.1.1. Glycogen Detection in Histological Section of the Liver Stained with PAS

Figure 1 shows PAS-positive granules indicating glycogen accumulation within hepatocytes. In adult rats (90 PND) of the F1:Fin group, we observed steatosis of the liver (Figure 1, insert on J). No signs of steatosis were visible in control rats of the same age (Figure 1, insert on I). Generally steatosis was characterized by fat accumulation, which is most prominent in the centrilobular zone.

2.1.2. Percentage of PAS-Positive Glycogen Area in Whole Hepatic Lobules, without Dividing the Liver Parenchyma into Zones

The percentage of PAS-positive glycogen area in the liver (without dividing the lobules into zones) from F1:Control and F1:Fin groups of rats was differentiated by individual age groups. This value was statistically significantly higher in the F1:Fin rats in all age groups (Figure 2).

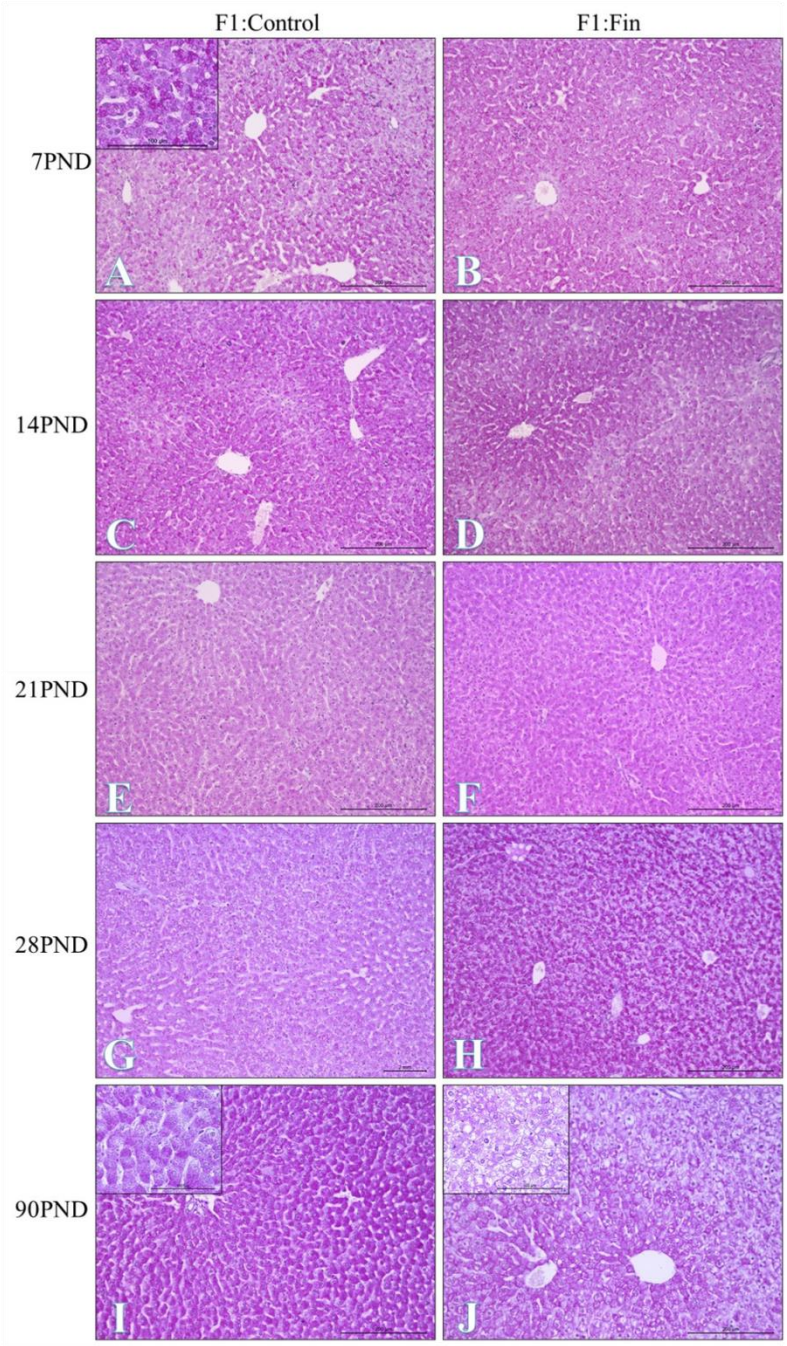

Figure 1. PAS-positive glycogen granules (magenta) within hepatocytes of rats offspring born from females fertilized by control (F0:Control) and finasteride-administrated (F0:Fin) male rats in their postnatal life (F1:Control/F1:Fin: 7 PND, 14 PND, 21 PND, 28 PND, 90 PND). Periodic Acid Schiff staining. Objective magnification: (**A**–**J**) ×20, scale bar 200 µm and insertion on (**A**, **I**, **J**) ×40, scale bar 100 µm.

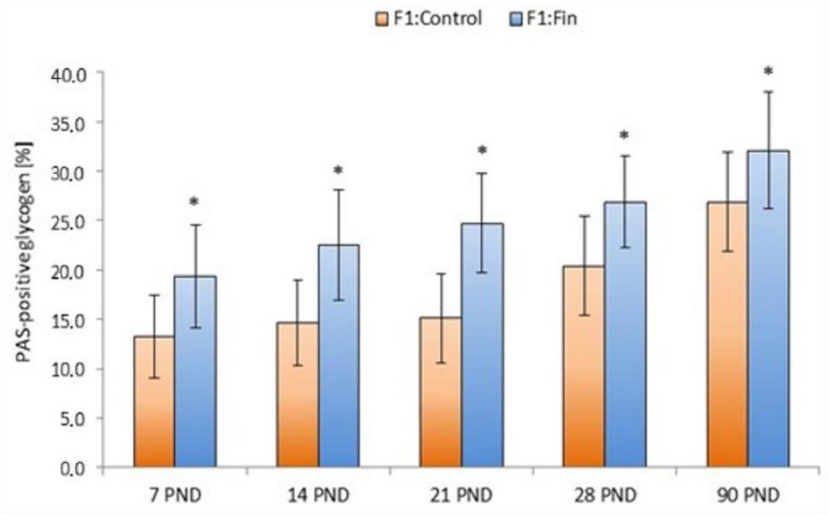

Figure 2. The percentage of PAS-positive glycogen areas in the livers of rats according to the days of development (7 PND, 14 PND, 21 PND, 28 PND, 90 PND) in the control (F1:Control) and in the F1 generation after finasteride administration (F1:Fin), * $p < 0.001$ vs. control in each group.

2.1.3. Percentage of PAS-Positive Glycogen Area in the Zones of the Hepatic Lobules

The same relationship was observed in each of the individual liver zones (zones 1, 2, and 3). The percentage of PAS-positive glycogen area in each zone (periportal, intermediate, perivenous) of hepatic lobules in the livers of F1:Fin rats was statistically significantly higher in comparison to control animals (Figure 3). Moreover in the livers of F1:Fin as well as F1:Control, zone 1 contains less glycogen than zone 2. Zone 3 has the highest content of glycogen.

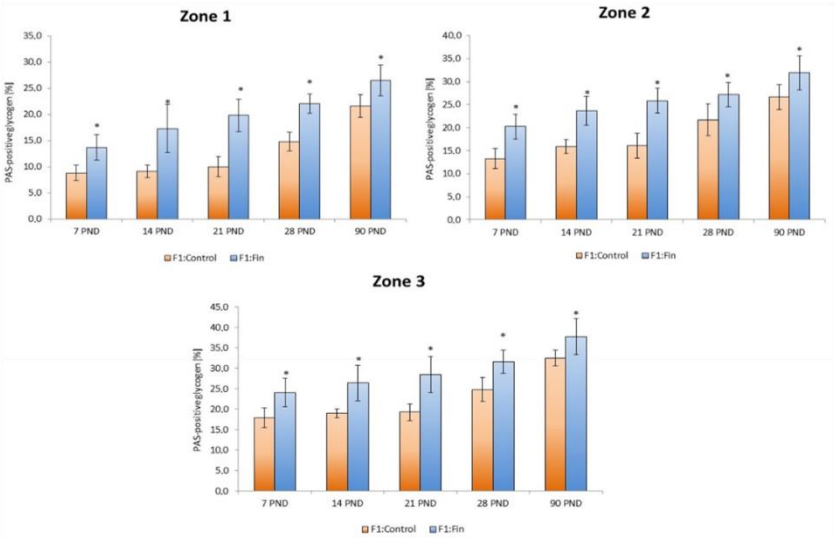

Figure 3. The percentage of PAS-positive glycogen areas in zones 1, 2, and 3 of the hepatic lobules of rats according to the days of development (7 PND, 14 PND, 21 PND, 28 PND, 90 PND) in the control (F1:Control) and in the F1 generation after finasteride administration (F1:Fin), * $p < 0.001$ vs. control in each group.

2.2. Relationship between Immunoexpression of GLUT-2 and Percentage of PAS-Positive Glycogen Area

Figure 4 shows the comparison between F1:Control and F1:Fin (in all age groups) of GLUT-2 immunoexpression according the IHC reaction intensity (weak, moderate, strong) within hepatocytes.

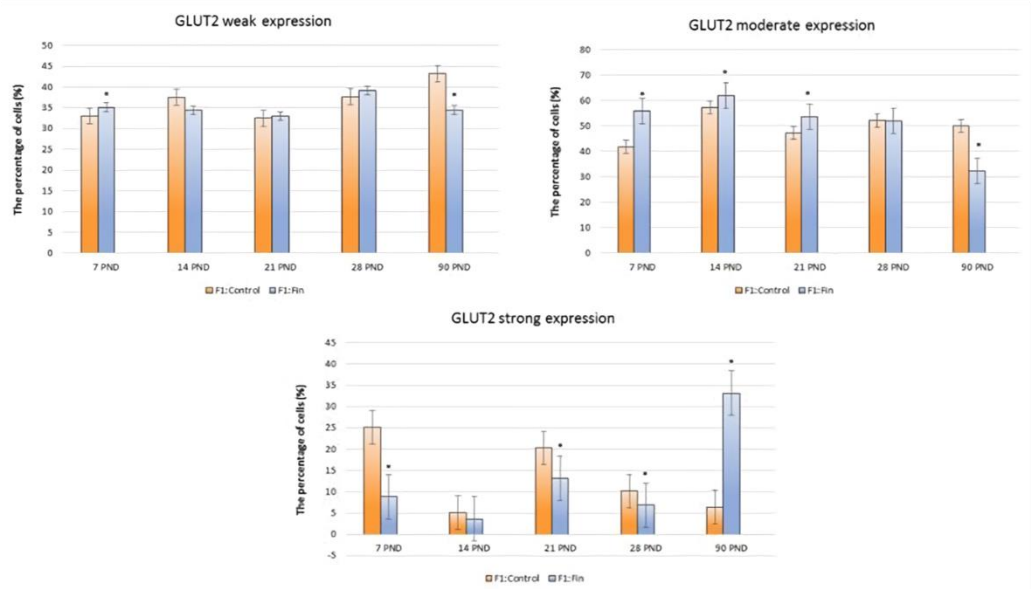

Figure 4. The percentage of GLUT-2-positive cells (divided into weak, moderate, and strong immunoexpression) in the livers of rats according to days of development (7 PND, 14 PND, 21 PND, 28 PND, 90 PND) in the control (F1:Control) and in the F1 generation after finasteride administration (F1:Fin), * $p < 0.001$ vs. control in each group.

Statistically significant changes in the weak GLUT-2 expression was observed in 7 PND and 90 PND (higher and lower in F1:Fin vs. F1:Control, respectively). Taking into account the moderate expression of GLUT-2, the differences between F1:Fin vs. F1:Control rats were noticeable in 7 PND, 14 PND, 21 PND (higher), and 90 PND (lower). In the case of cells with a strong of GLUT-2 expression, the relationship changed. In the groups of 7 PND, 21 PND and 28 PND F1:Fin rats, the percentage of cells with a strong of GLUT-2 expression was significantly decreased vs. F1:Control, as opposed to 90 PND where F1:Fin animals showed a much greater pattern of expression than adult control rats (Figure 4).

Figure 5 shows liver with weak, moderate and strong GLUT-2 immunoexpression.

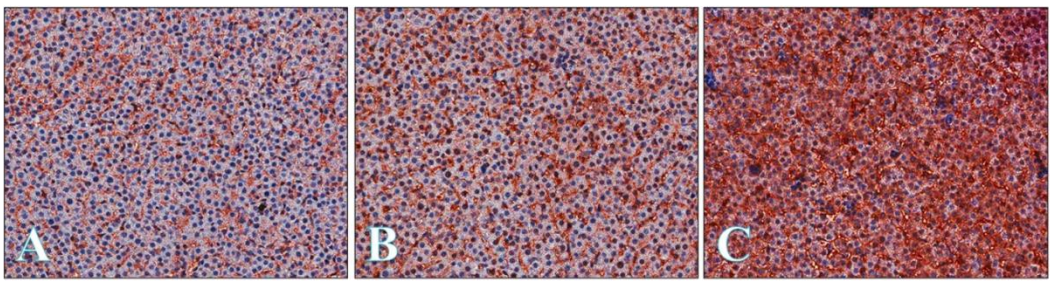

Figure 5. Representative microphotography showing GLUT-2 expression in the livers at the weak (**A**), moderate (**B**), and strong (**C**) level. Objective magnification: ×20.

The Spearman's rank correlation coefficient showed no correlation between percentage of PAS-positive glycogen area and a strong level of GLUT-2 expression.

2.3. qRT-PCR and IHC Analysis of GLUT-2, IR, and AR Expression

2.3.1. GLUT-2, IR, and AR mRNA Expression without Dividing into Age Groups

Regardless of the age of the animals, the transcripts of GLUT-2, IR, and AR levels in homogenates of F1:Fin rats' livers were changed in comparison to F1:Control animals (Figure 6). A noticeably statistically significant decrease in mRNAs concerned GLUT-2 and IR.

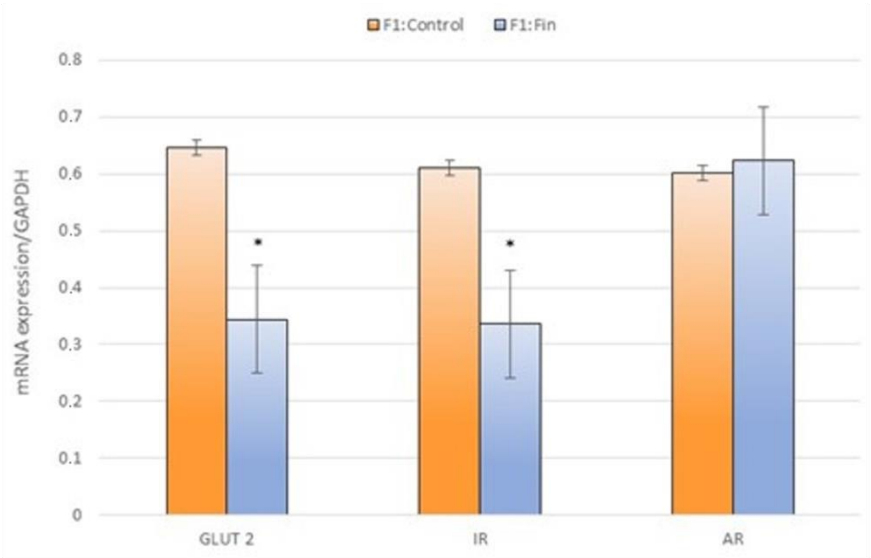

Figure 6. The glucose transporter 2 (GLUT-2), insulin receptor (IR) and androgen receptor (AR) mRNA levels (normalized to GAPDH) in the homogenates of hepatic tissue of the control offspring (F1:Control) and those born from females fertilized by finasteride-treated male rats (F1:Fin) without taking into account postnatal age. Values are expressed as arithmetic means ± SD; differences were evaluated using the Mann–Whitney *U*-test (n = 25 per each F1:control and F1:Fin,, $p \leq 0.05$).

2.3.2. GLUT-2, IR, and AR Expression with Dividing into Age Groups

Taking the age of the animals into account, decreased levels of GLUT-2 transcript were noticed at postnatal day 7 and 28 (Figure 7A). An IHC reaction confirmed this tendency (Figure 7B).

The immature animals of F1:Fin group in 7 PND, 21 PND, and mature in 90 PND have statistically lower levels of IR mRNA (Figure 8A), which was consistent with the immunoexpression of this marker at the protein level (Figure 8B).

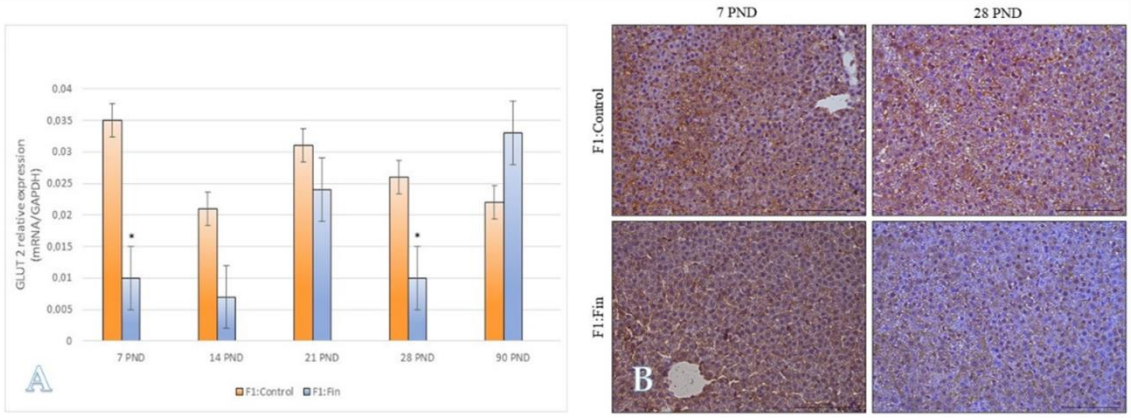

Figure 7. (**A**) The glucose transporter 2 mRNA levels (normalized to GAPDH) in the homogenates of hepatic tissue of control offspring (F1:Control) and those (F1:Fin) born from females fertilized by finasteride-treated male rats in postnatal days 7, 14, 21, 28, and 90. Values are expressed as arithmetic means ± SD; differences were evaluated using the Mann–Whitney *U*-test ($n = 5$ per each age group; $p \leq 0.05$). (**B**) Representative microphotography comparing differences (according the qRT-PCR analysis of 7 PND and 28 PND) in immunoexpression of GLUT-2 within the liver of F1:Control vs. F1:Fin animals. IHC reaction. Objective magnification ×20, scare bar 100 µm.

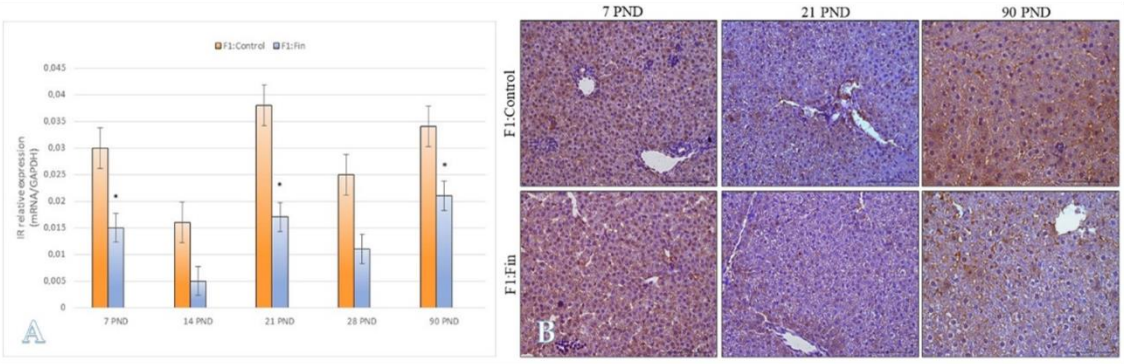

Figure 8. (**A**) The insulin receptor mRNA levels (normalized to GAPDH) in homogenates of hepatic tissue of control offspring (F1:Control) and those born (F1:Fin) from females fertilized by finasteride-treated male rats in postnatal days 7, 14, 21, 28, and 90. Values are expressed as arithmetic means ± SD; differences were evaluated using the Mann–Whitney *U*-test ($n = 5$ per each age group, $p \leq 0.05$). (**B**) Representative microphotography comparing differences (according the qRT-PCR analysis of 7 PND, 21 PND, and 90 PND) in immunoexpression of IR within the liver of F1:Control vs. F1:Fin animals. IHC reaction. Objective magnification ×20, scare bar 100 µm.

The expression of androgen receptors on mRNA level was statistically reduced only in adult F1:Fin rats (Figure 9A), and its decreased intensity in IHC reaction was not so evident (Figure 8B), however some hepatocytes in the livers of experimental rats show cytoplasmic immunoreactivity (brown coloration of hepatocytes, Figure 9B) with only a nuclear localization of the receptor in control animals.

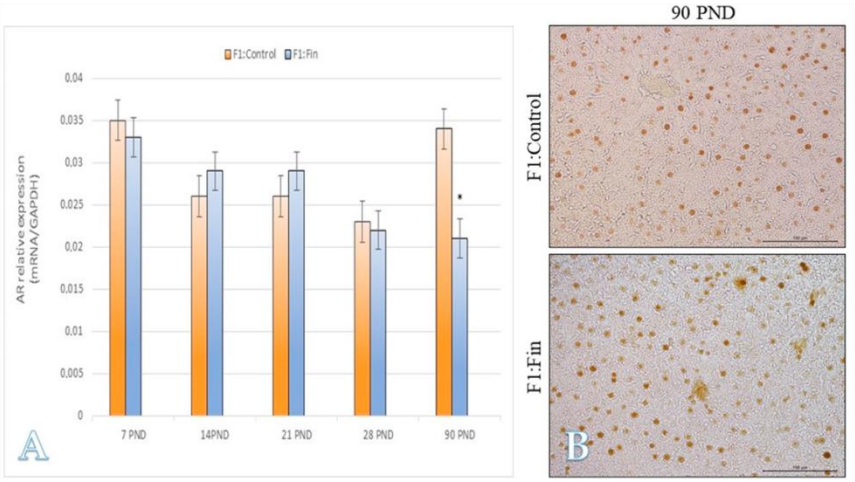

Figure 9. (**A**) The androgen receptor mRNA levels (normalized to GAPDH) in homogenates of hepatic tissue of control offspring (F1:Control) and those (F1:Fin) born from females fertilized by finasteride-treated male rats in postnatal day 7, 14, 21, 28, and 90. Values are expressed as arithmetic means ± SD; differences were evaluated using the Mann–Whitney *U*-test ($n = 5$ per each age group, $p \leq 0.05$). (**B**) Representative microphotography comparing differences (according the qRT-PCR analysis of 7 PND and 28 PND) in immunoexpression of AR within the liver of F1:Control vs. F1:Fin animals. IHC reaction. Objective magnification ×20, scare bar 100 µm.

2.4. Relationship between Serum Androgen Levels and GLUT-2, IR, and AR Transcripts

In our previous reports [32,33] conducting on the same experimental model we showed that, the male rats from F1:Fin group were characterized by a differing serum testosterone and dihydrotestosterone concentration in comparison to the F1:Control.

Within the F1:Control group of rats, the Spearman's rank correlation coefficient did not show any statistically significant correlation between the serum T concentration and the levels of GLUT-2, IR, and AR transcripts. Any correlation with the above mentioned markers was not shown in the serum DHT level either (Table 1).

Table 1. Correlation between serum androgens (T, DHT) and mRNAs for glucose transporter 2, insulin receptor and androgen receptor in the liver homogenates of male control rats' offspring (F1:Control) and finasteride-treated male rats' offspring (F1:Fin).

	F1:Control		**F1:Fin**	
	T	**DHT**	**T**	**DHT**
GLUT2	$r_s = -0{,}000$ $p = 1.000$	$r_s = -0.096$ $p = 0.655$	$r_s = 0.009$ $p = 0.974$	$r_s = -0.088$ $p = 0.689$
IR	$r_s = 0.449$ $p = 0.081$	$r_s = 0.012$ $p = 0.951$	$r_s = -0.353$ $p = 0.235$	$r_s = 0.067$ $p = 0.761$
AR	$r_s = 0.143$ $p = 0.583$	$r_s = 0.342$ $p = 0.102$	$r_s = -0.762$ $p = 0.000375$	$r_s = -0.562$ $p = 0.005253$

Values are express as the Spearman's rank coefficient (r_s) with statistically significant probability $p \leq 0.05$.

In the F1:Fin group of rats, a statistically significant strong negative correlation was only shown between T and AR mRNA, and similarly DHT was also negatively correlated with the AR transcript level (Table 1).

2.5. Serum Glucose Level and It Correlation with GLUT-2 mRNA

The male rats from F1:Fin group were characterized by a differing serum glucose level in comparison to the F1:Control (Table 2). The statistical differences for glucose were on development day 14, 21, and 28.

Table 2. Glucose concentrations in blood serum of rat offspring (F1:Control, F1:Fin, respectively) born from females fertilized by control (F0:Control) or finasteride-treated (F0:Fin) male rats.

Age	Glucose [mg/dL]	
PND	F1:Control	F1:Fin
14	118.0 ± 8.6	162 ± 29.3 ** vs. F1:Control
21	138.2 ± 72.0	177.9 ± 17.3 * vs. F1:Control
28	187.9 ± 19.1	200.6 ± 19.8 * vs. F1:Control
90	189.3 ± 13.3	190.0 ± 27.3

F1:Control, F1:Fin—rat offspring born from females fertilized by the control or finasteride-treated male rats, respectively; PND: postnatal day. Values are expressed as arithmetic means ± SD (n = 5 per age group) evaluated by the Mann–Whitney U-test; * $p \leq 0.05$, ** $p < 0.001$.

The Spearman's rank correlation coefficient did not show any statistically significant correlation between the serum glucose concentration and the age of animals (F1:Control: $r_s = -0.096$, $p = 0.58$; F1:Fin: $r_s = -0.18$, $p = 0.29$). Similarly, there were no correlation between the serum glucose concentration and GLUT-2 mRNA (F1:Control: $r_s = 0.09$, $p = 0.59$; F1:Fin: $r_s = 0.27$, $p = 0.12$).

3. Discussion

The bi-directional passive transport of glucose across plasma membranes [34] via GLUT-2 is responsible for the glucose balance in the cell. GLUT-2 up-regulation plays a more important role in the export of glucose than in its import to the liver [2]. GLUT-2 expression is required for the physiological control of glucose-sensitive genes [34]. To ensure the proper expression of glucose-dependent genes in the liver, it is necessary to maintain a proper balance between intracellular and extracellular GLUT-2-dependent glucose concentrations [35]. Mutations in the GLUT-2 gene not only cause transient neonatal diabetes mellitus (which disappears after approximately 18 months) [36] but also may lead to very rare Fanconi–Bickel syndrome, a condition associated with hepatomegaly (increased liver mass) and glycogen accumulation, growth retardation, and renal Fanconi syndrome [37,38].

In this study, a statistically increased level of glycogen in the liver of F1:Fin rats did not correlate with an increased level of GLUT-2, but in addition to high glycogen accumulation, we also observed steatosis in the livers of the adult (90 PND) offspring from males receiving finasteride. Therefore, it can be hypothesized that, in addition to 'the glycogen storage disease', rats of F1:Fin generation could also suffer from fatty liver disease, as a metabolic syndrome [4]. Moreover, the elevated glycogen accumulation in the liver of F1:Fin rats could be the result of increased glucose concentration in their blood serum. As it is common knowledge, that glycogenesis (the synthesis of glycogen from glucose) is stimulated inter alia by insulin in response to elevated blood glucose levels [3]. However the exact cause of elevated glycogen content in the liver of F1:Fin rats—lack of degradation or increased synthesis—have to be evaluated in the future (see Appendix A Figures A1 and A2).

It is well known that androgen receptors are expressed in the livers of male and female humans and rodents, and its hepatic expression is sex-dependent: AR expression is 20 times higher in the liver of adult male rats than in females [39]. AR expression is noted as age dependent: lower before puberty and higher in postpubescent life [40], which is partially compatible with our results. Lower AR expression were found at 14 PND, 21 PND and 28 PND, than in 90 PND of F1:Control; in F1:Fin rats, the expression of AR gradually decreased during pre-puberty, and the expression of AR transcripts in this group of rats was negatively correlated with circulating T and DHT. Since many liver diseases are

associated with steroid hormones like androgens and their receptors, the male F1:Fin rats may have developed some kind of liver disease.

Androgen-deficient rats, e.g., due to castration, have significantly increased mRNA and protein levels of GLUT-2 in the liver [20]. In our experiment, the F1:Fin rats had an increased level of serum T, decreased serum DHT levels, an elevated expression of GLUT-2, and much higher hepatic glycogen accumulation than the control F1 generation. These findings suggest that DHT, similarly to testosterone in Shen et al. [13], is crucial for glucose homeostasis by regulating hepatic glucose output, and that testosterone deprivation due to castration increases hepatic glucose output, induces hyperglycaemia, and develops symptoms seen in type 2 diabetes and metabolic syndrome. On the other hand, this greater content of glycogen in the livers of F1:Fin rats could have been a result, as was reported previously by Muthusamy et al. [20], of the up-regulation of GLUT-2 which plays a more important role in controlling glucose export out of the liver than into it. Moreover, the direction of glucose transport depends on glucose concentration and is regulated by hormonal factors [3]. Therefore, it is highly likely that the rats from the F1:Fin group in our study could develop a metabolic disease in the future.

Androgens achieve the genomic effect via activation of nuclear receptors, followed by binding to a specific DNA region, known as the androgen response element (ARE) motif, localized in its target gene [41]. Testosterone replacement therapy restores GLUT-2 expression in castrated rat livers suggesting that testosterone may have a direct effect on GLUT-2 transcription and translation [20]. In the promoter region of the GLUT-2 gene, the presence of androgen response elements (ARE) has not been identified yet—this is probably why we did not observe any correlation between androgen concentration and GLUT-2 mRNA expression. However, according to McEwan et al. [42], AR acts as an independent ligand-activated transcription factor or it may bind to some other coactivators [43,44] to increase GLUT-2 expression. Androgens could also achieve a biological effect via a receptor associated with the plasma membrane of the cell, a mechanism that has not been thoroughly researched [13]. Although in our study F1:Fin rats in each age group had increased concentrations of circulating testosterone, the level of AR transcript was decreased, and adult rats in this group (90 PND) also had cytoplasmic immunoexpression of AR in some hepatocytes. This is in line with the conclusion of Shen et al. [13], who stated that "the integration of nongenomic effects via membrane receptor signaling and genomic effects via nuclear receptor signaling of sex hormones is critical to produce the final sex hormone cellular outcomes".

In our study, the expression of insulin receptors, both at the mRNA and protein level was reduced in the liver of rat offspring born from females fertilized by the finasteride-treated male rats. On the other hand, there were no correlations between IR transcript and the level of serum androgens. It is known that MetS is characterized by the inability of insulin to adequately suppress hepatic gluconeogenesis, leading to hyperglycaemia, hyperinsulinemia and eventually to T2D [4]. In our experiment, in addition to the insulin resistance (decrease expression of insulin receptor), we showed hyperglycaemia of F1:Fin rats, that has been not age- and GLUT-2 mRNA level-dependent, so this elevated serum glucose concentration could have been caused by the trans-generational influence of finasteride. This is why it is likely that F1:Fin rats developed symptoms similar to MetS or even T2D during their lifetime, the origin of which might have been manifested by an increased body weight of F1:Fin vs. F1:Control that we find in a previous study on the same experimental model [32]. However this mechanism will be in the future evaluated by us (see Appendix A). The observed overweight could have been also associated with the modulation of adipose tissue metabolism by androgens (mainly DHT), as indicated by microarrays analysis by Zhang et al. [45], and therefore could be the result of a changed T–DHT ratio [33].

In summary, disturbed glucose transport into the cell (change of GLUT2 expression at the mRNA and protein level) and its utilization (increased glycogen accumulation in hepatocytes) in the liver of offspring from male rats receiving finasteride could result from

decreased insulin receptor expression, elevated serum glucose concentration, as well as from the dysfunction of androgen regulation and signalling (changed T–DHT ratio and decreased AR expression).

4. Experimental Section

4.1. Animals

The study was conducted on albino Wistar rats in accordance with Polish law and with the approval of the Local Ethics Committee for Scientific Experiments on Animals in Szczecin, Poland (Resolution no. 23/2010). Parent generation F0 produced male generation F1. Paternal rats in the F0:Fin group (n = 5) were treated with finasteride Proscar, MSD, Cramlington, UK) in daily doses of 5 mg/kg/bw, the same as in our previous studies [46–48] and experiments by other researchers [49,50]. The period of finasteride treatment before mating lasted 56 days (this changed the morphology and function of the testis) [46–48]. The mating period lasted a period of 5 months and not longer, to avoid the effect of ageing of the parent generation. Male rats (F0:Fin) received finasteride up until the end of the experiment (for 4–5 months). Once a week, the animals were weighed and the finasteride dose adjusted.

4.2. Generation of Filial Animals

The control group of offspring (F1:Control, n = 25) consist of male rats born from females fertilized by untreated control male rats. The experimental group of offspring (F1:Fin, n = 25) consists of male rats born from females fertilized by finasteride-treated male rats. The objective of the experiment was to sample the liver from both the treated and untreated offspring (F1) at 7, 14, 21, 28, and 90 postnatal days (PND) of life. Detailed information on the design of the animal treatment, mating, and collection of newborn offspring has been presented in our previous report [32]. After thiopental anesthesia (120 mg/kg bw, i.p., Biochemie GmbH, Vienna, Austria), the livers were divided into two parts, the first part was fixed in formalin and used for Periodic Acid-Schiff staining and immunohistochemical (IHC) reactions, the second tissue samples were frozen and used for qRT-PCR analyses.

4.3. Histological and Immunohistological Methods

The dissected livers were fixed in 10% formalin for at least 24 h and then washed with absolute ethanol (3 times over 3 h), absolute ethanol with xylene (1:1) (twice over 1 h) and xylene (3 times over 20 min). Then, after 3 h of saturation of the tissues in liquid paraffin, the samples were embedded in paraffin blocks. Using a microtome (Microm HM340E), 3–5 µm serial sections were taken and placed on polysine microscope slides (Thermo Scientific, UK; cat. no. J2800AMNZ). The sections of the livers were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol, and then used for PAS staining and IHC reaction.

PAS staining was made to demonstrate glycogen within the cell, because PAS reaction breaks 1,2-glycol linkages to form aldehydes, which are then revealed by Schiff's reagent [51]. To perform this procedure was used commercial kit (Bio-Optica, cat. no. 04-130802). Positive staining was determined microscopically (Leica DM5000B, Wetzlar, Germany) by visual identification of magenta color granules within cytoplasm of hepatocytes.

In order to expose the epitopes to IHC procedure, the deparaffinized and rehydrated sections were boiled twice in Target Retrieval Solution (DacoCytomation, S2367, S2369) in a microwave oven (700 W twice for 5 min). Once cooled and washed with PBS, the endogenous peroxidase was blocked using a 3% solution of perhydrol in methanol, and then the slides were incubated over night at 4 °C with primary antibodies against: GLUT-2 (Invitrogen, PA5-77459, final dilution 1:250), AR (Santa Cruz Biotechnology, sc-7305; final dilution 1:50), and IR (Abcam, ab 60946, final dilution 1:20). Antibodies were diluted in Antibody Diluent with Background Reducing Components (Dako, S3022). To visualize

the antigen-antibody complex, a Dako LSAB+System-HRP was used (DakoCytomation, K0679), based on the reaction of avidin-biotin-horseradish peroxidase with DAB as a chromogen, according to the staining procedure instructions included. Sections were washed in distilled H_2O and counterstained with hematoxylin. For a negative control, specimens were processed in the absence of a primary antibodies. Positive staining was determined microscopically (Leica DM5000B, Wetzlar, Germany) by visual identification of brown pigmentation.

4.4. Quantitative Computer Image Analysis Histological Slides

PAS-stained and GLUT-2-immunostained slides were scanned at a magnification of 400× (resolution of 0.25 µm/pixel) using the ScanScope AT2 scanner (Leica Microsystems, Wetzlar, Germany). The obtained digital images of the slides were analysed using the ImageScope viewer (Version 11.2.0.780; Aperio Technologies, Inc., Vista, CA, USA).

For the automatic computer analysis of PAS-positive glycogen in rat livers, a positive pixel count algorithm (version 9.1; Aperio Technologies, Inc.) was used. Other parameters were set to achieve compliance with the visual evaluation of colour intensity, including the intensity thresholds for positive results. The areas of analyses were manually determined. Using the algorithm, the number of PAS-positive and PAS-negative pixels were counted. The total number of PAS-positive pixels was counted in 30 random fields for each of the three zones of the hepatic lobule in each studied group with an average area of 0.049 mm^2 (6 fields per rat), Subsequently, the percentage of PAS-positive glycogen areas was calculated.

For the automatic computer analysis of GLUT-2 expression in the membrane of hepatocytes, a membrane v9 algorithm (version 9.1; Aperio Technologies, Inc.) was used. Similar to the PAS-positive glycogen analysis, other parameters were set to achieve compliance with the visual evaluation. The areas of analyses were also manually determined. Using the algorithm, the percentage of hepatocytes with weak, medium, and strong GLUT-2-positive immunostaining in the plasma membranes were counted. The percentage of cells with the GLUT-2 expression was counted in 30 random fields in each group with an average area of 0.217 mm^2 (6 fields per rat).

4.5. Quantitative Real-Time Reverse Transcription PCR (qRT-PCR) Analysis

Quantitative analysis of mRNA expression of GLUT-2, IR, and AR were performed in a twostep reverse transcription PCR. Tissues were suspended in 600 µL of RLT buffer and homogenized for 4 min on ice. Next, the sample was digested with proteinase K for 15 min at 55 °C and isolated on spin columns according to the manufacturer's protocol. All RNA was extracted from 50–100 mg tissue samples using an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). The RNA was then treated with DNase I (Qiagen) to eliminate genomic DNA contamination. All isolated RNA was quantified by spectrophotometry using a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, USA) and the optical density 260–280 nm ratio was determined; the 260:280 ratios were 1.8–2.0. Next, cDNA was prepared from 1 µg of total cellular RNA in a 20 µL reaction volume, using a FirstStrand cDNA synthesis kit and oligo-dT primers (Fermentas, USA). Quantitative assessment of mRNA levels was performed by real-time RT-PCR using an ABI 7500 Fast instrument with a Power SYBR Green PCR Master Mix reagent. Real-time conditions were as follows: 95 °C (15 s), 40 cycles at 95 °C (15 s), and 60 °C (1 min). The specificity assessment was conducted by performing a melting curve analysis (60 to 95 °C in temperature ramp melting); only one PCR product was amplified under these conditions. Reaction mixtures contained 10 µL of 2× SYBR Green supermix, 3 µL of primers (0.4 µmol/L each), 2 µL of cDNA template and 3 µL of water Each sample was analyzed in two technical replicates, and mean *Ct* values were used for further analysis. The relative quantity of a target, normalized to the endogenous control Gapdh gene and relative to a calibrator, is expressed as $2^{-\Delta\Delta Ct}$ (-fold difference), where *Ct* is the threshold cycle, ΔCt = (*Ct* of target genes) − (*Ct* of endogenous control gene), and $\Delta\Delta Ct$ = (ΔCt of samples for target gene) −

(ΔCt of calibrator for the target gene). The following primer pairs were used: for GLUT-2 (F: TCA GAA GAC AAG ATC ACC GGA; R: GCT GGT GTG ACT ATG AGT GGG), for IR (F: ATG GGC TCC GGG AGA GGA T; R: CTT CGG GTC TGG TCT TGA ACA), and for AR (F: TCC AAG ACC TAT CGA GGA GCG; R: GTG GGC TTG AGG AGA GCC AT). The primer sequences used in the study were obtained according to the sequence information obtained from the NCBI database, and were synthesized by Oligo.pl (IBB PAN, Poland).

4.6. Hormone Assays

The procedure of measurement of T and DHT in blood serum is the same as in our previously published work [33]. A standard sandwich ELISA assay was performed on the serum using a rat specific T and DHT ImmunoAssay System kit (CUSABIO; CBS-E05100r and CBS-E07879r), according to the manufacturer's instructions, and an Asys UVM 340 microplate reader (Asys Hitech Gmbh, Austria). The detection range for T was 0.13–25.6 ng/mL with a sensitivity of 0.06 ng/mL; for DHT, the detection range was 10–2000 pg/mL and the sensitivity 5 pg/mL.

4.7. Glucose Assesment in Blood

The levels of glucose in blood serum were measured in the Central Laboratory of The Independent Public Clinical Hospital No. 2 of Pomeranian Medical University in Szczecin (SPSK2, PUM). Glucose was measured by the UV test (700/340 nm), the reference enzymatic method with hexokinase (GLUC-3, Roche/Hitachi, cobas c 311, cobas c 501/502). The detection range was 2 to 750 mg/dL and the sensitivity 2 mg/dL.

4.8. Statistical Analysis

The results of the qRT-PCR analysis, serum glucose and androgens concentrations were analyzed using Statistica 6.1 software (StatSoft, Kraków, Poland). The arithmetical means and SDs ($X \pm SD$) were calculated for each of the parameters. The normal distribution of the results for the individual variables was obtained using the Shapiro–Wilk test. As most of the distributions deviated from a normal distribution, nonparametric tests were used for further analysis. To assess the differences between the groups, the nonparametric Mann–Whitney *U*-test and Kruskal–Wallis test with Dunn's multiple comparison test for post hoc analysis were used. A probability of $p \leq 0.05$ was considered statistically significant. Additionally, the serum levels of T and DHT, presented in our previous study [33], were correlated with the levels of GLUT-2, IR, and AR transcripts by Spearman's rank correlation coefficient (r_s). The same test was used to correlate serum glucose concentration with age of animals and hepatic GLUT-2 mRNA.

5. Conclusions

Rats in the F1:Fin group have a higher accumulation of hepatic glycogen as a result of hyperglycaemia, a lower expression of GLUT-2, IR, and AR in the liver, and liver steatosis and higher body weight (documented it in a previous study on the same experimental model [32]).

It can be concluded that finasteride has trans-generational consequences and probably epigenetic side effects that could lead to some metabolic syndromes such as hyperglycaemia, insulin resistance, type 2 diabetes or a fatty liver in males. Moreover, finasteride should become the focus of research in the relatively new field of pharmacology—pharmacoepigenomics.

Author Contributions: Conceptualization: A.K.-W.; data curation: A.K.-W., P.K.; formal analysis: M.G., A.K., I.B.-B., M.T., S.R., M.P.; investigation: A.K.-W., P.K.; resources: PMU (see funding); writing and original figures: A.K.-W., P.K.; writing and review and editing: A.K.-W., B.W. All authors have read and agreed to the published version of the manuscript.

Funding: The currying out the research, the publishing and English editing of the publication was financed by funds for research and development (statutory activity of the Pomeranian Medical University in Szczecin, WMS-167-02/S/16/2020).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Local Ethics Committee for Scientific Experiments on Animals in Szczecin, Poland (Resolution no. 23/2010, date of approval: 21.07.2010).

Data Availability Statement: The data presented in this study are contained within this article and Appendix A.

Acknowledgments: We would like to thank Andrzej Kram for enabling the slides to be scanned, and then the obtained images densitometrically assessed.

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

Abbreviations

$5\alpha R1^{-/-}$	5α-reductase type 1-knockout mice
$5\alpha R2^{-/-}$	5α-reductase type 2-knockout mice
ACACA	acetyl-CoA carboxylase alpha
AGA	androgenic alopecia
Ala	alanine
ALIOS	American lifestyle-induced obesity syndrome
AR	androgen receptors
ARE	androgen response elements
BPH	benign prostatic hyperplasia
DHT	dihydrotestosterone
E2	estradiol
F1:Control	male rats born from females fertilized by untreated control male rats
F1:Fin	male rats born from females fertilized by finasteride-treated male rats
FASN	fatty acid synthase
GK	glucokinase
GLUT2	glucose transporter 2
HFD	high-fat diet
IR	insulin receptor
MetS	metabolic syndromes
PAS	Periodic Acid Schiff
PC	pyruvate carboxylase
PND	postnatal day
PPARG	peroxisome proliferator activated receptor gamma
PTP1B	protein-tyrosine phosphatase 1B
T	testosterone
T2D	type 2 diabetes

Appendix A. Supplementary Data Concerning Lipid Metabolism within the Liver of F1:Fin Rats

F1:Fin rats showed a statistically significant decrease ($p = 0.011$) in serum insulin levels (0.37 ± 0.19 ng/mL) in comparison to F1:Control (0.42 ± 0.24 ng/mL). In the F1:Fin rats the serum levels of triglycerides was statistically significantly increased (77.8 ± 30.9 mg/dL) in comparison to F1:Control (59.4 ± 9.4 mg/dL) ($p = 0.005$). Moreover, adult F1:Fin rats had decreased sensitivity to insulin, as confirmed by the general decreased expression of IR at the mRNA and protein level, accompanied by decreased GLUT-2 and a decrease in the expression of the androgen receptor which could be associated with the regulation of IR and GLUT-2. Insulin resistance is known to result in the uncontrolled production of glucose in the liver (i.e., enhanced hepatic gluconeogenesis) and a decrease in its uptake by peripheral tissues, resulting in hyperglycaemia [52]. This elevated level of glucose have been documented in F1:Fin rats (Table 2).

In our study, intensification of gluconeogenesis in hyperglycaemic F1:Fin rats was confirmed by statistically significant increase (p = 0.000171) in the mRNA expression of hepatic pyruvate carboxylase (PC), a key regulator of gluconeogenesis (Figure A1) due to insulin acting as its repressor. The decrease in insulin levels could be also responsible for the statistically significant (p = 0.0453) decrease in the expression of glucokinase (GK) in the liver of F1:Fin rats observed in our study (Figure A1), which may have been associated with the regulatory function of insulin, an inducer of this enzyme.

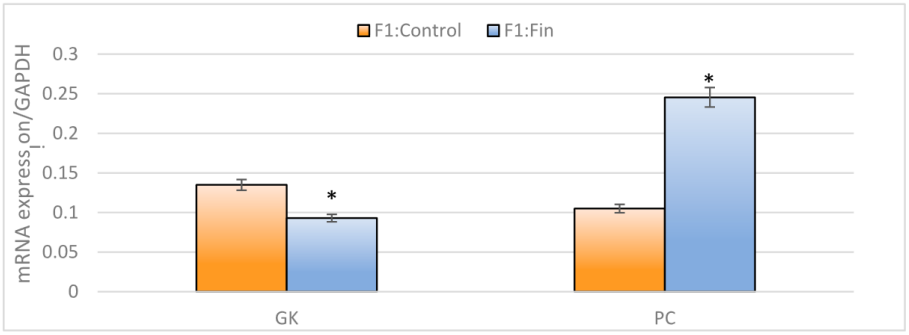

Figure A1. The glucokinase (GK) and pyruvate carboxylase (PC) mRNA levels (normalized to GAPDH) in the homogenates of hepatic tissue of the control offspring (F1:Control) and those born from females fertilized by finasteride-treated male rats (F1:Fin) without taking into account postnatal age. Values are expressed as arithmetic means ± SD; differences were evaluated using the Mann–Whitney *U*-test (n = 25 per each F1:control and F1:Fin, $p \leq 0.05$).

A lowered insulin level also increases the breakdown of glycogen in the liver because this activates glycogen phosphorylase. Then, the produced glucose-6-phosphate (G-6-P) is hydrolyzed by glucose-6-phosphatase, which results in the release of glucose into the bloodstream and exacerbated hyperglycemia [52]. The elevated glucose levels in the serum of F1:Fin rats may also be caused by the breakdown of muscle glycogen which occurs when blood insulin levels are decreased. As muscle cells do not produce glucose-6-phosphatase, they use glucose-6-phosphatase for their own energy metabolism, which leads to the production and accumulation of pyruvate. Much of pyruvate is transaminated to alanine (Ala). The products of transamination, alanine and keto-acids, are transported to the liver where Ala is then transaminated back to pyruvate—the main substrate for gluconeogenesis—thus increasing blood glucose levels [52].

The Spearman rank test did not show any correlation of acetyl-CoA carboxylase alpha (ACACA), fatty acid synthase (FASN), and peroxisome proliferator activated receptor gamma (PPARG) expression with the age of the animals (similar to GK and PC). Therefore, we checked the significance of the differences in the expression of these enzymes between F1:Control and F1:Fin, without division into age groups. This analysis only revealed statistically significant differences for ACACA (p = 0.026850), and a decrease in FASN which was on the verge of significance (p = 0.063840) (Figure A2).

The reduction in insulin concentration in the serum of F1:Fin rats could also result in the inhibition of lipogenesis and activation of lipolysis. This is confirmed by a reduction in mRNA expression of FASN and ACACA (enzymes responsible for the synthesis of fatty acids, for which insulin is an inductor) and PPARG in F1:Fin rats vs. F1:Control. Increased lipolysis leads to a release of increased amounts of glycerol, another substrate for gluconeogenesis in the liver, and to a release of free fatty acids utilized in the liver (but also in the heart and skeletal muscle) as the preferred energy substrates for beta-oxidation. However, an excess of free fatty acids and acylo-CoA is used in the synthesis of ketone bodies [52]. Decreased insulin levels may have affected the utilization of ketone bodies by the muscles and other cells in F1:Fin rats due to a lack of oxaloacetate necessary for the tricarboxylic acid cycle which leads to ketonaemia. Decreased insulin levels result in the

intensification of lipolysis and then an increase in free fatty acid levels in the blood which in turn results in an elevated release of VLDL from the liver and increased chylomicron levels in the blood. All these events contribute to the hypertriglyceridemia observed in F1:Fin rats.

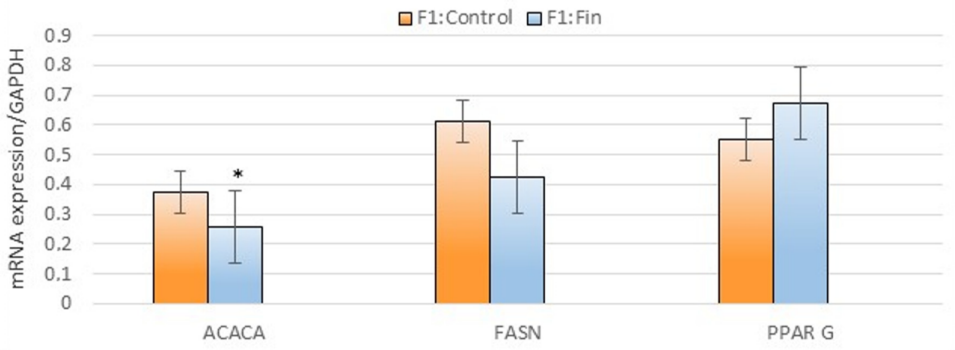

Figure A2. The acetyl-CoA carboxylase alpha (ACACA), fatty acid synthase (FASN) and peroxisome proliferator activated receptor gamma (PPARG) mRNA levels (normalized to GAPDH) in the homogenates of hepatic tissue of the control offspring (F1:Control) and those born from females fertilized by finasteride-treated male rats (F1:Fin) without taking into account postnatal age. Values are expressed as arithmetic means ± SD; differences were evaluated using the Mann–Whitney *U*-test (n = 25 per each F1:control and F1:Fin, $p \leq 0.05$).

The Spearman test analysis showed, a correlation between mRNA of AR with FASN and PPARG in both groups (F1:Control and F1:Fin) (Table A1).

Table A1. Correlation between mRNA for androgen receptor (AR) and mRNAs for acetyl-CoA carboxylase alpha (ACACA), fatty acid synthase (FASN) and peroxisome proliferator activated receptor gamma (PPARG) in the liver homogenates of male control rats' offspring (F1:Control) and finasteride-treated male rats' offspring (F1:Fin).

	F1:Control	**F1:Fin**
	AR	**AR**
ACACA	$r_s = -0.3423$ $p = 0.179$	$r_s = -0.2670$ $p = 0.284$
FASN	$r_s = -0.4243$ $p = 0.043$	$r_s = -0.4717$ $p = 0.031$
PPARG	$r_s = 0.890$ $p = 0.0000$	$r_s = 0.7397$ $p = 0.000024$

Values are express as the Spearman's rank coefficient (r_s) with statistically significant probability $p \leq 0.05$.

In prostate cancer cells (LNCaP), it has been proven that the coordinated regulation of the expression of genes involved in cholesterol and fatty acid synthesis were identified in response to androgen [53]. An androgen induction of SREBP2 was shown. This outcome was explained by researchers as a result of the presence of androgen responsive element (ARE)-like sequences in the promoter region of SREBP2, and it suggests a potentially direct activation of SREBP2–FASN pathway by androgen [53], which is in line with previous studies [54,55]. Therefore, the correlation between the transcript levels for AR and FASN could have resulted in fatty liver due to AR–SREBP2–FASN pathway.

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8. DODADKOWE, NIEOPUBLIKOWANE WYNIKI BADAŃ STANOWIĄCE UZUPEŁNIENIE ROZPRAWY DOKTORSKIEJ

Ekspresja receptora estrogenowego alfa i indeks mitotyczny hepatocytów wątroby męskiego potomstwa samców szczurów otrzymujących finasteryd

Wstęp

Wątroba jest jednym z narządów estrogeno-zależnych, bowiem hepatocyty wykazują ekspresję receptorów estrogenowych [57]. Badania wykazały, że wiele zaburzeń metabolicznych i chorób wątroby jest zależnych od hormonów płciowych, zatem
od sygnalizacji poprzez receptory dla hormonów płciowych SHR (ang. *sex hormone receptor*), do których należą zarówno ERs i AR [58].

Wiele chorób wątroby opisuje się jako schorzenia, zależne od płci, z różną częstotliwością występowania i odmiennym rokowaniem dla mężczyzn i kobiet [59]. Przykładowo, częstym powikłaniem terapii tamoksyfenem (selektywny modulator ER, o działaniu antyestrogennym) u kobiet z rakiem piersi, jest stłuszczenie wątroby, wynikające z nieprawidłowej β-oksydacji kwasów tłuszczowych [60]. Myszy z nokautem ERα w komórkach wątroby (LKO, ang. *liver ERα knockout*) [61] i myszy z nokautem Gpr30 (błonowy receptor estrogenowy sprzężony z białkiem G, ang. *G protein-coupled estrogen receptor*) [62] będące na diecie wysokotłuszczowej charakteryzowały się podwyższonym poziomem trójglicerydów (TG, ang. *triglyceride*) i diacyloglicerydów
w wątrobie, ale tylko samce miały obniżony poziom HDL, podwyższoną akumulację lipidów w wątrobie i insulinooporność. Z kolei w mysim modelu nadekspresji aromatazy cytochromu P450, zmieniony wątrobowy metabolizm lipidów, będący konsekwencją wzrostu koncentracji estrogenów obserwowano, także tylko u zwierząt płci męskiej [63].

Wiele danych literaturowych przemawia za tym, że to właśnie estrogeny sprzyjają rozwojowi nowotworów wątroby, związanych ze wzrostem aktywności mitogennej hepatocytów [64], jednakże *Mishkin i wsp.* [65] wykazali, że u zwierząt z indukowanymi acetyloaminofluorem guzkami przerostowymi wątroby, egzogenny

estradiol
w połączeniu z tamoksyfenem prowadził do regresji guzków. Wzrost aktywności receptora estrogenowego, wykazano w ogniskowym przeroście guzkowym wątroby, spowodowanym stosowaniem doustnych środków antykoncepcyjnych, a w gruczolaku wątroby, odnotowano natomiast obniżoną zdolność wiązania estrogenów przez hepatocyty [66].

W omawianiu zaburzeń funkcjonowania wątroby, często podnosi się kwestię jej androgenowo-estrogenowej zależności. Przykładowo, wykazano, że kastracja prowadziła do zwiększenia poziomu aromatyzacji androgenów do estrogenów w wątrobie, wzrostu stężenia estrogenów w surowicy, nadekspresji ER w hepatocytach równolegle z obniżeniem poziomu androgenów we krwi i spadkiem ekspresji AR w wątrobie [67]. Zatem obydwa hormony płciowe mogą determinować hepatokarcynogenezę, dodatkowo wykazano iż była ona częstsza u zwierząt płci męskiej, a kastracja opóźniała wystąpienie HCC [68].

Omówione powyżej kwestie są ciekawe, cenne z punktu widzenia chorób rozrostowych/nowotworowych wątroby, jednakże nie są one jednoznaczne. Wskazują na liczne, wielopoziomowe kaskady zdarzeń oraz zależności pomiędzy estrogenami/ERs oraz/lub androgenami/AR w tkance zdrowej, czy zmienionej chorobowo. Brak jest również jednoznacznej odpowiedzi na pytanie czy, analogicznie jak w przypadku raka prostaty [69], w chorobowo zmienionej tkance wątrobowej, kluczowa jest równowaga pomiędzy ekspresją AR a ERα oraz równowaga androgenowo-estrogenowa.

Z powyższych względów oraz z uwagi na fakt, że podawanie finasterydu zmienia równowagę androgenową [70, 6], androgenowo-estrogenową [71, 72, 73], ekspresję AR [74] oraz ERα i ERβ [70], zbadanie poziomu ekspresji ERα oraz aktywności mitotycznej hepatocytów w wątrobach zwierząt z zaburzoną homeostazą androgenową (T/DHT) wydaje się być ciekawym aspektem w odniesieniu do interferowania, czy współdziałania różnych hormonów steroidowych w fizjologię hepatocytów.

Cel pracy

Celem pracy badawczej była analiza poziomu proliferacji hepatocytów w korelacji z ekspresją ERα w wątrobie pokolenia potomnego (F1:Fin), uzyskanego

od samic, krytych szczurami (F0:Fin) z wywołanym farmakologicznie (finasterydem) deficytem DHT w odniesieniu do grupy zwierząt kontrolnych (F1:Control).

Materiał i metody

Materiał Badawczy

Zwierzęcy model badawczy został szczegółowo omówiony w rozdziale 4. tej dysertacji.

Analiza immunoekspresji ERα i PCNA z zastosowaniem reakcji immunohistochemicznej (IHC)

Do reakcji IHC wykorzystano komercyjny zestaw Dako LSAB+System-HRP (DakoCytomation, K0679), natomiast przeciwciała pierwszorzędowe skierowane były przeciwko ERα (SantaCruz Biotechnology, sc-7305, 1:250) i PCNA (Abcam, ab 60946, 1:250). Szczegółową metodykę reakcji IHC omówiono w publikacji *Kur i wsp.* [75].

Analiza ilościowa immunoekspresji PCNA i ERα

Po reakcji IHC preparaty zostały zeskanowane przy użyciu skanera ScanScope AT2 (Leica Microsystems, Wetzlar, Niemcy) przy powiększeniu 400x (rozdzielczość 0,25 μm/pikseli). Uzyskane w ten sposób obrazy cyfrowe wątroby analizowano za pomocą przeglądarki ImageScope (wersja 11.2.0.780; Aperio Technologies, Vista, CA, USA). Do analizy ilościowej PCNA-dodatnich i ERα-dodatnich zastosowano algorytm jądrowy v9 (wersja 9.1; Aperio Technologies, Vista, CA, USA). Pozostałe parametry zostały ustawione w celu osiągnięcia zgodności z oceną wizualną. Poszczególne obszary analiz zostały określone ręcznie. Stosując algorytm, zliczono odsetek komórek PCNA-dodatnich i ERα-dodatnich niezależnie w 30 polach o dużej mocy w każdej grupie (6 pól na szczura) o średniej powierzchni 1,5 mm^2 (w przypadku PCNA) i 1,9 mm^2 (w przypadku ERα).

Analiza statystyczna

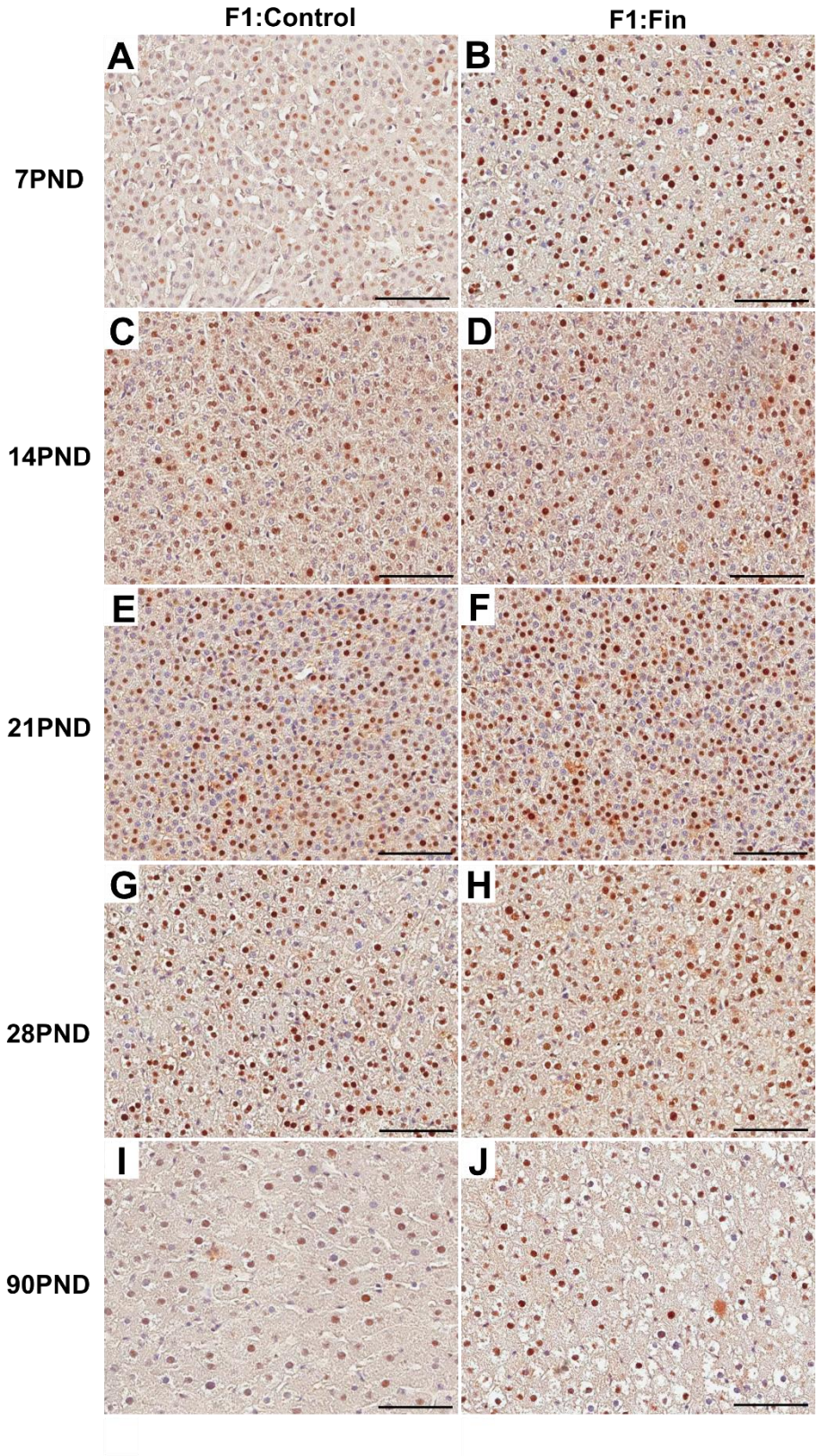
Uzyskane dane liczbowe analizowano za pomocą programu Statistica 13.1 (StatSoft, Kraków, Polska). Obliczono średnie arytmetyczne, odchylenia standardowe, mediany oraz wartości minimalne i maksymalne. Uzyskane wyniki poddano analizie na

rozkład normalny za pomocą testu Shapiro-Wilka. Następnie zastosowano testy nieparametryczne, ponieważ większość rozkładów odbiegała od rozkładu normalnego. Do oceny różnic pomiędzy grupami zastosowano test Kruskal'a–Wallis'a z testem wielokrotnych porównań Dunn'a do analizy post hoc oraz test U Mann'a–Whitney'a. Różnice międzygrupowe uznano za istotne przy $p<0,05$. Zależność pomiędzy immunoekspresją ERα a PCNA oceniono współczynnikiem korelacji Rang Spearman'a (Rs).

Wyniki

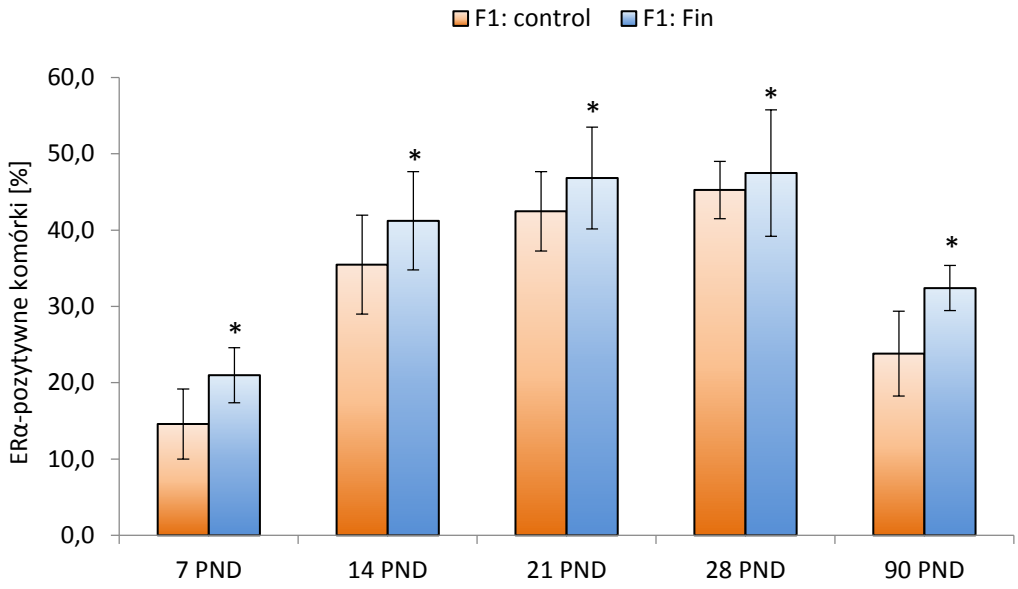
Immunoekspresja ERα

Immunoekspresję ERα zaobserwowano w jądrach hepatocytów (brązowy odczyn) (Rycina 1).



Rycina 1. Reprezentatywna mikrofotografia przedstawiająca immunoekspresję ERα w wątrobach samców szczurów F1:Control *vs*F1:Fin w poszczególnych okresach życia postnatalnego: 7PND (A, B), 14PND (C, D), 21PND (E, F), 28PND (G, H), 90PND (I, J). Skala: 50 µm. Reakcja IHC.

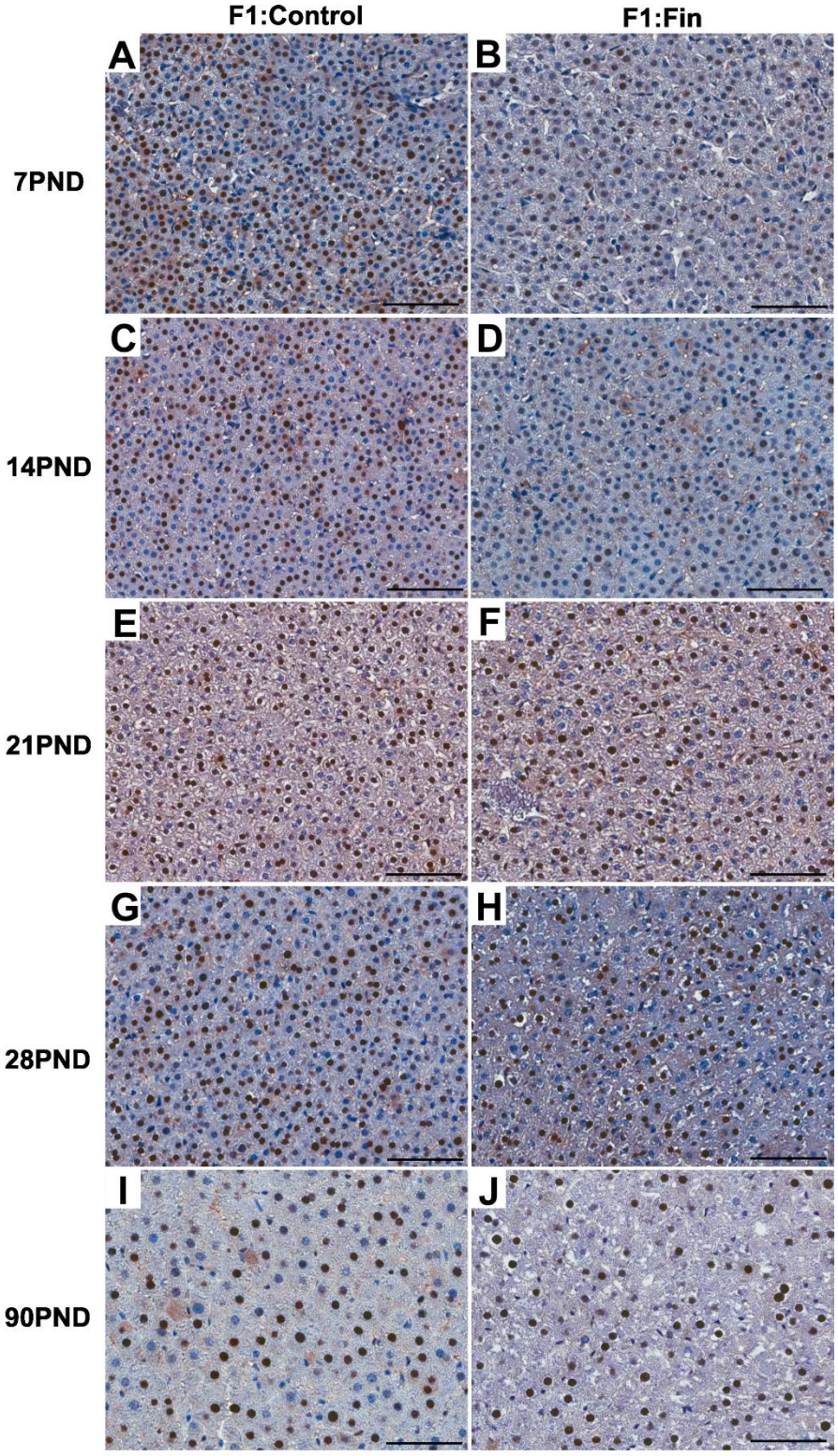
We wszystkich grupach wiekowych (7PND, 14PND, 21PND, 28PND, 90PND) odsetek komórek ERα-dodatnich w wątrobach szczurów grupy F1:Fin był statystycznie istotnie wyższy (odpowiednio p<0,001, p<0,003, p<0,004, p<0,007, p<0,001), w porównaniu ze zwierzętami kontrolnymi. Najwyższy odsetek komórek ERα-dodatnich zaobserwowano u 28-dniowych zwierząt kontrolnych (45,26%), jak i szczurów
grupy F1:Fin (47,48%) (Wykres 1).



Wykres 1. Odsetek komórek ERα-dodatnich w wątrobie szczurów według dni rozwoju (7PND, 14PND, 21PND, 28PND, 90PND) w kontroli (F1:Control) oraz w pokoleniu F1:Fin, od pokolenia ojcowskiego, otrzymującego finasteryd,* p < 0,05.

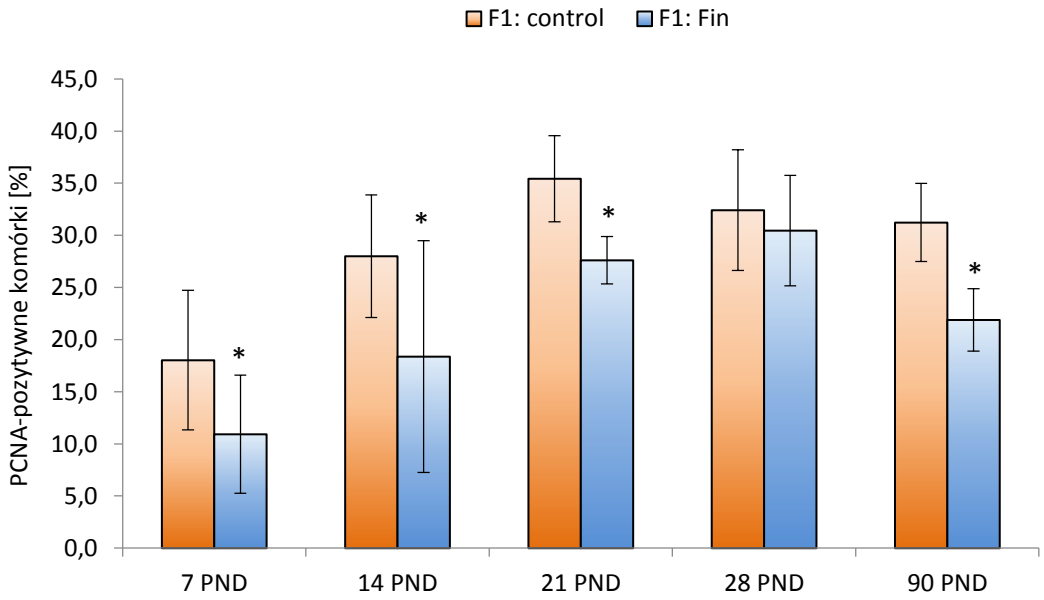
Immunoekspresja PCNA

W hepatocytach wszystkich analizowanych grup szczurów, immunolokalizacja PCNA była jądrowa (brązowy odczyn) (Rycina 2).



Rycina 2. Reprezentatywna mikrofotografia przedstawiająca immunoekspresję PCNA w wątrobach samców szczurów F1:Control *vs* F1:Fin w poszczególnych okresach życia postnatalnego: 7PND (A, B), 14PND (C, D), 21PND (E, F), 28PND (G, H), 90PND (I, J). Skala: 50 μm. Reakcja IHC.

W grupach wiekowych 7PND, 14PND, 21PND i 90PND odsetek komórek PCNA-dodatnich w wątrobach szczurów F1:Fin był statystycznie istotnie niższy ($p<0,001$) w porównaniu do tkanek zwierząt kontrolnych. Jedynie w 28. dniu życia postnatalnego nie było znamiennie statystycznie istotnej różnicy. Najwyższy odsetek komórek PCNA-dodatnich wykazano w grupie F1:Control w 21. dniu życia (35,44%), a u szczurów grupy F1:Fin w 28. dniu po narodzeniu (30,46%) (Wykres 2).



Wykres 2. Odsetek komórek PCNA-dodatnich w hepatocytach wątrób szczurów według dnia rozwoju (7PND, 14PND, 21PND, 28PND, 90PND) w kontroli (F1:Control) oraz w pokoleniu F1:Fin, od pokolenia ojcowskiego, otrzymującego finasteryd, * $p < 0,001$.

Korelacja Rang Spearmana

Tylko w dwóch grupach wiekowych zwierząt kontrolnych wykazano dodatnią (F1:Control 7PND, $Rs = 0,405$, $p = 0,027$) i ujemną (F1:Control 90PND, $Rs = - 0,363$, $p = 0,05$) korelację pomiędzy immunoekspresją ERα a PCNA.

Dyskusja

Wiadomo, że wątroba jest tkanką docelową dla sygnalizacji estrogenowej w warunkach fizjologicznych, jak i patologicznych, przy czym w tym drugim aspekcie nie są w pełni poznane mechanizmy i zależności tej interakcji [56], a wyniki różnych badań są czasami sprzeczne [76]. Oba podtypy ERs (α i β) są obecne w komórkach wątroby, i co ciekawe, mężczyźni mają wyższą ekspresję ERα niż kobiety [77]. Możliwe, że ta cecha leży u podstaw wyższej zachorowalności i śmiertelności wśród mężczyzn na HCC [76].

W swoim badaniu udokumentowałam jądrową lokalizację ERα w hepatocytach, niezależnie od wieku zwierząt, zarówno w grupie F1:Control, jak i F1:Fin. Wczesne badania *Fisher'a i wsp.* [78] wykazały, że około 55-60% komórek miąższowych z nieuszkodzonej wątroby szczurów zawiera ERs całkowicie zlokalizowane
w cytoplazmie, przy ich jednoczesnym braku w nukleoplazmie. Co ciekawe, podczas regeneracji wątroby szczura (hepatektomia, jako model badawczy dla niektórych szybko rosnących nowotworów), dynamicznie zmieniało się rozmieszczenie ER z cytoplazmy
do nukleoplazmy, prowadząc do podwyższenia syntezy kwasów dezoksyrybonukleinowych, podwyższenia indeksu mitotycznego i regeneracji; a jeszcze szybszy proces translokacji ER do nukleoplazmy obserwowano w hepatocytach zwierząt, którym w trakcie częściowej resekcji wątroby podawano 17β-estradiol. Ten sam zespół badaczy, opisał również obniżenie ilości ER-pozytywnych hepatocytów w wyniku hepatektomii (w porównaniu do wątrób zwierząt których, nie poddano tej procedurze) [78]. Było to w przeciwieństwie do badań *Uebi i wsp.* [56] oraz *Tsugawa i wsp.* [79], którzy u myszy po częściowej hepatektomii (PH, ang. *partial hepatectomy*) obserwowali nadekspresję ERα w hepatocytach przestrzeni okołoportalnej oraz nadprodukcję estradiolu promujące w ten sposób proliferację hepatocytów, w trakcie regeneracji narządu. Podobnie, podwyższoną zawartość receptorów estrogenowych w kompartymencie jądrowym obserwowano u osób z marskością wątroby oraz u chorych cierpiących na raka wątrobowokomórkowego, indukowanego HCV,
w porównaniu z prawidłowymi próbkami wątrób [77].

Badania *in vitro* prowadzone na komórkach HepG2 (linia ludzkiego raka wątroby) stymulowanych estradiolem, wykazały, że E2 wpływa na proliferację hepatocytów [56], która jest mediowana przez ERα [56, 79]; a *Tu i wsp.* [80] prowadząc badania na innej linii komórkowej, wykazali, że nadekspresja ERα mediuje apoptozę w transfekowanych, ERα-ujemnych komórkach Hep3B, poprzez wiązanie ERα z białkiem SP1
(ang. *specificity protein 1*, czynnik transkrypcyjny, aktywujący transkrypcję genów docelowych dla ER).

Te odmienne wyniki mogą być spowodowane różnicami gatunkowymi, modelem badawczym (*in vivo*/*in vitro*), bądź wynikać mogą z jakości materiału biologicznego (tkanka zdrowa/tkanka chora), czy metody badawczej (WB, RT-PCR, IHC, ect.).

Istnieją także dowody na to, że estrogen może mieć działanie przeciwnowotworowe [76, 81], może hamować przerzuty w raku wątrobowokomórkowym szczura, poprzez obniżanie ekspresji IL-6 i czynnika wzrostu hepatocytów [82] i nadekspresja ER α może wprowadzać komórki Hep3B w samobójczą śmierć [80].

Zatem, sygnalizacja poprzez receptory estrogenowe prowadzić może komórkę na rozdroża – albo komórka uruchomi procesy adaptacyjne, przeżyje, a następnie doprowadzi do regeneracji tkanki/narządu, albo wejdzie w apoptozę. Zapewne badania nad molekularnymi mechanizmami up-/down-regulacji ERs w różnych chorobach wątroby potrwają jeszcze wiele lat.

Wzrost ekspresji ERα, idący w parze ze spadkiem aktywności proliferacyjnej hepatocytów u zwierząt grupy F1:Fin w porównaniu do F1:Control jest przeciwny do badań innych [56, 79], prawdopodobnie ze względu na użyte metody analiz – *Tsugawa i wsp*. [79] oraz *Uebi i wsp*. [56] proliferację hepatocytów *in vitro* sprawdzali metodą
z wykorzystaniem bromodeoksyurydyny (BrdU, ang. *bromodeoxyuridine*) i z wykorzystaniem przeciwciała przeciwko Ki67.

Ze względu na obniżoną ekspresję PCNA w hepatocytach zwierząt grupy F1:Fin moje wyniki badań są raczej zbliżone do wyników badań dotyczących chorób rozrostowych wątroby, w których wykazano, że estrogeny, poprzez receptory estrogenowe hamują powstawanie nowotworów i obniżają ich inwazyjność.

Naugler i wsp. [22] wykazali, że estrogeny hamują produkcję IL-6 poprzez zmniejszenie aktywacji NF-B indukowanej przez Myd-88. Zatem, fizjologiczne dawki estrogenów mogą hamować przerzuty HCC nie tylko poprzez zmniejszenie ekspresji IL-6, ale także poprzez obniżenie poziomu czynnika wzrostu hepatocytów [82]. Wykazano,
że podawanie finasterydu, prowadziło do zmiany stosunku apoptozy do proliferacji komórek dystalnych kanalików krętych nefronu, infiltrację miąższu nerki przez limfocyty B i T oraz zmiany poziomu immunoekspresji IL-6 [73].

Ponieważ, w zasadzie nie wykazałam korelacji pomiędzy immunoekspresją ERα, a PCNA, obniżona aktywność proliferacyjna hepatocytów zwierząt grupy F1:Fin, mogła być związana z wykazaną przeze mnie obniżoną wrażliwością na insulinę (spadek ekspresji receptora dla insuliny na poziomie mRNA, jak i białka) [75]. Okazuje się bowiem, że regeneracja wątroby indukowana jest także insuliną [83]. Związanie insuliny z receptorem insulinowym prowadzi do aktywacji i internalizacji InR przez endosomy,
a następnie ich translokacji do jądra, gdzie aktywowana jest fosfolipaza C. Skomplikowana kaskada zdarzeń, zależna od fosfolipazy C finalnie prowadzi do inicjacji sygnalizacji jądrowej (związanie jądrowego receptora dla inozytolu (InsP3) z kanałami bramkowanymi jonami wapnia) [84]. Inny mechanizm pośredniczący w działaniu mitogennym insuliny w wątrobie polega na aktywacji i fosforylacji kaskady PI3K-KB-mTOR [84]. W sumie te czynniki mają szerokie implikacje kliniczne i mogą być ukierunkowane na kontrolowanie wzrostu komórek wątrobowych i zwiększenie zdolności regeneracyjnych hepatocytów po PH lub przeszczepieniu wątroby od żywego dawcy (LDLT, ang. *living donor liver transplant*) [85].

U dojrzałych płciowo szczurów F1:Fin obserwowano stłuszczenie wątroby, jako jeden ze skutków ubocznych, transpokoleniowej konsekwencji stosowania finasterydu [75]. Chirurgiczna resekcja i przeszczepienie stłuszczonej wątroby, są problematyczne, ponieważ stłuszczenie hepatocytów często powoduje niewydolność w wątrobie szczątkowej, jak również pierwotny brak podjęcia funkcji przeszczepionej wątroby [86]. W stłuszczonych hepatocytach, które wchodzą w zaprogramowaną śmierć komórki, dochodzi do regulacji w górę syntazy kwasów tłuszczowych i liganda dla tej syntazy (FasL, ang. *Fas-ligand*) [86]. Odmiennie, w swoim badaniu obserwowałam

statystycznie istotny/bądź na granicy istotności spadek transkryptu dla acylo-CoA karboksylazy alfa i dla syntazy kwasów tłuszczowych, u zwierząt grupy F1:Fin w porównaniu do kontroli (F1:Control) [75]. Możliwe, że obniżony indeks proliferacyjny, wykazany w moim badaniu, wynikał z innego mechanizmu molekularnego. Dowiedziono bowiem, że różne zmodyfikowane mechanizmy sygnalizacji, powstające w wyniku adaptacji do przewlekłych nieprawidłowości metabolicznych i zmniejszonej produkcji ATP są prawdopodobnymi przyczynami zwiększonej śmiertelności i upośledzonej regeneracji [86]. Ponadto uważa się, że stłuszczenie wątroby zmniejsza tolerancję na uszkodzenie niedokrwienne i stres oksydacyjny. Dlatego stłuszczenie hepatocytów przyczynić się może do upośledzenia regeneracji wątroby ze względu na zwiększoną podatność na uszkodzenia, jak również słabą odpowiedź mitotyczną [86]. To może być zgodne z moimi wynikami, które pokazują wyższą aktywność mitotyczną w hepatocytach wątrób zwierząt niedojrzałych płciowo (F1:Fin 21PND, 28PND) w porównaniu do dojrzałych osobników (F1:Fin 90PND), u których obserwowano stłuszczenie wątroby [75].

Na podstawie uzyskanych wyników badań można sugerować, iż podawanie finasterydu samcom pokolenia ojcowskiego spowodowało (i) podwyższenie ekspresji receptora estrogenowego alfa w hepatocytach; (ii) obniżenie aktywności mitogennej hepatocytów w wątrobach pokolenia F1:Fin w porównaniu do F1:Control.

Z jednej strony nadekspresja ERα z jednocześnie obniżonym indeksem mitotycznym hepatocyów zwierząt z grupy F1:Fin może sugerować podobieństwo warunków panujących w wątrobie tych zwierząt do warunków patologicznych, gdzie jak wykazano w niektórych badaniach, estrogeny poprzez receptory estrogenowe hamują powstawanie nowotworów i obniżają ich inwazyjność. Z drugiej jednak strony, skorelowanie wyników tej części pracy doktorskiej z opublikowanymi danymi [75], czyli insulinoopornością (obniżona ekspresja InR u zwierząt F1:Fin) wskazuje na insulinozależne obniżenie aktywności proliferacyjnej [83] zwierząt grupy F1:Fin, a stłuszczenie wątroby obserwowane u dojrzałych osobników grupy F1:Fin, może skutkować obniżeniem tolerancji na czynnik uszkadzające i upośledzać proces regeneracji [86].

9. WNIOSKI

Najważniejszym, oryginalnym i nowatorskim osiągnięciem badań tej dysertacji było udokumentowanie, że stosowanie finasterydu przez pokolenie ojcowskie (w wieku reprodukcyjnym) może prowadzić do efektu międzypokoleniowego, negatywnego dla czynności wątroby ich męskiego potomstwa. Analiza tego problemu może mieć istotne znaczenie z punktu widzenia chorób cywilizacyjnych takich, jak: globalny wzrost otyłości i insulinooporności, cukrzycy, a także niealkoholowego stłuszczenia wątroby – chorób m.in. zależnych od hormonów.

10. STRESZCZENIE ROZPRAWY DOKTORSKIEJ W JĘZYKU POLSKIM

Niniejsza rozprawa doktorska opiera się na dwóch opublikowanych pracach. Pierwsza z nich szeroko omawia rolę hormonów płciowych w metabolizmie wątroby, zarówno w warunkach fizjologicznych, jak i patologicznych; omawia choroby wątroby w aspekcie zależności od płci. Druga publikacja dostarcza danych, wskazujących na to, że podawanie finasterydu dorosłym samcom szczurów powoduje u męskiego pokolenia potomnego zmiany w ekspresji GLUT2, InR i AR w hepatocytach, które korelują z poziomami krążących androgenów (T i DHT), prowadząc w ten sposób do zmian w wątrobowym metabolizmie węglowodanów i lipidów, skutkując wzrostem magazynowania glikogenu w komórkach, hiperglikemią, insulinoopornością, a także niektórymi objawami zespołu metabolicznego czy stłuszczeniem wątroby.

Dodatkowo niniejsza dysertacja została wzbogacona o nieopublikowane wyniki badań, które wskazują na zmianę wrażliwości hepatocytów na estrogeny (nadekspresja ERα) i obniżenie aktywności mitogennej (obniżona ekspresja PCNA) hepatocytów w wątrobach pokolenia F1:Fin w porównaniu do F1:Control. Może to sugerować podobieństwo warunków panujących w wątrobie tych zwierząt do warunków patologicznych, gdzie estrogeny, poprzez receptory estrogenowe hamują powstawanie nowotworów i obniżają ich inwazyjność. Bądź może dowodzić tego, że obniżenie aktywności proliferacyjnej wynikało z insulinooporności tych zwierząt, a obserwowane stłuszczenie wątroby u zwierząt F1:Fin 90PND, może skutkować obniżeniem tolerancji na czynnik uszkadzające i dalej upośledzać proces regeneracji.

11. STRESZCZENIE ROZPRAWY DOKTORSKIEJ W JĘZYKU ANGIELSKIM

This doctoral dissertation is based on two published publications. The first one extensively discusses the role of sex hormones in liver metabolism, both in physiological and pathological conditions; discusses sex-related liver disease. The second publication provides data showing that administration of finasteride to adult male rats causes changes in the expression of GLUT2, InR and AR in hepatocytes in male offspring, which correlate with levels of circulating androgens (T and DHT), thus leading to change in hepatic metabolism of carbohydrates and lipids, resulting in an increase in glycogen storage in hepatocytes, hyperglycemia, insulin resistance, as well as some symptoms of metabolic syndrome or hepatic steatosis.

In addition, this dissertation has been enriched with unpublished research results that indicate a change in hepatocyte sensitivity to estrogens (ERα overexpression) and a decrease in mitogenic activity (reduced PCNA expression) of hepatocytes in F1:Fin livers compared to F1:Control. This may suggest the similarity of the conditions in the liver of these animals to pathological conditions, where estrogens, through ERs, inhibit the formation of tumors and reduce their invasiveness. Or, prove that the decreased proliferative activity resulted from insulin resistance in these animals, and hepatic steatosis observed in F1:Fin 90PND animals may result in a decrease tolerance to the damaging factor and further impair the regeneration process.

12. OŚWIADCZENIA WSPÓŁAUTORÓW PUBLIKACJI

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Potwierdzam mój wkład pracy w przygotowanie następującej publikacji naukowej:

Sex Hormone-Dependent Physiology and Diseases of Liver. **Int J Environ Res Public Health. 2020 Apr 11;17(8):2620. doi: 10.3390/ijerph17082620.**

Mój wkład pracy polegał na zaplanowaniu tematu pracy przeglądowej oraz przygotowaniu manuskryptu do publikacji.
Oceniam swój wkład na 50%.

The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, IR, and AR Expression in the Liver. **Int J Mol Sci. 2021 Jan 27;22(3):1242. doi: 10.3390/ijms22031242.**

Mój wkład pracy polegał na przeprowadzeniu reakcji immunohistochemicznych na skrawkach wątrób oraz ich barwienie metodą PAS, izolacji RNA z homogenatów wątrób, a następnie na przygotowaniu manuskryptu do publikacji.
Oceniam swój wkład na 50%.

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Sex Hormone-Dependent Physiology and Diseases of Liver. **Int J Environ Res Public Health. 2020 Apr 11;17(8):2620. doi: 10.3390/ijerph17082620.**

Mój wkład pracy polegał na zaplanowaniu tematu pracy przeglądowej, pisaniu oraz redagowaniu manuskryptu, jako autor korespondencyjny umieściłam manuskrypt na platformie czasopisma, poprawiałam tekst manuskryptu zgodnie z uwagami Recenzentów oraz przygotowałam odpowiedzi na uwagi recenzentów. Oceniam swój wkład na 40%

The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, IR, and AR Expression in the Liver. **Int J Mol Sci. 2021 Jan 27;22(3):1242. doi: 10.3390/ijms22031242.**

Mój wkład pracy polegał na zaplanowaniu eksperymentu, analizowaniu otrzymanych wyników, nadzorowaniu pisania manuskryptu, przygotowaniu manuskryptu do publikacji, jako autor korespondencyjny umieściłam manuskrypt na platformie czasopisma oraz poprawiałam tekst manuskryptu zgodnie z uwagami Recenzentów, na prośbę Recenzentów nadzorowałam wykonanie dodatkowych analiz oraz przygotowałam odpowiedzi na uwagi Recenzentów. Oceniam swój wkład na 15%.

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Potwierdzam mój wkład pracy w przygotowanie następującej publikacji naukowej:

***Sex Hormone-Dependent Physiology and Diseases of Liver*. Int J Environ Res Public Health. 2020 Apr 11;17(8):2620. doi: 10.3390/ijerph17082620.**

Mój wkład pracy polegał na formalnej analizie przygotowywanego do publikacji manuskryptu.
Oceniam swój wkład na 5%.

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Potwierdzam mój wkład pracy w przygotowanie następującej publikacji naukowej:

Sex Hormone-Dependent Physiology and Diseases of Liver. **Int J Environ Res Public Health. 2020 Apr 11;17(8):2620. doi: 10.3390/ijerph17082620.**

Mój wkład pracy polegał na merytorycznym nadzorze w trakcie pisania pracy przeglądowej oraz poprawieniu tekstu manuskryptu po uwagach Recenzentów. Oceniam swój wkład na 5%

The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, IR, and AR Expression in the Liver. **Int J Mol Sci. 2021 Jan 27;22(3):1242. doi: 10.3390/ijms22031242.**

Mój wkład pracy polegał na merytorycznym nadzorze w trakcie pisania manuskryptu oraz poprawieniu tekstu manuskryptu po uwagach Recenzentów. Oceniam swój wkład na 5%.

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Potwierdzam mój wkład pracy w przygotowanie następującej publikacji naukowej:

The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, IR, and AR Expression in the Liver. **Int J Mol Sci. 2021 Jan 27;22(3):1242. doi: 10.3390/ijms22031242.**

Mój wkład pracy polegał na wykonaniu komputerowej analizy uzyskanych odczynów, opisaniu tych wyników i ich analizie statystycznej.
Swój wkład oceniam na 5%.

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The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, IR, and AR Expression in the Liver. **Int J Mol Sci. 2021 Jan 27;22(3):1242. doi: 10.3390/ijms22031242.**

Mój wkład pracy polegał na zeskanowaniu preparatów histologicznych.
Swój wkład oceniam na 5%.

28.10.2022
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Potwierdzam mój wkład pracy w przygotowanie następującej publikacji naukowej:

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Mój wkład pracy polegał na wykonaniu reakcji qRT-PCR.
Oceniam swój wkład na 5%.

27.10.2022
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Potwierdzam mój wkład pracy w przygotowanie następującej publikacji naukowej:

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Mój wkład pracy polegał na wykonaniu analiz statystycznych dotyczących oznaczonych parametrów w surowicy krwi i danych liczbowych uzyskanych z analiz qRT-PCR.
Swój wkład oceniam na 5%.

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OŚWIADCZENIE

Potwierdzam mój wkład pracy w przygotowanie następującej publikacji naukowej:

The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, IR, and AR Expression in the Liver. **Int J Mol Sci. 2021 Jan 27;22(3):1242. doi: 10.3390/ijms22031242.**

Mój wkład pracy polegał na formalnej analizie przygotowywanego do publikacji manuskryptu.
Oceniam swój wkład na 5%.

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OŚWIADCZENIE

Potwierdzam mój wkład pracy w przygotowanie następującej publikacji naukowej:

The Postnatal Offspring of Finasteride-Treated Male Rats Shows Hyperglycaemia, Elevated Hepatic Glycogen Storage and Altered GLUT2, IR, and AR Expression in the Liver. **Int J Mol Sci. 2021 Jan 27;22(3):1242. doi: 10.3390/ijms22031242.**

Mój wkład pracy polegał na formalnej analizie wyników dotyczących komputerowej analizy uzyskanych odczynów.
Swój wkład oceniam na 5%.

27.10.2022r

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