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# Small non-coding RNAs as a potential diagnostic and therapeutic tool in insulin resistance

Małe niekodujące RNA jako potencjalne narzędzie diagnostyczne oraz terapeutyczne w insulinooporności

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**Wykaz skrótów**

ALT- (ang. *alanino aminotransferase*) - aminotransferaza alaninowa

ANG1- (ang. *Angiopoietin 1*) – angiopoetyna 1

ASC- (ang. *Adipose Stem Cells*) – komórki macierzyste z tkanki tłuszczowej

AST- (ang. *aspartate aminotransferase*) – aminotransferaza asparaginianowa

Ccl2- (ang. *chemokine ligand 2*) - ligand chemokiny 2

CD200- (ang. *OX-2 membrane glycoprotein*) - glikoproteina błonowa OX-2

CD29- (ang. *integrin beta 1*) – integryna beta 1

CVD- (ang. *cardiovascular disease*) – choroba układu sercowo naczyniowego

ECM – (ang. *extracellular matrix*) – macierz pozakomórkowa

EMS- (ang. *Equine Metabolic Syndrome*) – syndrom metaboliczny koni

FOXO1- (ang. *forkhead box protein O1*)

GGT- (ang. *gamma-glutamyl transferase*) - gamma-glutamylotranspeptydaza

HDL-c- (ang. *high-density lipoprotein cholesterol*) - cholesterol lipoproteinowy o dużej gęstości

HPC- (ang. *Hoof Progenitor Cells*) – komórki progenitorowe kopyt

IL-1- (ang. *interleukin 1*) – interleukina 1

IL1β- (ang. *interleukin 1β*) – interleukina 1β

IL-6- (ang. *interleukin 6*) – interleukina 6

INSR- (ang. *Insulin Receptor*) – receptor insuliny

IPSCs- (ang. *Induced Pluripotent Stem Cells*) – indukowane pluripotentne komórki macierzyste

ISCT- (ang. *International Society of Cellular Therapy*) – Międzynarodowe Stowarzyszenie Terapii Komórkowej

K14- (ang. *keratin 14*) – keratyna 14

K15- (ang. *keratin 15*) – keratyna 15

lncRNA- (ang. *long non-coding RNA*) – długie niekodujące RNA

MI- (ang. *myocardial infraction*) – zawał mięśnia sercowego

miRNA- (ang. *micro RNA*) – micro RNA

MSCs- (ang. *Mesenchymal Stem Cells*) – mezenchymalne komórki macierzyste

NAFLD- (ang. *nonalcoholic fatty liver disease*) – niealkoholowe stłuszczenie wątroby

NANOG- (ang. *Nanog Homeobox*)

ncRNAs- (ang. *non-coding RNAs*) – niekodujące RNA

OCT4- (ang. *octamer-binding transcription factor 4*) - czynnik transkrypcyjny 4 wiążący oktamer

PBMC- (ang. *peripheral blood mononuclear cell*) - komórki jednojądrzaste krwi obwodowej

PTEN- (ang. *phosphatase and tensin homolog deleted on chromosome ten*) - homolog fosfatazy i tensyny

SOX2- (ang. *sex determining region Y box 2*) – region Y determinujący płeć pole 2

SREBP-1c- (ang. *sterol response element binding protein 1c*) - białko wiążące sekwencję odpowiedzi na sterole 1c

TNF-p- (ang. *tumor necrosis factor p*) - czynnik martwicy nowotworów p

TNFα- (ang. *tumor necrosis factor α*) - czynnik martwicy nowotworów α

VEGFA- (ang. *vascular endothelial growth factor*) - czynnik wzrostu śródbłonka naczyniowego A

VLDL- (ang. *very low density lipoprotein*) - lipoproteina o bardzo niskiej gęstości

WHO- (ang. *World Health Organization*) – Światowa Organizacja Zdrowia

**Streszczenie**

Syndrom metaboliczny koni (EMS; ang. *Equine Metabolic Syndrom*) to schorzenie, u podstaw którego leży insulinooporność oraz deregulacja szlaku sygnałowego insuliny. Kluczowym organem w rozwoju insulinooporności jest wątroba, która ze względu na swoją funkcję często określana jest jako centrum metaboliczne organizmu. W obrazie klinicznym syndromu metabolicznego koni obserwuje się insulinooporność, upośledzenie metabolizmu wątrobowego, otyłość oraz regionalne depozyty tkanki tłuszczowej, hiperinsulinemię, hipoadiponektynemię, hiperleptynemię, zapalnie ogólnoustrojowe i nadciśnienie tętnicze. Jednym z najdotkliwszych objawów insulinooporności oraz EMS jest ochwat. W ostatnich latach znacznie wzrosło zainteresowanie komórkami macierzystymi, pod względem ich udziału w patofizjologii syndromu metabolicznego koni oraz ochwatu a także ze względu na ich obiecujący potencjał terapeutyczny. Ponadto, przedmiotem wielu badań dotyczących insulinooporności stała się grupa relatywnie nowo odkrytych biomarkerów, w tym niekodujących RNA (ncRNAs; *non-coding RNAs*), takich jak miRNA (micro-RNA) oraz lncRNA (długie niekodujące RNA; ang. *long non-coding RNA*).

Celem badań prowadzonych w ramach realizowanej pracy doktorskiej było wyselekcjonowanie potencjalnych markerów miRNA oraz lncRNA, które mogą być wykorzystane jako specyficzne i precyzyjne biomarkery schorzeń powiązanych z insulinoopornością. Dodatkowo, postawiona hipoteza badawcza, zakładała, że komórki macierzyste korony rogotwórczej (ang. *coronary corium*) – HPC (ang. *Hoof Progenitor Cells*) biorą udział w patofizjologii ochwatu. Ponadto, analiza reakcji tych komórek na proces zapalny oraz hiperinsulinemię pod względem ich unikalnego sekretomu miRNA może dostarczyć wartościowych informacji dotyczących przebiegu ochwatu, jego potencjalnej diagnostyki oraz terapii. Na podstawie najnowszej literatury wybrano 6 obiecujących niekodujących miRNA oraz lncRNA, które potencjalnie mogą stanowić czułe i specyficzne biomarkery insulinooporności wątrobowej oraz schorzeń metabolicznych. W przebiegu przeprowadzonych badań opracowano również protokół izolacji komórek HPC z korony rogotwórczej. Pozwoliło to na scharakteryzowanie morfologii, procesu proliferacji i różnicowania, stresu oksydacyjnego, metabolizmu mitochondrialnego, odpowiedzi zapalnej, apoptozy oraz unikalnego sekretomu miRNA komórek HPC w porównaniu do modelu komórek ASC (ang. *Adipose Stem Cells*). Co więcej, przeprowadzono ocenę reakcji komórek HPC na środowisko prozaplane oraz hiperinsulinemię, ze względu na ich potencjalny udział w patofizjologii ochwatu.

Wyniki przeprowadzonych badań wykazały, że niekodujące RNA stanowią precyzyjne biomarkery związane z insulinoopornością, natomiast nowo wyizolowana oraz scharakteryzowana populacja komórek HPC może odgrywać istotną rolę w regeneracji urazów skóry, naskórka, a także uszkodzeń naczyń krwionośnych zachodzących podczas ochwatu w obrębie puszki kopytowej. Co więcej, uzyskane wyniki pokazały, że wybrane miRNA mogą wykazywać potencjał diagnostyczny oraz terapeutyczny w przebiegu ochwatu, ze względu na zmianę ich ekspresji w komórkach HPC w przebiegu zapalenia oraz hiperinsulinemii.

**Streszczenie w języku angielskim**

Equine Metabolic Syndrome (EMS) is a disease stemming from insulin resistance and deregulation of the insulin signalling pathway. The key organ in the development of insulin resistance is the liver, which due to its function is often referred to as the metabolic centre of the organism. The clinical symptoms of equine metabolic syndrome include insulin resistance, impaired hepatic metabolism, obesity and regional fat deposits, hyperinsulinemia, hypoadiponectinemia, hyperleptinemia, systemic inflammation and hypertension. Laminitis is one of the most severe manifestations of insulin resistance and equine metabolic syndrome. In recent years, the interest in stem cells has increased significantly, in terms of their participation in the pathophysiology of equine metabolic syndrome and laminitis, as well as due to their significant therapeutic potential,. Additionally, a new group of relatively newly discovered biomarkers is the subject of many studies on insulin resistance, as well as other diseases, namely non-coding RNAs (ncRNAs - non-coding RNAs), such as miRNAs (micro-RNAs) and lncRNAs (long non-coding RNAs - long non-coding RNAs).

The aim of the research conducted as part of the doctoral thesis was to select potential miRNA and lncRNA markers that can be used as specific and precise biomarkers of diseases associated with insulin resistance. In addition, the research hypothesis assumed that stem cells of the coronary corium – HPC (*Hoof Progenitor Cells*) are involved in the pathophysiology of laminitis and that the analysis of their response to the inflammatory process and hyperinsulinemia in terms of their unique miRNA secretome can provide valuable information on the pathophysiology of laminitis, its potential diagnosis and therapy. Based on the most recent literature, 6 promising non-coding miRNAs and lncRNAs were selected, which may be sensitive and specific biomarkers of hepatic insulin resistance and metabolic diseases. As part of the conducted research, the process of isolating HPC cells from the area of the coronary corium was also developed. The morphology, proliferation, differentiation, oxidative stress, mitochondrial metabolism, inflammatory response, apoptosis and unique miRNA secretome of HPC cells were characterized in comparison to the ASC cell model (*Adipose Stem Cells*). Moreover, an assessment of the response of HPC cells to the inflammatory environment and hyperinsulinemia was performed, due to their potential participation in the pathophysiology of the laminitis.

The obtained results of the latest research reports analysis as well as the conducted research indicate that non-coding RNAs are precise biomarkers related to insulin resistance and that the newly isolated and characterized population of HPC cells may play a role in the

regeneration of the dermis and epidermis injuries as well as regeneration of the damage to blood vessels occurring during laminitis. Moreover, the results of the conducted research show that the selected miRNAs may have diagnostic and therapeutic potential in the course of laminitis, due to the change in their expression in HPC cells in the course of inflammation and hyperinsulinemia.

## 1. Wstęp

Insulina jest hormonem peptydowym produkowanym oraz wydzielanym przez komórki β zlokalizowane w wysepkach Langerhansa w trzustce. Jej podstawową funkcją jest utrzymywanie odpowiedniego poziomu glukozy we krwi poprzez aktywację wychwytu glukozy z krwi, a także poprzez regulację metabolizmu węglowodanów, białek oraz lipidów. Wiele komórek somatycznych posiada receptory insulinowe, jednak obniżenie poziomu glukozy we krwiobiegu jest głównie zależne od bezpośredniego oddziaływania insuliny na komórki mięśni szkieletowych, hepatocyty oraz adipocyty [1]. Insulinooporność jest definiowana jako stan, w którym tkanki docelowe tj. wątroba, tkanka tłuszczowa czy mięśnie szkieletowe wykazują obniżoną wrażliwość na insulinę. Skutkuje to brakiem możliwości utrzymania prawidłowego poziomu glukozy mimo prawidłowego lub podwyższonego poziomu insuliny w krwiobiegu. Już w 1936 roku Himsworth zaobserwował, że równoległe podanie insuliny oraz glukozy u pacjentów z cukrzycą skutkowało jednym z dwóch rezultatów: (1) u części z pacjentów po podaniu glukozy wraz z insuliną obserwowano stabilny bądź obniżony poziom glukozy w krwiobiegu, a pacjenci ci zostali zakwalifikowani jako wrażliwi na insulinę; (2) pozostali pacjenci, u których po podaniu glukozy wraz z insuliną stwierdzono znacznie podwyższony poziom glukozy we krwi zostali określeni jako niewrażliwi na insulinę [2]. Obecnie wiemy, że zaobserwowany przez Himsworth brak wrażliwości na insulinę jest obrazem typowym dla schorzenia znanego jako syndrom metaboliczny, w którym pomimo prawidłowego poziomu insuliny w krwiobiegu, tkanki docelowe nie są w stanie przeprowadzić skoordynowanych procesów mających na celu obniżenie poziomu glukozy we krwi.

Skomplikowana patofizjologia insulinooporności związana jest z upośledzoną aktywnością insuliny w komórkach docelowych. Szczegółowe określenie właściwego zaburzenia w fizjologii komórkowej, skutkującego upośledzeniem działania insuliny było tematem wielu badań, szczególnie na skutek identyfikacji nowych efektorów insuliny [3–7]. Pierwotnie, gdy INSR (ang. *Insulin Receptor*) był jedynym znanym efektorem insuliny, podejrzewano że insulinooporność może być wynikiem obniżonej ilości tych receptorów w błonie komórek docelowych. Kluczowym pytaniem było, czy insulinooporność faktycznie jest wynikiem niedoboru czy też defektu receptorów insulinowych na powierzchni błony komórkowej, czy może wynika ona z zaburzeń w przekazywaniu sygnału na dalszych etapach szlaku sygnałowego [6,8]. Dalsze badania wykazały jednak, że do zaburzeń w odpowiedzi na insulinę dochodzi dopiero, gdy ilość receptora INSR w błonie komórkowej zostanie obniżona poniżej 5-10% zawartości fizjologicznej. Zjawisko to wynika z faktu, że komórki posiadają zapasowe

receptory INSR. Na podstawie tych obserwacji wywnioskowano, że insulinooporność jest wynikiem kombinacji dwóch zjawisk, zarówno defektu receptorów błony komórkowej jak i zaburzeń w przekazywaniu sygnału na dalszych etapach szlaku sygnałowego[5,6].

Insulinooporność jest jednym z głównych objawów schorzeń metabolicznych takich jak cukrzyca typu 2, syndrom metaboliczny czy niealkoholowe stłuszczenie wątroby. Co więcej, pojawienie się insulinooporności jest często jednym z pierwszych objawów poprzedzających rozwój tych schorzeń. Choroby metaboliczne stanowią jedno z większych wyzwań dla medycyny, gdyż liczebność pacjentów cierpiących na schorzenia powiązane z insulinoopornością stale wzrasta. Z raportu Międzynarodowej Federacji Cukrzycy (ang. *International Diabetes Federation*) wynika, iż w roku 2000 szacowana prewalencja cukrzycy na świecie u pacjentów w wieku 20-79 lat wynosiła 151 milionów. Liczba ta potroiła się jednak i w roku 2019 stwierdzono 463 miliony przypadków tego schorzenia [9]. Szacuje się, że do roku 2045 około 700 milionów pacjentów zachoruje na cukrzycę. Poważny problem stanowi też wysoka śmiertelność będąca skutkiem cukrzycy. Raport WHO wskazuje, iż w 2019 roku aż 4.2 miliona pacjentów zmarło w wyniku cukrzycy. Istniejące dane sugerują, że prewalencja syndromu metabolicznego jest jeszcze wyższa. W większości krajów w regionie Azji i Pacyfiku prawie jedna piąta populacji dorosłych choruje na syndrom metaboliczny [10]. W USA częstość występowania tego schorzenia zmieniała się na przestrzeni lat. W latach 1988-1994 wynosiła 25,3%, natomiast w okresie 2007-2012 sięgnęła aż 34,2% [11]. Z racji tego, że syndrom metaboliczny jest co najmniej trzy razy bardziej powszechny niż cukrzyca, szacuje się, że około 25% globalnej populacji jest dotknięta tym schorzeniem, co przekłada się na ponad bilion zachorowań [12].

Kluczowym organem w rozwoju insulinooporności jest wątroba, która ze względu na swoją funkcję często określana jest jako centrum metaboliczne organizmu. W warunkach fizjologicznych odpowiada ona za metabolizm węglowodanów, lipidów oraz białek. W odpowiedzi na insulinę komórki wątroby przeprowadzają procesy glikogenezy, glikogenolizy, glikolizy, glukoneogenezy oraz lipogenezy, w zależności od statusu metabolicznego organizmu tj. w odpowiedzi na posiłek lub post. W przypadku insulinooporności, szlak sygnałowy insuliny zostaje jednak upośledzony, co prowadzi do kumulacji defektów w metabolizmie wątrobowym. Tym samym, wątroba staje się kluczowym narządem odgrywającym zasadniczą role w rozwoju hiperglicemii, ogólnoustrojowego zapalenia oraz w lipogenezie de novo [13–15]. Co więcej, wiele badań wskazuje na upośledzenie metabolizmu wątrobowego oraz wynikającą z tego insulinooporność wątrobową jako kluczową w rozwoju zarówno cukrzycy typu 2 jak i syndromu metabolicznego u ludzi [13,16]. Prewalencja

niealkoholowego stłuszczenia wątroby (ang. *NAFLD - nonalcoholic fatty liver disease*) wśród pacjentów z cukrzycą typu 2 wynosi 40-60%, podczas gdy u pacjentów z syndromem metabolicznym jest to aż 79% [17,18]. Zaproponowano również hipotezę, iż niealkoholowe stłuszczenie wątroby jest nie tylko objawem syndromu metabolicznego, ale też istotnym prekursorem tego schorzenia [16]. W związku z powyższym, wczesna diagnoza wątrobowej insulinooporności jest bardzo istotna, gdyż stan ten często poprzedza pojawienie się innych objawów.

Syndrom metaboliczny koni to schorzenie, u podstaw którego leży insulinooporność oraz deregulacja szlaku sygnałowego insuliny. W obrazie klinicznym EMS obserwuje się insulinooporność, upośledzenie metabolizmu wątrobowego, otyłość oraz regionalne depozyty tkanki tłuszczowej, hiperinsulinemię, hipoadiponektynemię, hiperleptynemię, zapalnie ogólnoustrojowe, nadciśnienie tętnicze oraz ochwat [19]. Jednym z głównych czynników ryzyka dla rozwoju EMS jest otyłość, chociaż dane kliniczne wskazują na to, że schorzenie to dotyka również koni o właściwej masie ciała. Charakterystyczne depozyty tkanki tłuszczowej można zaobserwować na szyi w pobliżu więzadła karkowego (ang. *cresty neck*), na łopatkach, w okolicach gruczołu mlekowego, napletka i nasady ogona [20]. Tkanka tłuszczowa jest aktywnym endokrynnym organem, wydzielającym adipokiny oraz cytokiny, które mogą wywierać silne działanie lokalne oraz systemowe [21]. Dotychczas, u koni przebadano dwie adipokiny - leptynę oraz adiponektynę. Leptyna jest często określana jako hormon sytości, ponieważ jest wydzielana przez tkankę tłuszczową, podczas gdy zapasy energii w organizmie są wysokie [22]. Receptory na neuronach jądra łukowatego podwzgórza reagują na poziom leptyny w krwiobiegu poprzez stymulację apetytu lub poczucie nasycenia. Podwyższone poziomy leptyny zostały zaobserwowane u koni oraz kucy z insulinoopornością, a hiperleptynemia może również stanowić komponent syndromu metabolicznego u tych zwierząt [23,24]. Adiponektyna jest uważana za adipokinę uwrażliwiającą na insulinę, a jej poziomy w krwiobiegu korelują z wrażliwością na insulinę u ludzi oraz w modelach zwierzęcych [25,26]. Badania wykazały, że stężenie adiponektyny we krwi było odwrotnie proporcjonalne do masy ciała u koni, a niższe stężenia adiponektyny obserwowano u koni otyłych oraz u koni z toczącym się ogólnoustrojowym procesem zapalnym [24,27]. Kolejnym kluczowym elementem w rozwoju insulinoorponości u koni otyłych jest ogólnoustrojowe zapalenie. Otyłe konie z EMS charakteryzują się podwyższoną ekspresją cytokin prozapalnych (TNFA, IL1B, CCL2), a także hipertrofią adipocytów [28,29]. Wykazano również podwyższoną ekspresję genów Il-1β oraz IL-6 w tkance tłuszczowej pobranej z okolic więzadła karkowego u koni z EMS [30]. Dodatkowo, u koni z EMS oraz u kuców z historią ochwatu pastwiskowego stwierdzono podwyższony poziom

TNFα w serum oraz w osoczu [31,32]. Zaobserwowano także korelację między otyłością, obniżoną wrażliwością na insulinę oraz podwyższoną ekspresją genów TNF-p oraz IL-1 [33]. Syndrom metaboliczny koni objawia się również upośledzeniem metabolizmu wątrobowego na poziomie komórkowym, gdyż u koni z EMS zaobserwowano podwyższoną apoptozę, stres retikulum endoplazmatycznego, stres oksydacyjny, akumulację lipidów oraz stan zapalany [34,35]. Badania wskazują również, że hiperinsulinemia towarzysząca EMS może także częściowo wynikać z obniżonego klirensu wątrobowego. Sekrecja insuliny przez trzustkę może być oceniania na bazie pomiaru stężenia C-peptydu w serum, ponieważ białko to jest uwalniane równolegle z insuliną. Podczas gdy około 70% wydzielonej insuliny jest przechwytywane i metabolizowane w wątrobie, C-peptyd pozostaje w krwiobiegu, tym samym stosunek C-peptydu do insuliny dostarcza informacji o klirensie wątrobowym insuliny. U koni otyłych zaobserwowano wysokie stężenia insuliny oraz C-peptydu, jednak stosunek C-peptydu do insuliny był obniżony, sugerując tym samym podwyższoną sekrecję insuliny i jej obniżony klirens wątrobowy [36]. Ponadto, u koni z EMS zaobserwowano podwyższony poziom enzymów wątrobowych, aminotransferazy asparaginowej (AST), aminotransferazy alaninowej (ALT) oraz γ- glutamylotranspeptydazy (GGT) [37]. Zarówno u ludzi jak w modelach zwierzęcych obserwowano korelację między podwyższoną wątrobową lipogenezą de novo a hiperinsulinemią i insulinoopornością [38]. Podwyższony poziom trójglicerydów w osoczu został również stwierdzony u kucyków walijskich z EMS [39]. Co więcej, niektóre badania wskazują również na podwyższony poziom wolnych kwasów tłuszczowych u insulinoopornych otyłych koni [40]. Wyniki dotyczące podwyższonego poziomu wolnych kwasów tłuszczowych są jednak rozbieżne i nie posiadały wartości różnicowej podczas diagnozowania fenotypu syndromu metabolicznego koni [39–41]. Jedno badanie wskazało również na podwyższony poziom lipoprotein o bardzo niskiej gęstości (ang. *VLDL- very low density lipoprotein*) u koni z EMS [40]. W 2009 roku Międzynarodowa Federacja Cukrzycy przedstawiła nowe wytyczne w celu usprawnienia diagnostyki syndromu metabolicznego u ludzi. Aby pacjent został zakwalifikowany jako cierpiący na syndrom metaboliczny, musiał spełniać co najmniej 3 z 5 kryteriów. Kryteria te to: otyłość brzuszna, podwyższony poziom triglicerydów, obniżone stężenie HDL-c, podwyższone stężenie glukozy na czczo oraz podwyższony poziom ciśnienia krwi. W badaniu PAMELA (*Pressioni Arteriose Monitorate E Loro Associazioni*), podwyższone ciśnienie krwi występowało aż u 80% pacjentów z syndromem metabolicznym. Syndrom metaboliczny był również związany z podwyższonym ryzykiem występowania chorób układu krążenia (*CVD – cardiovascular disease*), zawału mięśnia sercowego (*MI-myocardial infraction*) oraz udaru [42]. Konie z syndromem metabolicznym są jednak mniej

podatne na rozwój powikłań układu sercowo-naczyniowego, ze względu na różnice w diecie, metabolizmie lipoprotein oraz długości życia [43]. Nie mniej jednak, podczas lata kucyki ze skłonnością do ochwatu oraz insulinoopornością wykazywały znacznie podwyższone ciśnienie krwi, podwyższony poziom insuliny oraz triglicerydów w osoczu [44]. Co więcej, u kucy z EMS stwierdzono przerost mięśnia sercowego, który był skorelowany z odpowiedzią na insulinę podczas doustnego testu obciążenia glukozą oraz z ciśnieniem krwi [43].

Jednym z najistotniejszych powikłań towarzyszących syndromowi metabolicznemu koni jest ochwat. Ochwat to śmiertelna choroba koni, związana z obniżoną jakością życia, wyniszczającym bólem oraz kulawiznami [45]. Różne badania wskazują na częstotliwość występowania tego schorzenia między 1,5% do 34% populacji lub 1,5% do 23,8% [46]. Podczas ochwatu dochodzi do niedokrwienia tkanek palca, na skutek działania mediatorów naczynioaktywnych (endotelina, histamina) oraz równoczesnej wymiany tlenu między naczyniami doprowadzającymi i odprowadzającymi, poprzez anastomozy tętniczo-żylne. Rozwijająca się hipoksja doprowadza do uszkodzenia i śmierci komórek skóry oraz naskórka w aparacie zawieszającym kość kopytową. Badania histologiczne wskazują natomiast na rozpad jąder komórkowych w naskórku oraz odłączenie błony podstawnej, która stanowi połączenie między tworzywem kopyta a naskórkiem [47]. Wskazuje się również na udział metaloproteinaz, które w warunkach fizjologicznych oraz patologicznych odpowiadają za degradację macierzy zewnątrzkomórkowej (ECM; ang. *extracellular matrix*)[48,49]. Opisane zmiany stanowią początek ochwatu, następnie dochodzi do rozwoju procesu zapalnego, któremu towarzyszy przekrwienie, agregacja erytrocytów, nieznaczna neutrofilia, apoptoza komórek oraz zwiększona perfuzja naczyniowa. Na skutek tych procesów w puszce kopytowej rozwija się obrzęk. W przebiegu ochwatu można wyszczególnić następujące stadia: bezobjawowe stadium wstępne, stadium nadostre, ostre, podostre i przewlekłe [47,50]. Szacuje się, że faza wstępna ochwatu trwa od 24 do 60 godzin, a podczas jej trwania nie stwierdza się żadnych objawów klinicznych oraz zmian radiograficznych [51,52]. Ochwat ostry trwa od 24 do 72 godzin, lecz okres ten może wydłużyć się nawet do 7 dni. W fazie ostrej ochwatu obserwuje się podwyższoną temperaturę puszki kopytowej, wzmożony puls wyczuwalny na tętnicach palcowych, kulawiznę oraz przenoszenie ciężaru na niebolesną kończynę [51]. Na tym etapie proces zapalny może ulec wyciszeniu lub dochodzi do dalszego rozwoju choroby w formę podostrą lub przewlekłą. W formie podostrej nie obserwuje się zerwania aparatu zawieszającego kość kopytową, natomiast w formie przewlekłej obserwuje się obniżenie oraz rotację kości kopytowej. Gdy proces chorobowy trwa ponad 3 dni, ochwat klasyfikuje się jako formę przewlekłą, w której można zaobserwować zmiany radiograficzne. W przypadku formy

nadostrej schorzenia może dojść do zzucia puszki kopytowej [47]. Ochwat może także wystąpić na skutek sepsy, chorób gastroenterologicznych, operacji na jamie brzusznej, endotoksemii, zapalenia płuc, stanu zapalnego macicy, nadmiernej podaży węglowodanów czy w wyniku bolesnych chorób lub urazów kończyny przeciwległej. Najnowsze badania wskazują jednak, że najczęstszą forma ochwatu jest ochwat o podłożu hormonalnym [53,54]. Bazując na danych z USA oraz Europy, endokrynopatie występują aż u 90% przypadków ochwatu [55,56]. Z tego względu obok choroby Cushinga, syndrom metaboliczny koni jest drugim schorzeniem leżącym u podstawy większości przypadków ochwatu u koni. W wielu przypadkach ochwat ma tendencję do nawracania, niestety często prowadzi również do eutanazji zwierzęcia. Mimo postępów w dziedzinie medycyny weterynaryjnej wciąż brakuje w pełni skutecznych metod terapeutycznych w leczeniu tego schorzenia. Niektórzy badacze proponowali terapie oparte o komórki macierzyste oraz osocze bogatopłytkowe. Co ciekawe wynik takiej terapii był obiecujący [57]. Zainteresowanie komórkami macierzystymi, pod względem ich udziału w patofizjologii syndromu metabolicznego koni oraz ochwatu, a także ze względu na ich znaczny potencjał terapeutyczny znacznie wzrosło w ostatnich latach [57–62]. Rozwój terapii opartych na komórkach macierzystych jest bezpośrednią odpowiedzią na potrzebę spersonalizowanych i skutecznych metod terapeutycznych i diagnostycznych w różnych schorzeniach ludzi oraz zwierząt [63–65]. Podstawowym założeniem terapii z zastosowaniem różnych populacji komórek macierzystych jest nasilenie procesów naprawczych w komórkach, tkankach oraz organach pacjentów, poprzez przywrócenie homeostazy [64,65]. Dotychczas w różnych badaniach klinicznych przebadano i zastosowano indukowane pluripotencjalne komórki macierzyste (IPSCs- ang. *Induced Pluripotent Stem Cells*) oraz mezenchymalne komórki macierzyste (MSCs- ang. *Mesenchymal Stem Cells*). Ze względu na brak zastrzeżeń dotyczących etyki, a także względnie prosty proces izolacji oraz hodowli, mezenchymalne komórki macierzyste są szeroko stosowane, zarówno w medycynie ludzkiej jak i weterynaryjnej [63,64]. Mogą one być izolowane z różnych tkanek takich jak szpik kostny, tkanka tłuszczowa, łożysko oraz pępowina. Różne badania wykazały specyficzne cechy molekularne oraz potencjał terapeutyczny mezenchymalnych komórek macierzystych, w zależności od tkanki źródłowej, z której zostały wyizolowane. Z tego względu skuteczność terapii komórkowych może być bezpośrednio zależna od właściwości danej populacji komórek macierzystych oraz ich unikalnego sekretomu. Kluczowym wydaje się więc być zatem dobranie odpowiedniej populacji do danego schorzenia [66]. Co ważne, pochodzenie komórek macierzystych, a także status zdrowotny dawcy, od którego zostały pobrane odgrywa znaczną rolę w ich późniejszym potencjale terapeutycznym [67]. Badania wskazują także, że wiele

różnych schorzeń prowadzi do upośledzenia fizjologii oraz funkcji komórek macierzystych, co w następstwie przyczynia się do dalszego rozwoju choroby [68–71]. Przykładowo, rozległe badania zostały przeprowadzone na komórkach ASC, w celu oceny wpływu syndromu metabolicznego koni na ich fizjologię oraz potencjał terapeutyczny [37,60,61,72,73]. Komórki ASC stanowią bardzo obiecującą populację komórek macierzystych, jako że są izolowane z tkanki tłuszczowej, która jest łatwo dostępna [74]. Były one używane w różnych celach terapeutycznych, mogą służyć między innymi do regeneracji kości, usprawnienia procesu gojenia ran oraz leczenia schorzeń neurologicznych [64,74]. Badania wykazały jednak, że komórki ASC izolowane od koni chorujących na syndrom metaboliczny posiadały obniżony potencjał do proliferacji, obniżoną żywotność, obniżony potencjał klonogenny, cechy starzenia, podwyższoną akumulację czynników stresu oksydacyjnego oraz nasiloną apoptozę [75]. Nie ma więc wątpliwości, że dalsze badania w celu pełniejszego zrozumienia patofizjologii syndromu metabolicznego koni oraz ochwatu i wpływu tych schorzeń na populacje komórek macierzystych a także poszukiwania nowych metod terapeutycznych i diagnostycznych są konieczne. Obecnie, nowa grupa relatywnie nowo odkrytych biomarkerów jest przedmiotem wielu badań dotyczących insulinooporności, syndromu metabolicznego, a także innych schorzeń. Mowa tutaj o niekodujących RNA, takich jak miRNA oraz lncRNA. W przebiegu insulinooporności, cukrzycy typu 2 oraz syndromu metabolicznego zaobserwowano deregulację licznych niekodujących RNA, co biorąc pod uwagę funkcję regulacyjną tych cząsteczek jest w pełni zrozumiałe [76,77]. miRNA to duża grupa małych (15-22 nukleotydów), nie-kodujących sekwencji o strukturze „spinki do włosów", wysoce zachowanych między różnymi gatunkami. Od momentu ich odkrycia, ich biogeneza, aktywność i funkcja stanowiły temat wielu badań, jednak ich znaczenie było długo pomijane, ze względu na ograniczenia możliwości technologicznych metod badawczych. Dopiero odkrycie dwóch miRNA, *lin-4* oraz *let-7*, które kontrolują przebieg rozwoju nicienia *Caenorbabditis elegans* pozwoliło na rozpoznanie kluczowej funkcji miRNA w regulacji ekspresji genów [78]. Ekspresja miRNA jest zazwyczaj wysoce specyficzna tkankowo i regulowana na poziomie transkrypcji. Sekwencje miRNA mogą być zlokalizowane między sekwencjami kodującymi białka lub w sekwencjach kodujących, a ich transkrypcja jest przeprowadzana przez polimerazę RNA. Główny mechanizm działania cząsteczek miRNA polega na potranskrypcyjnej regulacji ekspresji genów, co jest możliwe dzięki komplementarności par zasad z cząsteczkami informacyjnego RNA [79]. Potranskrypcyjna regulacja ekspresji mRNA opiera się na destabilizacji lub represji transkryptu [80]. Sprawia to, że miRNA stanowią część praktycznie każdego procesu komórkowego - od rozwoju poprzez różnicowanie, metabolizm, aktywność komórkową oraz apoptozę. Jedno miRNA może

regulować ekspresję wielu genów, natomiast jeden gen może podlegać regulacji przez wiele różnych miRNA, co wyraźnie podkreśla kluczową rolę miRNA w regulacji procesów komórkowych. W szczególności, ze względu na fakt, że całe ścieżki sygnałowe nierzadko mogą podlegać regulacji przez grupę miRNA lub przez pojedyncze miRNA [80]. Rola oraz funkcje niedawno odkrytych lncRNA, nie są jeszcze w pełni zdefiniowane, jednak wzajemna regulacja między miRNA oraz lncRNA jasno wskazuje, że lncRNA wpływają znacząco na aktywność i działanie miRNA. Podobnie jak w przypadku miRNA, ich udział w regulacji różnych procesów biologicznych jest znaczący. Wykazano, ze biorą udział w imprintingu genomowym, allosterycznej regulacji aktywności enzymów oraz w konformacji chromosomów [81,82]. Złożone sieci regulacyjne lncRNA zostały opisane w wielu mechanizmach komórkowych, a ich dysregulacja została opisana w różnych schorzeniach takich jak cukrzycowa choroba nerek, rak jelita grubego, choroba Parkinsona czy choroby układu krążenia [83–87]. lncRNA mogą być ogólnie skategoryzowane w trzy obszerne grupy, w oparciu o ich funkcję. Pierwsza grupa stanowi niefunkcjonalne lncRNA, które najprawdopodobniej są produktem szumu transkrypcyjnego. Druga grupa składa się lncRNA, których transkrypty mają mniejsze znaczenie i sam proces transkrypcji jest wystarczający dla ich funkcji. Trzecią grupę stanowią natomiast funkcjonalne lncRNA, które posiadają zdolność działania w orientacji *cis* i/lub *trans* [88]. W niektórych aspektach lncRNA są podobne do mRNA, ponieważ m. in. podlegają transkrypcji przez II polimerazę, często posiadają czapeczkę-5′, podlegają splicingowi oraz poliadenylacji. Spośród szerokiego zakresu funkcji lncRNA, poosiadają one również zdolność interakcji z miRNA. Wykazano, że niektóre miRNA mogą obniżać stabilność cząsteczek lncRNA. Co więcej, lncRNA mogą regulować poziom miRNA w cytoplazmie poprzez wiązanie specyficznych miRNA. Ponadto lncRNA mogą również konkurować z miRNA o miejsca wiązania w mRNA [89–91]. Wraz z odkryciem niekodujących RNA została poznana całkowicie nowa płaszczyzna mechanizmów molekularnych. Ich funkcja w regulacji ekspresji genów sprawia, że stanowią one część każdego procesu komórkowego, a ich dysregulacja została zaobserwowana w wielu różnych schorzeniach. Zarówno miRNA jak i lncRNA zostały rozpoznane jako uniwersalne biomarkery w chorobach takich jak Alzheimer [92], rak piersi [93,94], rak żołądka [95], choroby układu sercowo-naczyniowego [96], osteoporoza postmenopauzalna [97] i wiele innych. Ze względu na swoją funkcję regulacyjną, miRNA oraz lncRNA są postrzegane jako potencjalne cele terapeutyczne. Dodatkowo, ze względu na ich obecność w krwiobiegu istnieje możliwość wykorzystania tych cząsteczek jako precyzyjnych i specyficznych biomarkerów.

## 2. Cel pracy

Celem badań w ramach realizowanej pracy doktorskiej było wyselekcjonowanie potencjalnych markerów miRNA oraz lncRNA, mogących służyć do wczesnej diagnostyki inulinooporności wątrobowej. Dodatkowo, celem pracy było opracowanie protokołu izolacji oraz charakterystyka komórek progenitorowych korony rogotwórczej kopyta HPC, ze względu na ich możliwy udział w patofizjologii ochwatu oraz potencjał terapeutyczny.

Jako szczegółowe cele przedstawionej pracy można wyróżnić:

1. Wybór spośród miRNA oraz lncRNA najbardziej obiecujących biomarkerów insulinooporności wątrobowej na bazie analizy literaturowej.
2. Opracowanie protokołu procesu izolacji komórek HPC z okolic koronki kopyt końskich.
3. Charakterystyka morfologii, proliferacji, różnicowania, stresu oksydacyjnego, metabolizmu mitochondrialnego, odpowiedzi zapalnej, apoptozy oraz unikalnego sekretomu miRNA komórek HPC w porównaniu do modelu komórek ASC.
4. Ocena reakcji komórek HPC na środowisko prozaplane, ze względu na ich potencjalny udział w patofizjologii ochwatu.
5. Analiza proliferacji, stresu oksydacyjnego, metabolizmu mitochondrialnego, odpowiedzi zapalnej, apoptozy, ścieżki sygnałowej insuliny oraz unikalnego sekretomu miRNA komórek HPC w warunkach hiperinsulinemii w porównaniu do komórek ASC.

## 3. Hipoteza badawcza

Główna hipoteza badawcza przedstawionej rozprawy doktorskiej zakładała, że miRNA oraz lncRNA mogą być wykorzystane jako specyficzne i precyzyjne biomarkery schorzeń powiązanych z insulinoopornością, a w szczególności mogą służyć jako wartościowe markery we wczesnej diagnostyce insulinooporności wątrobowej. Ponadto, założono, że komórki macierzyste korony rogotwórczej kopyta HPC biorą udział w patofizjologii ochwatu oraz, że analiza ich reakcji na proces zapalny oraz hiperinsulinemię pod względem ich unikalnego sekretomu miRNA może dostarczyć wartościowych informacji dotyczących przebiegu ochwatu, jego potencjalnej diagnostyki oraz terapii.

**4.1. Komentarz do publikacji: Pielok Ariadna, Marycz Krzysztof: Non-Coding RNAs as Potential Novel Biomarkers for Early Diagnosis of Hepatic Insulin Resistance, International Journal of Molecular Sciences, 2020, vol. 21, nr 11, s.1-19, Numer artykułu:4182. DOI:10.3390/ijms21114182**

W ostatnich latach prewalencja schorzeń metabolicznych takich jak cukrzyca typu 2 czy syndrom metaboliczny stale wzrasta. Upośledzenie metabolizmu wątrobowego, skutkujące insulinoopornością wątrobową jest zarazem powszechnym symptomem, jak również kluczowym etapem w rozwoju cukrzycy typu 2 oraz syndromu metabolicznego. Wątroba, jako centrum metaboliczne organizmu, odgrywa kluczową rolę w utrzymywaniu homeostazy glukozy w organizmie. W przypadku cukrzycy typu 2 oraz syndromu metabolicznego insulinooporność wątrobowa jest często możliwa do wykrycia zanim pojawią się pozostałe objawy. Z tego względu opracowanie metod diagnostycznych, umożliwiających wczesną diagnozę insulinooporności wątrobowej pozwoliłoby na wdrożenie odpowiedniego leczenia zanim rozwiną się pozostałe symptomy oraz komplikacje związane ze wspomnianymi schorzeniami. Niekodujące RNA takie jak miRNA oraz lncRNA są postrzegane jako nowe, obiecujące biomarkery oraz cele terapeutyczne, szczególnie ze względu na ich funkcję regulacyjną. Dysregulacja miRNA oraz lncRNA była obserwowana u pacjentów insulinoopornych. Znaczna ilość tych transkryptów bierze udział w regulacji ścieżki sygnałowej insuliny w wątrobie. Celem artykułu przeglądowego była selekcja najbardziej obiecujących transkryptów miRNA oraz lncRNA pod względem ich potencjału we wczesnej diagnostyce insulinooporności wątrobowej. Wyselekcjonowanych zostało 6 transkryptów: 3 miRNA (miR-802, miR-499-5p, and miR-122) oraz 3 lncRNA (H19, MEG3, MALAT1), ze względu na ich wyraźnie zmieniony poziom w krwioobiegu u pacjentów w stanie przedcukrzycowym, a także u pacjentów z cukrzycą typu 2 oraz syndromem metabolicznym.

Badania opisane w pracy przeglądowej wykazały, że miR-499-5p bierze udział w regulacji ekspresji PTEN (ang. *Phosphatase and Tensin Homolog*), podczas gdy miR-802 oraz lnc MEG3 uczestniczą w regulacji procesu glukoneogenezy. Niektóre niekodujące RNA mogą także regulować więcej niż jeden etap szlaku sygnałowego insuliny, przykładowo miR-122, lnc MALAT1 oraz lnc H19 wpływają na proces wątrobowej lipognezy de novo. Ponadto, w artykule przedstawiona została rola wspomnianych niekodujących RNA w insulinooporności wątrobowej, a także ich aktywność regulacyjna w ścieżce sygnałowej insuliny w wątrobie. Dodatkowo, na bazie dostępnej literatury przeanalizowana została wartość prognostyczna wybranych transkryptów.

**4.2. Komentarz do publikacji: Marycz Krzysztof, Pielok Ariadna, Kornicka-Garbowska Katarzyna: Equine Hoof Stem Progenitor Cells (HPC) CD29 + /Nestin + /K15 + – a Novel Dermal/epidermal Stem Cell Population With a Potential Critical Role for Laminitis Treatment, Stem Cell Reviews and Reports, 2021, vol. 17, nr 4, s.1478-1485. DOI:10.1007/s12015-021-10187-x**

Ochwat to poważna oraz niezwykle bolesna choroba kopyt koni. Schorzenie to ma tendencję do nawracania i niestety, często wymaga eutanazji zwierzęcia. Jest konsekwencją niedokrwienia tkanek palca, co może skutkować zmianami w obrębie aparatu zawieszającego kość kopytową, a także jej oddzieleniem od puszki kopytowej. Patofizjologia tego schorzenia wciąż nie jest jednak w pełni poznana, głównie ze względu na jej wieloczynnikowy charakter. W świetle przedstawionych danych, możliwe, że nowa populacja komórek macierzystych bierze udział w regeneracji tkanek uszkodzonych w przebiegu ochwatu. W przedstawionej publikacji zaprezentowano proces izolacji oraz charakterystykę komórek HPC wyizolowanych z korony rogotwórczej kopyt końskich. Fenotyp komórek został zbadany przy użyciu cytometrii przepływowej oraz metody RT-qPCR. Wyniki cytometrii przepływowej wykazały, ze komórki HPC wykazują ekspresję dwóch markerów powierzchniowych, Nestin oraz CD29. Analiza RT-qPCR ujawniła, ze komórki HPC charakteryzują się również wysoką ekspresją markerów komórek progenitorowych, takich jak SOX2, OCT4 oraz NANOG. Morfologia komórek HPC została zbadana przy użyciu mikroskopii świetlnej, konfokalnej oraz elektronowej. Wizualizacja komórek wykazała, że komórki HPC są wrzecionowatego kształtu, z centralnie położonym jądrem komórkowym oraz wydłużonymi mitochondriami. Plastyczność komórek HPC potwierdzono poprzez przeprowadzenie procesu różnicowania w kierunku osteoblastów oraz chondroblastów. Uzyskane wyniki wykazały, że komórki HPC cechują się wysoką proliferacją in vitro oraz plastycznością podobną do macierzystych komórek mezenchymalnych. Podejrzewamy, że ze względu na swoją lokalizację komórki HPC mogą brać udział w patofizjologii ochwatu, tym samym mogą być użyteczne w dalszych badaniach dotyczących ochwatu oraz nowych metod terapeutycznych dla tego schorzenia, również takich, które oparte są o zastosowanie komórek macierzystych.

**4.3. Komentarz do publikacji: Pielok Ariadna, Kępska Martyna, Steczkiewicz Zofia, Grobosz Sylwia, Bourebaba Lynda, Marycz Krzysztof: Equine Hoof Progenitor Cells Display Increased Mitochondrial Metabolism and Adaptive Potential to a Highly Pro-Inflammatory Microenvironment, International Journal of Molecular Sciences, 2023, vol. 24, nr 14, s.1-25, Numer artykułu:11446.DOI:10.3390/ijms241411446**

Mezenchymalne komórki macierzyste wykazują unikalny fenotyp molekularny oraz możliwości biologiczne w zależności od tkanki, z której zostały wyizolowane. W poprzednim artykule opisany został proces izolacji oraz wstępna charakterystyka i fenotyp nowej populacji komórek macierzystych, rezydujących w koronie rogotwórczej kopyt końskich – komórki HPC, które mogą posiadać obiecujący potencjał pod względem leczenia ochwatu u koni. Celem przeprowadzonych badań było porównanie populacji komórek HPC z dobrze poznanymi oraz scharakteryzowanymi komórkami ASC. Obie populacje komórkowe były utrzymywane i porównane w standardowych warunkach hodowli oraz poddawane primingowi z użyciem koktajlu cytokinowego w celu stworzenia warunków prozapalnych, pozwalających na odtworzenie środowiska ochwatu. Komórki zostały zbadane pod kątem ekspresji kluczowych markerów regulujących fenotyp, metabolizm, dynamikę mithochondrialną, stres oksydacyjny, apoptozę oraz immunomodulację przy użyciu metody RT-qPCR. Morfologia badanych komórek została oceniona z zastosowaniem barwienia immunofluorescencyjnego. Oceniona została również ich zdolność do migracji, przy użyciu barwienia z pararozaliną. Do oceny cyklu komórkowego, potencjału błony mitochondrialnej oraz stresu oksydacyjnego została wykorzystana metoda cytometrii mikrokapilarnej. Uzyskane wyniki pokazały, że w warunkach standardowych komórki HPC wykazywały podobną morfologię do komórek ASC, ale charakteryzował je odmienny fenotyp. Komórki HPC w standardowych warunkach hodowlanych posiadały niższą zdolność migracji oraz inny rozkład procentowy komórek w cyklu komórkowym. Dodatkowo, w warunkach standardowych, komórki HPC wykazywały odmienną dynamikę mitochondrialną oraz poziom stresu oksydacyjnego. W środowisku prozapalnym komórki HPC wykazywały inne poziomy ekspresji markerów apoptozy oraz immunomodulacji w porównaniu do ASC. Co ważne, komórki HPC charakteryzowały się również odmienną ekspresją wybranych miRNA. W warunkach prozapalnych, zaobserwowano, że komórki HPC wykazują różnice względem komórek ASC w ekspresji markerów dynamiki mitochondrialnej, jednak ekspresja markerów apoptozy oraz immunomodulacji była podobna w obu populacjach. W warunkach prozapalnych ekspresja wybranych markerów miRNA była znacznie obniżona w komórkach HPC, co wskazuje na ich

potencjalny udział w patofizjologii ochwatu a także na ich możliwe wykorzystanie diagnostyczne.

### 4.4. Komentarz do publikacji: Pielok Ariadna, Króliczewski Jarosław, Kępska Martyna, Marycz Krzysztof: A comparative Study of Equine Hoof Progenitor Cells and Adipose-Derived Stem Cells in Hyperinsulinemia.

Najbardziej powszechną formą ochwatu jest ochwat o podłożu hormonalnym, często towarzyszący syndromowi metabolicznemu koni. Syndrom metaboliczny objawia się insulinoopornością, hiperinsulinemią, otyłością regionalną, uogólnionym procesem zapalnym, nadciśnieniem tętniczym, zaburzeniami metabolizmu wątrobowego oraz dysregulacją adipokin. W ostatnich latach terapie oparte o komórki macierzyste są coraz powszechniej stosowane zarówno w medycynie ludzkiej jak i weterynaryjnej. Komórki mezenchymalne wykazują unikalny fenotyp molekularny oraz potencjał terapeutyczny, w zależności od tkanki, z której zostały wyizolowane. Ostatnio, wyizolowaliśmy oraz scharakteryzowaliśmy nową populację macierzystych komórek mezenchymalnych, komórki HPC pochodzące z korony rogotwórczej kopyt końskich. Komórki te, ze względu na swoją lokalizację mogą być zaangażowane patofizjologię ochwatu oraz w proces regeneracji skóry, naskórka oraz uszkodzonych naczyń krwionośnych.

W przedstawionej pracy porównano komórki HPC do dobrze scharakteryzowanego modelu komórek ASC, w standardowych warunkach hodowli oraz w warunkach imitujących hiperinsulinemię. Przeanalizowano proliferację, metabolizm oraz dynamikę mitochondrialną, stres oksydacyjny, apoptozę, zapalenie, ścieżkę insulinową oraz ekspresję wybranego panelu miRNA (miR-21-5p, miR-27a, miR-30c-5p, miR-34a-5p, miR34c, miR-96-5p, miR-125a, miR-125b-5p, miR-218, miR-451). Proliferacja, potencjał błony mitochondrialnej oraz stres oksydacyjny zostały zbadane w oparciu o techniki cytometrii mikrokapilarnej. Dodatkowo, metabolizm oraz dynamika mitochondrialna, stres oksydacyjny, apoptoza, zapalenie, ścieżka insulinowa oraz ekspresja miRNA zostały poddane analizie z wykorzystaniem metody RT-qPCR.

Wyniki przeprowadzonych badań wykazały, że w standardowych warunkach hodowli komórki HPC charakteryzują się odmiennym potencjałem proliferacyjnym oraz odmiennym potencjałem błony mitochondrialnej. Co więcej, poziom stresu oksydacyjnego również różnił się między badanymi populacjami komórek w standardowych warunkach hodowli. Ekspresja markerów związanych z metabolizmem oraz dynamiką mitochondrialną, apoptozą, zapaleniem,

ścieżką insulinową oraz ekspresja miRNA różniła się między populacjami HPC oraz ASC w standardowych warunkach hodowlanych. Co więcej, zarówno komórki HPC oraz ASC wykazały podobną dynamikę w reakcji na hiperinsulinemię pod względem proliferacji, potencjału błony mitochondrialnej, stresu oksydacyjnego, metabolizmu oraz dynamiki mitochondrialnej, apoptozy, zapalenia oraz ścieżki insulinowej. Ostatecznie, pod względem ekspresji miRNA komórki HPC wykazały jednak unikalną odpowiedź i ekspresja wszystkich badanych markerów obniżyła się w odpowiedzi na hiperinsulinemię.

Uzyskane wyniki badań sugerują, że komórki HPC oraz komórki ASC wykazują podobną dynamikę oraz odpowiedź w warunkach hiperinsulinemii, jednak komórki HPC charakteryzuje unikalny wzór ekspresji wybranego panelu miRNA. Tym samym, możliwe, że badane miRNA mogą stanowić istotny czynnik diagnostyczny w przebiegu ochwatu i wskazane są dalsze badania, w celu określenia ich unikalnego wpływu na patofizjologię tego schorzenia.

## 5. Dyskusja

Schorzenia związane z insulinoopornością takie jak cukrzyca typu 2, syndrom metaboliczny, niealkoholowe stłuszczenie wątroby oraz syndrom metaboliczny koni stanowią ogromne wyzwanie dla medycyny ludzkiej oraz weterynaryjnej. W ostatnich latach, znacznie wzrastająca prewalencja tych chorób powoduje, że ciągle poszukuje się nowych oraz skuteczniejszych metod diagnostycznych oraz terapeutycznych. Wczesna diagnoza insulinoopornosci pozwala uchronienić pacjentów przed rozwojem licznych komplikacji. Analiza najnowszych doniesień naukowych oraz badania przeprowadzone w ramach prezentowanej rozprawy wskazują, że niekodujące RNA mogą stanowić nową pulę specyficznych i precyzyjnych biomarkerów. Nie można pominąć również ich potencjału terapeutycznego w schorzeniach związanych z insulinoopornością.

Analiza dotychczasowych doniesień literaturowych wykazała, że miRNA oraz lncRNA mogą służyć jako markery insulinooporności wątrobowej, która jest znaczącym komponentem schorzeń o podłożu metabolicznym. Wybrano 6 niekodujących RNA, bazując ich funkcji regulacyjnej w metabolizmie wątrobowym, szlaku sygnałowym insuliny, a także ze względu na korelację między poziomem ich ekspresji w wątrobie i w krwiobiegu w toku insulinooporności wątrobowej. Podwyższona ekspresja miR-802, powiązana z otyłością oraz insulinoopornością, została zaobserwowana w różnych tkankach, takich jak nerki, biała tkanka tłuszczowa, mięśnie szkieletowe oraz wątroba [98]. Co więcej, wykazano również związek między podwyższoną ekspresją miR-802 a stresem oksydacyjnym oraz insulinoopornością wątrobową [99]. Zaobserwowano także, że podwyższona ekspresja miR-802 powiązana jest z regulacją procesu glukoneogenezy, a poziom miR-802 w krwiobiegu pacjentów z cukrzycą typu 2 był znacznie podwyższony [98,100]. Badania wykazały, że miR-499 ma kluczowe znaczenie w regulacji metabolizmu glukozy w wątrobie oraz w rozwoju insulinooporności wątrobowej, poprzez regulację ekspresji PTEN [101]. Dodatkowo, wykazano związek pomiędzy poziomem ekspresji miR-499 a rozwojem niealkoholowego stłuszczenia wątroby [102]. Obserwacje przeprowadzone na pacjentach ujawniły, że poziom miR-499 w erytrocytach był znacznie obniżony u pacjentów w stanie przedcukrzycowym [103]. miR-122 jest wysoce specyficznym miRNA dla wątroby i stanowi około 52% wątrobowego miRNA. Odgrywa on kluczową rolę w regulacji rozwoju hepatocytów oraz w regulacji ekspresji genów wątroby, przez co bierze udział w różnych procesach komórkowych, związanych np. ze stresem oksydacyjnym czy zapaleniem [104–108]. Badania wskazywały również na udział miR-122 w regulacji metabolizmu lipidowego wątroby. Zaobserwowano również, że dysregulacja ekspresji miR-122 towarzyszy

insulinooporności wątrobowej [109,110]. Najnowsze doniesienia wskazują także na wysoką wartość prognostyczną miR-122. Wykazano, że poziom miR-122 w krążeniu był znacznie wyższy u pacjentów z insulinoopornością [111]. Najnowsze badania wykazały, że lncRNA MALAT1 ma kluczowy wpływ na metabolizm glukozy oraz lipidów, a podwyższoną ekspresję MALAT1 zaobserwowano w różnych modelach cukrzycy [112]. Dodatkowo wykazano, że poprzez regulację ekspresji SREBP-1c, MALAT1 odgrywa znaczną rolę w lipogenezie de nowo, toteż podwyższona ekspresja MALAT1 koreluje z stłuszczeniem wątroby oraz insulinoopornością wątrobową [113]. Co więcej, zaobserwowano, że poziom MALAT1 w osoczu pacjentów z cukrzycą ciążową był znacznie podwyższony [114]. Udział lncRNA MEG3 został wykazany w regulacji szlaku sygnałowego insuliny poprzez wpływ na ekspresję FOXO1 [115,116]. Podwyższona ekspresja MEG3 została również zaobserwowana w dwóch modelach eksperymentalnych insulinooporności wątrobowej [117]. MEG3 posiada również potencjał diagnostyczny, jako że jego nadekspresja wykazano w komórkach PBMC (ang. *peripheral blood mononuclear cell*) pacjentów z cukrzycą typu 2 [118]. Rozbieżne odkrycia zostały opublikowane na temat poziomu ekspresji lncRNA H19 oraz jego wpływu na metabolizm wątrobowy. Nie pozostawia jednak wątpliwości fakt, że lncRNA H19 odgrywa kluczową rolę w metabolizmie wątrobowym, poprzez regulację procesu glukoneogenezy oraz lipogenezy [119-121]. Dodatkowo, znacznie podwyższona ekspresja lncRNA H19 została zaobserwowana u pacjentów z cukrzycą typu 2 [122]. Analiza najnowszych doniesień naukowych jasno wskazuje, że niekodujące RNA, takie jak miRNA oraz lncRNA mogą stanowić wartościowe markery diagnostyczne przy wykrywaniu insulinooporności oraz towarzyszących jej schorzeń metabolicznych. Wiele badań wskazuje także, że w celu maksymalizacji potencjału diagnostycznego niekodujących RNA, korzystnym podejściem jest ustanowienie panelu składającego się z kilku markerów. Co więcej, poprzez interwencję w poziom ich ekspresji możliwe jest regulowanie rozmaitych procesów metabolicznych, co czyni niekodujące RNA obiecującymi celami terapeutycznymi. Bazując na najnowszych doniesieniach literaturowych, poszukiwanie miRNA, które podlegają dysregulacji w różnych jednostkach chorobowych, może być właściwym kierunkiem w celu ustalenia potencjalnych markerów diagnostycznych oraz celów terapeutycznych.

Ochwat stanowi jedną z najdotkliwszych manifestacji insulinooporności oraz syndromu metabolicznego koni. Schorzenie to jest znacznym wyzwaniem dla lekarzy weterynarii, ze względu na ciężki przebieg oraz tendencję do nawracania, często prowadząc do eutanazji zwierzęcia. Nadal prowadzone są badania mające na celu dokładniejsze poznanie patofizjologii tego schorzenia, a także poszukiwanie nowych, skuteczniejszych metod terapeutycznych.

W leczeniu rozmaitych schorzeń, między innymi również chorób o podłożu metabolicznym obecnie wykorzystuje się różne populacje komórek macierzystych [37,123]. Macierzyste komórki mezenchymalne stanowią unikalną populację komórek, które ze względu na stosunkowo prosty proces izolacji oraz hodowli, mogą być wykorzystywane w terapiach opartych na komórkach macierzystych. Jednak, potencjał terapeutyczny danej populacji komórkowej w dużej mierze zależy od jej fenotypu molekularnego, unikalnego sekretomu oraz tkanki z której dana populacja została wyizolowana [124,125]. W toku badań przeprowadzonych w ramach przedstawionej rozprawy doktorskiej, po raz pierwszy zostały wyizolowane, zidentyfikowane oraz opisane komórki HPC, rezydujące w koronie rogotwórczej kopyta. Komórki te wykazywały wysoki potencjał proliferacyjny, adherencję do podłoża naczynia hodowlanego oraz zdolność różnicowania się w różne typu komórek docelowych, między innymi w osteoblasty oraz chondroblasty. Na bazie analizy przy użyciu cytometrii przepływowej wykazano, że komórki HPC charakteryzują się ekspresja dwóch markerów powierzchniowych: Nestin oraz CD29. Co więcej, poprzez porównanie dwóch populacji komórek MSC, tj. komórek ASC oraz komórek HPC, zaobserwowano, że komórki HPC wykazują wysoką ekspresję genów Nestin, K14, K15, VEGFA, CD200, ANG1, OCT4, SOX2 oraz NANOG. Postawiona hipoteza badawcza zakłada, że ze względu na swoja lokalizację, komórki HPC mogą być zaangażowane w patofizjologię ochwatu, a także, że mogą posiadać potencjał terapeutyczny w leczeniu tego schorzenia. Wysoka ekspresja CD29, Nestin, K15, CD200, VEGFA, and ANG1 może sugerować, że komórki HPC uczestniczą w regeneracji naczyń krwionośnych podczas ochwatu. Zarówno VEGFA oraz ANG1 odgrywają kluczową rolę w indukcji angiogenezy, poprzez aktywację proliferacji komórek endotelialnych, a także promowanie ich migracji i procesu neowaskularyzacji. Kwestie te mogą być kluczowe podczas regeneracji uszkodzeń naczyń krwionośnych wynikających z ochwatu [126]. Co więcej, wysoka ekspresja keratyny 15 (K15) może wskazywać na potencjalna rolę komórek HPC w regeneracji urazów skóry oraz naskórka, które stanowią częsty element w przebiegu ochwatu [127–130]. Dalsze badania miały na celu dokładną analizę morfologii, proliferacji, stresu oksydacyjnego, metabolizmu oraz dynamiki mitochondrialnej, apoptozy, a także odpowiedzi zapalnej komórek HPC w porównaniu do komórek ASC w warunkach standardowych oraz w środowisku prozapalnym. Dodatkowo, wykonano również szczegółową analizę sekretomu miRNA komórek HPC oraz komórek ASC. Badania wykazały, że komórki HPC posiadaj podobną morfologię do komórek ASC, jednak charakteryzuje je niższy potencjał proliferacyjny oraz migracyjny, co sugeruje, że w przypadku użycia komórek HPC do celów terapeutycznych, ich aktywność może być raczej lokalna. Co więcej, komórki HPC wykazywały niższą ekspresję metaloproteinaz w warunkach

standardowych, co sugeruje ich potencjalnie pozytywny wpływ na remodelowanie macierzy zewnątrzkomórkowej, jako że podwyższona ekspresja metaloproteinaz stanowi jeden z komponentów ochwatu. Opublikowana praca jest pierwszym artykułem opisującym unikalny sekretom miRNA w komórkach HPC. W standardowych warunkach hodowli charakteryzowała je wyższa ekspresja miR-21-5p, miR-27-3p, miR-34c and miR-125b-5p w porównaniu do komórek ASC. Ekspresja badanych miRNA w komórkach HPC uległa jednak znacznemu obniżeniu w warunkach prozapalnych, co może wskazywać na potencjał tych niekodujących RNA jako biomarkerów diagnostycznych lub celów terapeutycznych w przebiegu ochwatu. Co więcej, przeprowadzono również analizę porównawczą komórek HPC oraz ASC w warunkach hiperinsulinemii. Uzyskane wyniki badań wskazują, że obie populacje wykazują podobne reakcje na hiperinsulinemię pod względem proliferacji, metabolizmu oraz dynamiki mitochondrialnej czy apoptozy. Komórki HPC charakteryzowały się jednak unikalną odpowiedzią na stres oksydacyjny w warunkach hiperinsulinemii. Co ciekawe, ponownie przeanalizowano panel wybranych 10 miRNA (miR-21-5p, miR-27a, miR-30c-5p, miR-34a-5p, miR34c, miR-96-5p, miR-125a, miR-125b-5p, miR-218, miR-451) i zaobserwowano ich obniżoną ekspresję w komórkach HPC w warunkach hiperinsulinemii. Wyniki te wskazują, że niekodujące RNA, takie jak miRNA oraz lncRNA mogą służyć jako czułe i precyzyjne markery służące do wykrywania insulinooporności wątrobowej, co więcej miRNA mogą również stanowić istotny komponent patofizjologii ochwatu i mogą służyć jako markery oraz potencjalne cele terapeutyczne w przebiegu tego schorzenia.

## 6. Wnioski

Celem analizy najnowszych doniesień lieteraturowych, a także badań przeprowadzonych w ramach przedstawionej pracy doktorskiej była ocena potencjału diagnostycznego oraz terapeutycznego małych niekodujących RNA w przebiegu insulinooporności. Jednym ze schorzeń wynikających z insulinooporności jest syndrom metaboliczny, w którym kluczową rolę odgrywa insulinooporność wątrobowa. Ponadto, jednym z najczęstszych objawów syndromu metabolicznego koni jest ochwat. Na bazie analizy najnowszych doniesień literaturowych, a także w wyniku przeprowadzonych badań wyciągnięto następujące wnioski:

1. Niekodujące RNA (miR-802, miR-499, miR-122, lncRNA MALAT1, lncRNA MEG3, lncRNA H19) stanowią precyzyjne biomarkery insulinooporności wątrobowej oraz schorzeń metabolicznych takich jak cukrzyca typu 2, syndrom metaboliczny oraz niealkoholowe stłuszczenie wątroby.
2. Po raz pierwszy wyizolowana oraz scharakteryzowana populacja komórek HPC spełnia wszystkie kryteria komórek macierzystych ustanowione przez Międzynarodowe Stowarzyszenie Terapii Komórkowej (ang. *ISCT- International Society of Cellular Therapy*).
3. Ze względu na swoją lokalizację oraz fenotyp, komórki HPC mogą odgrywać rolę w regeneracji urazów skóry, naskórka a także uszkodzeń naczyń krwionośnych zachodzących podczas ochwatu w obrębie kopyta.
4. Komórki HPC wykazują podobną morfologię do komórek ASC, ale charakteryzuje je niższy potencjał proliferacyjny oraz migracyjny, co może ograniczać ich potencjał terapeutyczny.
5. Komórki HPC wykazują niższą ekspresję metaloproteinaz oraz wyższą ekspresję proangiogennego VEGFA w porównaniu do komórek ASC, co może wskazywać na ich wyższy potencjał terapeutyczny w przypadku ochwatu, w porównaniu do innych populacji komórek macierzystych.
6. W warunkach prozapalnych, komórki HPC oraz komórki ASC wykazywały obniżoną dynamikę mitochondrialną, jednak w komórkach HPC nie zaobserwowano obniżenia ekspresji genów związanych z metabolizmem mitochondrialnym, co wskazuje na ich potencjał adaptacyjny w warunkach zapalnych.
7. Warunki prozapalne skutkują obniżeniem ekspresji wybranego panelu miRNA (miR-21-5p, miR-27a, miR-30c-5p, miR-34a-5p, miR34c, miR-96-5p, miR-125a, miR-125b-5p, miR-

218, miR-451) w komórkach HPC, co wskazuje na ich możliwą rolę w patofizjologii ochwatu, a także potencjał diagnostyczny oraz terapeutyczny.

8. Wybrany panel miRNA (miR-21-5p, miR-27a, miR-30c-5p, miR-34a-5p, miR34c, miR-96-5p, miR-125a, miR-125b-5p, miR-218, miR-451) może mieć potencjał diagnostyczny, a także zostać obrany jako cel terapeutyczny, ze względu na fakt, że ekspresja tych niekodujących RNA jest obniżona w komórkach HPC w warunkach hiperinsulinemii.
9. Komórki HPC mogą posiadać potencjał w terapii ochwatu, ze względu na lepszy metabolizm oraz dynamikę mitochondrialną a także większą odporność na stres oksydacyjny w porównaniu do komórek ASC.

## 7. Literatura


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**8. Oświadczenia określające udział Autora w publikacjach naukowych będących podstawią przedstawionej rozprawy doktorskiej.**

*Załącznik nr 3 do Trybu postępowania w sprawie nadania stopnia naukowego doktora*

Ariadna Pielok
imię i nazwisko

Wrocław, 22.09.2023r
miejscowość i data

Uniwersytet Przyrodniczy we Wrocławiu,
Wydział Biologii i Hodowli Zwierząt,
Katedra Biologii Eksperymentalnej,
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# OŚWIADCZENIE

Oświadczam, że w pracy: **Pielok Ariadna**, Marycz Krzysztof: Non-Coding RNAs as Potential Novel Biomarkers for Early Diagnosis of Hepatic Insulin Resistance, International Journal of Molecular Sciences, 2020, vol. 21, nr 11, s.1-19, Numer artykułu:4182. DOI:10.3390/ijms21114182 mój udział polegał na wyszukiwaniu oraz analizie literatury, przygotowaniu grafik oraz tabel, przygotowaniu tekstu manuskryptu oraz polemice z recenzentami.

22.09.23, Ariadna Pielok
data i podpis

Potwierdzam treść oświadczenia.

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data i podpis promotora

*Załącznik nr 3 do Trybu postępowania w sprawie nadania stopnia naukowego doktora*

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OŚWIADCZENIE

Oświadczam, że w pracy: Marycz Krzysztof, **Pielok Ariadna**, Kornicka-Garbowska Katarzyna: Equine Hoof Stem Progenitor Cells (HPC) CD29 + /Nestin + /K15 + – a Novel Dermal/epidermal Stem Cell Population With a Potential Critical Role for Laminitis Treatment, Stem Cell Reviews and Reports, 2021, vol. 17, nr 4, s.1478-1485. DOI:10.1007/s12015-021-10187-x mój udział polegał na pozyskaniu materiału badawczego, opracowaniu metody izolacji oraz hodowli komórek HPC (Hoof Progenitor Cells), hodowli komórek HPC, przeprowadzeniu komórek HPC przez proces osteogenezy oraz chondrogenezy, przeprowadzeniu analiz RT-qPCR, przeprowadzeniu analiz z użyciem cytometrii, nadzorze nad prowadzonymi eksperymentami, analizie danych uzyskanych w ramach prowadzonych eksperymentów, przygotowaniu tablic wynikowych, przygotowaniu tekstu manuskryptu (sekcja materiały i metody).

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Podpisane elektronicznie przez Krzysztof Mariusz Marycz (Certyfikat kwalifikowany) w dniu 2023-09-22.
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*Załącznik nr 3 do Trybu postępowania w sprawie nadania stopnia naukowego doktora*

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# OŚWIADCZENIE

Oświadczam, że w pracy: **Pielok Ariadna**, Kępska Martyna, Steczkiewicz Zofia [i in.] : Equine Hoof Progenitor Cells Display Increased Mitochondrial Metabolism and Adaptive Potential to a Highly Pro-Inflammatory Microenvironment, International Journal of Molecular Sciences, 2023, vol. 24, nr 14, s.1-25, Numer artykułu:11446. DOI:10.3390/ijms241411446 mój udział polegał na pozyskaniu materiału badawczego, izolacji badanych komórek HPC (Hoof Progenitor Cells), nadzorze nad prowadzonymi eksperymentami, analizie danych uzyskanych w ramach prowadzonych eksperymentów, przygotowaniu tablic wynikowych, przygotowaniu tekstu manuskryptu oraz polemice z recenzentami.

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Podpisane elektronicznie przez Krzysztof Mariusz Marycz (Certyfikat kwalifikowany) w dniu 2023-09-22.

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# OŚWIADCZENIE

Oświadczam, że w pracy: **Pielok Ariadna**, Jarosław Króliczewski, Kępska Martyna, Marycz Krzysztof: A Comparative Study of Equine Hoof Progenitor Cells and Adipose-Derived Stem Cells in Hyperinsulinemia (praca załączona do serwisu Preprints.org oraz załączona do recenzji w czasopiśmie International Journal of Molecular Sciences), mój udział polegał na pozyskaniu materiału badawczego, izolacji oraz hodowli badanych komórek HPC (Hoof Progenitor Cells), nadzorze nad prowadzonymi eksperymentami, analizie danych uzyskanych w ramach prowadzonych eksperymentów, przygotowaniu tablic wynikowych, przygotowaniu tekstu manuskryptu oraz polemice z recenzentami.

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## 9. Publikacje

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*Review*

# Non-Coding RNAs as Potential Novel Biomarkers for Early Diagnosis of Hepatic Insulin Resistance

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**Abstract:** In the recent years, the prevalence of metabolic conditions such as type 2 Diabetes (T2D) and metabolic syndrome (MetS) raises. The impairment of liver metabolism resulting in hepatic insulin resistance is a common symptom and a critical step in the development of T2D and MetS. The liver plays a crucial role in maintaining glucose homeostasis. Hepatic insulin resistance can often be identified before other symptoms arrive; therefore, establishing methods for its early diagnosis would allow for the implementation of proper treatment in patients before the disease develops. Non-coding RNAs such as miRNAs (micro-RNA) and lncRNAs (long-non-coding RNA) are being recognized as promising novel biomarkers and therapeutic targets—especially due to their regulatory function. The dysregulation of miRNA and lncRNA activity has been reported in the livers of insulin-resistant patients. Many of those transcripts are involved in the regulation of the hepatic insulin signaling cascade. Furthermore, for several miRNAs (miR-802, miR-499-5p, and miR-122) and lncRNAs (H19 imprinted maternally expressed transcript (H19), maternally expressed gene 3 (MEG3), and metastasis associated lung adenocarcinoma transcript 1 (MALAT1)), circulating levels were altered in patients with prediabetes, T2D, and MetS. In the course of this review, the role of the aforementioned ncRNAs in hepatic insulin signaling cascade, as well as their potential application in diagnostics, is discussed. Overall, circulating ncRNAs are precise indicators of hepatic insulin resistance in the development of metabolic diseases and could be applied as early diagnostic and/or therapeutic tools in conditions associated with insulin resistance.

**Keywords:** insulin resistance; hepatic; liver; miRNA; lncRNA; ncRNA; circulating; marker

## 1. Introduction

In recent years, the number of people and animals suffering from conditions correlated with insulin resistance such as type 2 diabetes (T2D) and metabolic syndrome (MetS) has been rapidly rising, making them some of the most burning medical challenges of 21st century. The report of International Diabetes Federation stated that in the year 2000, the global estimate of diabetes prevalence in patients between ages 20 and 79 was 151 million, but this number tripled to a total of 463 million in 2019 [1]. By the year 2045, around 700 million people are predicted to develop diabetes. The estimated number of deaths in 2019 due to diabetes was 4.2 million, which is very alarming, especially when compared to 2016 World Health Organization (WHO) report in which diabetes was stated as a cause of death for 1.6 million patients. The existing data imply that metabolic syndrome is even more common. In most countries in the Asia-Pacific region, nearly one fifth of the adult population has been reported to be affected by MetS [2], while in the United States, the overall prevalence of MetS has fluctuated

from 25.3% in 1988–1994 to 25.0% in 1999–2006, before it finally increased to 34.2% in 2007–2012 [3]. Since MetS is at least three times more common than diabetes, about 25% of world population is estimated to be affected by this disorder—which translates to over a billion people worldwide [4].

One of the main features of both T2D and MetS onset is insulin resistance, defined as an inability of the target tissues to orchestrate well-coordinated glucose-lowering processes such as the suppression of gluconeogenesis, lipolysis, net glycogen synthesis, and cellular glucose uptake in response to physiological insulin levels in plasma [5]. All of the above are a result of impaired insulin signaling at the cellular level. Glucose homeostasis in an organism is a state managed on multiple levels by various regulatory mechanisms. However, it is widely acknowledged that the liver, along with other tissues such as skeletal muscle and white adipose tissue, plays a crucial role in maintaining this balance [6]. The liver is recognized as the metabolic center of an organism, and, as such, it orchestrates the metabolism of carbohydrates, lipids, and proteins. In normal conditions, the liver reacts to insulin levels by facilitating the processes of glycogenesis, glycogenolysis, glycolysis, gluconeogenesis, and lipogenesis during the fasting/feeding state. However, if the hepatic insulin signaling cascade becomes impaired, as happens in the case of insulin resistance, pathologies in hepatic metabolism arise and the liver itself becomes a critical contributor to hyperglycemia, inflammation, and *de novo* lipogenesis [7–9]. Furthermore, mounting evidence suggests that the impairment of hepatic metabolism, resulting in hepatic insulin resistance, is a fundamental step in the development of both conditions [7,10]. The prevalence of nonalcoholic fatty liver disease (NAFLD) among T2D and MetS patients also supports this hypothesis, as 79% of MetS patients [11] and 40–60% of T2D subjects [12] are affected. Some researchers have gone even further and considered the NAFLD not only a manifestation of MetS but a crucial precursor of this condition [10].

Therefore, it is clear that the early diagnosis of hepatic insulin resistance could be beneficial, as it often precedes the arrival of other symptoms. However, the majority of clinically used diagnostic methods are currently based around blood glucose measurements. Metrics such as the fasting plasma glucose (FPG) value, the 2-h plasma glucose (2-h PG) value during a 75-g oral glucose tolerance test (OGTT), or HbA1C (hemoglobin A1c) criteria [13] are applied in order to determine alterations in glucose metabolism within an organism. Unfortunately, these criteria are often insufficient when performed alone, and, thus, in order to properly diagnose a patient, more than one is required. For example, according to Meijnikman et al. [14], out of a total of 581 Caucasian patients diagnosed with prediabetes, 44.2% subjects would be misdiagnosed if relying only on the HbA1c criterion without the support of the OGTT. Similar observations were made by Karnchanasorn et al. [15]. Furthermore, methods that target systemic insulin resistance have also been developed. Currently, the hyperinsulinemic-euglycemic glucose clamp technique and the frequently-sampled intravenous glucose tolerance test (FSIVGTT) [16] are recognized as the gold standards, yet their clinical application is limited [17,18]. Nevertheless, there are available methods such as homeostatic model assessment of insulin resistance (HOMA-IR), homeostatic model assessment 2 (HOMA2), the quantitative insulin sensitivity check index (QUICKI) [17], or Matsuda, which are generally accepted and considered reliable. Unfortunately, the lack of clear guidelines applicable in clinical use limits their potential for the early diagnosis of insulin resistance before system-wide alterations in carbohydrate metabolism develop. Furthermore, it has also been reported that some accompanying conditions may affect the aforementioned methods' sensitivity and specificity. For example, in patients with polycystic ovary syndrome (PCOS), the aforementioned surrogate tests have presented a high positive predictive value (90–96%) but a low negative predictive value (36–45%); therefore, a number of subjects have remained unrecognized by any of these methods [19]. Consequently, establishing universal diagnostic methods that would target hepatic insulin resistance, which is a hallmark of prediabetes, T2D, and MetS, might be the right approach.

Currently, a whole new group of relatively novel biomarkers is starting to resurface as early indicators of hepatic insulin resistance, namely ncRNAs (non-coding RNAs) such as lncRNA (long non-coding RNA) and miRNA (micro-RNA). Numerous lncRNAs and miRNAs have been reported

to be dysregulated in the state of insulin resistance [20,21] which, when considering their crucial regulatory function, is understandable. Kornfeld et al. [22] demonstrated that in the liver of high fat diet (HFD)-mice, the expression of 66 miRNAs was significantly altered; from which the expression of 90.1% of genes was increased and only 9.1% was decreased. Additionally, in the livers derived from Lepr db/db (homozygous for the diabetes db mutation of the leptin receptor) mice, the expression of 156 miRNAs was altered when compared to healthy controls.

The bioinformatics analysis performed by Yuan et al. [21] presented even more impressive data, as 4614 (2719 up-regulated and 1893 down-regulated) hepatic miRNAs and 2813 (818 up-regulated and 1995 down-regulated) lncRNAs were dysregulated in the livers of HFD male Sprague Dawley rats. Therefore, it has become clear that those non-coding RNAs possess an important diagnostic potential for the detection of hepatic insulin resistance. Especially since published evidence has suggested that both miRNAs and lncRNAs serve as very inclusive and sensitive markers among patients with varying ethnicities or medical conditions. For example, the circulating levels of the lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) were found to be increased in women with GDM (gestational diabetes mellitus) [23]. However, the value of a marker partly lies in the possibility of its easy acquisition for the purpose of further testing. Therefore, among the plethora of hepatic dysregulated miRNAs and lncRNAs, those in which circulating levels correlate with the hepatic state are of special interest because collecting a single blood sample from a patient is a clinically achievable practice performed on a daily basis.

In this review, the chosen miRNAs, including *miR-802*, *miR-499-5p*, *and miR-122-5p*, and lncRNA, including maternally expressed gene 3 (*MEG3*), *MALAT1*, and H19 imprinted maternally expressed transcript (*H19*) are discussed. Additionally, the prognostic potential of the described ncRNAs in circulation is addressed. The aforementioned ncRNAs were chosen because they play a crucial role in the regulation of the hepatic insulin signaling cascade, and, therefore, their hepatic expression patterns are significantly altered in subjects affected by hepatic insulin resistance. Furthermore, the levels of described ncRNAs in circulation vary in accordance with similar fluctuations in the liver, which makes them promising biomarkers in terms of future use in early hepatic insulin resistance diagnostics (Table 1).

**Table 1.** Circulating non-coding RNA (ncRNAs) altered in hepatic insulin resistance.

| ncRNA | Status in Liver | Status in Circulation | Reference |
|---|---|---|---|
| *miR-802* | ↑ | ↑ | [22,24,25] |
| *miR-499-5p* | ↓ | ↓ | [26,27] |
| *miR-122-5p* | ↑ | ↑ | [28–32] |
| *lnc MEG3* | ↑ | ↑ | [33–36] |
| *lnc MALAT1* | ↑ | ↑ | [23,37–39] |
| *lnc H19* | ↑/↓ * | ↑ | [40–44] |

* Findings differ between various research.

## 2. Hepatic Insulin Signaling Cascade

The liver is referred to as the metabolic center of the organism, and, as such, it plays a pivotal role in glucose homeostasis, along with white adipose tissue and skeletal muscles [5]. Within the hepatic tissue, insulin facilitates a number of different reactions, such as the reduction of hepatic glucose production and the promotion of glycogen synthesis. Additionally, it also affects the synthesis of numerous lipids and proteins including albumin and fibrinogen [45,46]. The insulin signaling cascade in hepatocytes begins in a manner universal to all cells—by binding to an INSR (insulin receptor). As a result of this binding, the INSR is activated. Next, the recruitment of various scaffold proteins—of which the insulin receptor substrate (IRS) family remains as the best-studied—occurs (Figure 1). IRS1 and IRS2 (insulin receptor substrate 1 and 2) are the two main isoforms expressed in hepatocytes. Pi3K (phosphatidylinositol 3-kinase) is recruited by the IRS and catalyzes the production of PIP3 (prolactin induced protein 3) from PIP2 (prolactin induced protein 2), which then binds to Akt (protein kinase B).

Simultaneously, insulin inhibits PTEN (phosphatase and tensin homolog) because it catalyzes a reverse reaction that enables the accumulation of PIP3. Subsequently to the PIP3 binding, Akt is activated by the phosphorylation of motifs in its activation loop—Thr308 and Ser473, in Akt1. The diversity of Akt substrates indicates that the ramifications of the insulin signaling pathway appear subsequently to Akt. FOXO (forkhead box), GSK3 (glycogen synthase kinase 3), or various regulators of mTOR (mechanistic target of rapamycin kinase) activity are among Akt substrates. FOXO1 (forkhead box O1) regulates gluconeogenic gene transcription, while GSK3 regulates glycogen synthesis. Upon activation, Akt phosphorylates FOXO1, which results in FOXO1's exclusion from nucleus, where it regulates the activity of G6PC (glucose-6-phosphatase catalytic subunit) and PCK1 (phosphoenolpyruvate carboxykinase 1)—two genes involved in hepatic gluconeogenesis. Upon entering the cytosol, FOXO1 undergoes deactivation via ubiquitination. In addition to the glucose metabolism, insulin also regulates lipids anabolism and catabolism within the liver, particularly by the SREBP-1c (sterol regulatory element-binding proteins) signaling pathway. Since insulin promotes net hepatic lipogenesis in standard conditions, insulin-resistant patients should be characterized by decreased lipogenesis, and such a phenotype was described in mice with total genetic insulin resistance (ablation of the hepatic insulin receptor) [47]. However, in normal humans and rodents, an opposite effect has been observed because insulin resistance is usually accompanied by hepatic steatosis and increased net hepatic lipogenesis, often resulting in NAFLD development. This anomaly is known as "pathway-selective insulin resistance and responsiveness" [5,48–50]. Conclusively, insulin resistance imposes a vast and diverse effect on hepatic metabolism and the liver's ability to maintain glucose and lipid homeostasis.

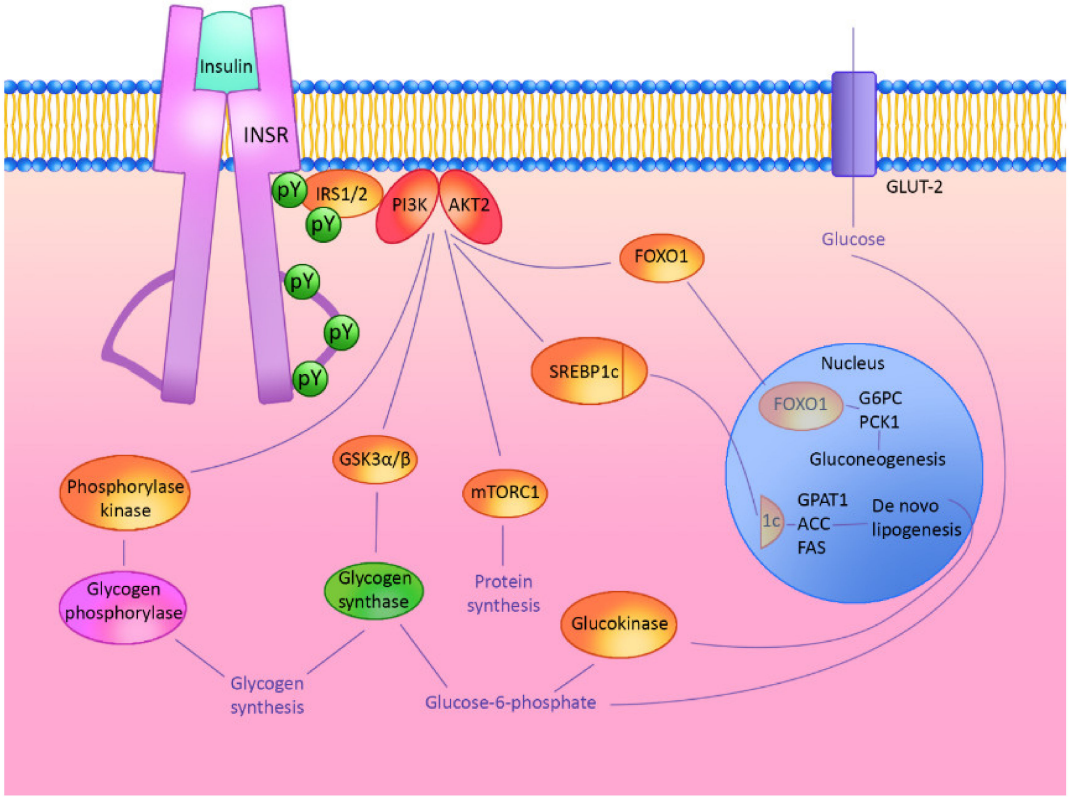


**Figure 1.** Hepatic insulin signaling cascade.

## 3. microRNAs and lncRNAs—Crucial Regulators of Cellular Pathways

miRNAs are a large group of small (15–22 nts), non-coding sequences with hairpin conformation, highly conserved among the species. Since their discovery 20 years ago, their biogenesis, activity, and function have been intensively studied and paved the path for much revolutionary research. However, their importance has been long underestimated due to technological limitations.

It was not until the discovery that two miRNAs—*lin-4* and *let-7*—control the timing in nematode (*Caenorbabditis elegans*) development that the significance of miRNA was fully recognized [51]. At this point, miRBase, which is an online miRNA database, lists 2654 mature miRNAs and 1917 precursor miRNAs (pre-miRNAs) in *Homo sapiens*. Simultaneously, around 60% of protein-coding human genes possess predicted miRNA target sites [52].

The dysregulation of miRNAs has been described in numerous diseases [53], including T2D, MetS, and NAFLD. miRNA expression tends to be largely tissue-specific and transcriptionally regulated. Usually, they are located within introns or lncRNAs, and their transcription is facilitated by RNA-polymerase. Importantly, miRNAs are organized into families based on the similarity of their seed sequences, which are two-to-eight nucleotides long (starting from the 5′ end) and determine the targeted mRNAs. Consequently, the main role of miRNA concerns the posttranscriptional regulation of mRNA expression via the destabilization or repression of the transcript [52]. This function makes miRNA a part of virtually any cellular process—including development determination, metabolism, cell activity, and apoptosis. One miRNA may target multiple genes, while one gene can be regulated by a number of miRNAs, which clearly underlines miRNAs' critical roles as regulators—especially since entire signaling pathways might be controlled by either clusters of miRNAs or a single miRNA [52]. In turn, the role and biological function of recently discovered lncRNAs (over 200 nts) is still not fully elucidated. However, the cross-talk between these two types of non-coding RNA clearly indicates that lncRNA has the potential to affect miRNA activity.

lncRNAs are loosely categorized together in a very diverse and large group, and, similarly to miRNA, their input in the regulation of numerous biological processes is crucial. They have been reported to partake in genomic loci imprinting, allosterically regulating enzymatic action or even chromosome conformation [54,55]. Intricate regulatory lncRNA patterns have been described in a variety of cellular mechanisms, and their dysregulation has also been demonstrated in different disorders [56]. In general, lncRNAs can be categorized into three broad subgroups based on their function. First, the non-functional lncRNAs, which are probably a product of transcriptional noise. The second comprises lncRNAs, the transcripts of which are of lesser importance and the act of transcription itself is sufficient for their action. Finally, the third group consists of the functional lncRNAs with the ability to act in *cis* and/or *trans* orientations [57]. In some aspects, lncRNAs are similar to mRNAs, as they are transcribed by polymerase II from the genomic loci, often 5′-capped, spliced, and polyadenylated. Some lncRNAs can even produce small peptides [58]. Among the wide repertoire of lncRNAs functions, they also pose the ability to interact with miRNAs in various ways. It has been described that miRNAs can target lncRNAs in order to reduce their stability. Furthermore, lncRNAs can act as molecular sponges or decoys of miRNAs and, consequently, regulate the miRNA level in the cytoplasm by binding specific miRNAs, and actively sequestrate them from their target mRNAs; such lncRNAs are defined as competing endogenous (ce) RNAs [59,60]. Moreover, lncRNAs can also compete with miRNAs for binding sites in shared target mRNAs [61]. Therefore, next to miRNAs, lncRNAs form another viable group of highly-specialized regulators of gene expression, and both of these ncRNAs have been reported to act as regulators of the hepatic insulin signaling cascade [62,63].

Consequently, *miR-499-5p* has been described to regulate *PTEN* expression, while *miR-802* and *MEG3* are involved in gluconeogenesis. Some ncRNAs can even affect more than one ramification of the insulin signaling pathway like *miR-122, MALAT1*, and *H19*, which are also correlated with hepatic *de novo* lipogenesis.

### 3.1. miR-802

microRNA-802, located on the 21st chromosome, has been intensively studied in different types of cancer, and it has been shown that it plays a crucial role during metastasis, progression, and invasion. Huang et al. [64] observed that *miR-802* not only inhibits the proliferation and invasion but also the epithelial–mesenchymal transition of glioblastoma multiforme cells. *miR-802's* role has also been

described in tongue squamous cell carcinoma [65], pancreatic cancer progression [66], and human cervical cancer [67]. Recently, Zhang et al. [68] demonstrated *miR-802's* ability to inhibit the aggressive behaviors of non-small cell lung cancer cells. However, it has also been described that *miR-802* can accelerate the growth of hepatocellular carcinoma (HCC) [69] and that its high concentration in blood is associated with a poor prognosis in HCC patients [70]. Furthermore, *miR-802* has been shown to partake in obesity-induced nephropathy in obese human and mice [71]. In fact, *miR-802* upregulation associated with obesity and insulin resistance has been observed in different tissues such as the kidney, white adipose tissue, skeletal muscle, and the liver [24]. As is widely known, the damage occurring in target organs during diabetes is multicausal, so next to high glucose and fatty acids levels, the increased production of reactive oxygen species (ROS) also accounts for the damage. Yang et al. [25] demonstrated a connection between *miR-802*, oxidative stress, and hepatic insulin resistance. In their study, they showed that in HFD mice, *miR-802* upregulation was associated not only with higher blood glucose and serum insulin levels but also with a lowered activity of oxidative-stress related enzymes such as SOD (superoxide dismutase), CAT (catalase), and GSH-Px (glutathione peroxidase). Following this thread, ROS generation was significantly greater upon *miR-802* upregulation and could be improved by *miR-802* inhibition. Subsequently, the expression levels of key proteins in the insulin signaling cascade were investigated in order to assess the influence of *miR-802's* up-regulation of hepatic insulin resistance. Interestingly, the expression of phosphorylated Akt1 was decreased, while the phosphorylated IRS1 (Ser307) level was increased. Conspicuously, IRS1 phosphorylation in the Ser307 site was found to result in the decreased phosphorylation of IRS-1 tyrosine residues, which, in turn, leads to the accelerated degradation of this protein and (as a consequence) the reduced phosphorylation of its target—Akt [72]. The vital role of *miR-802* upregulation in hepatic insulin resistance was also supported by Kornfeld et al. [22]. In their study, the up-regulation of *miR-802* in the liver was once again confirmed in both HFD mice and Lepr db/db mice. Furthermore, this up-regulation was shown to negatively affect *Hnf1b* (hepatocyte nuclear factor 1-beta) expression, which consequently resulted in the induction of *PPARγ* (peroxisome proliferator-activated receptor gamma coactivator) and its target genes: *G6pc* (glucose-6-phosphatase catalytic subunit) and *Pck1* (phosphoenolpyruvate carboxykinase 1), both involved in gluconeogenesis. Similar findings were described by Higuchi et al. [24], who also demonstrated that *miR-802* circulating levels were significantly increased in patients with T2D; therefore, *miR-802* could be used as a marker for T2D.

### 3.2. miR 499-5p

The microRNA-499 gene is located in the 20th intron of the *Myh7b* (beta-myosin heavy chain 7B) [73], and, therefore, it is often associated with cancer risk because it might affect *Myh7b* gene function. It has also been reported to downregulate a proto-oncogene *ETS1* in HepG2 cells (human liver hepatocellular carcinoma cells) by increasing *MMP-7* (matrix metallopeptidase 7) expression [74]. As was observed in a study by Ma et al. [75], *miR-499* polymorphism is also connected with a higher susceptibility to hepatocellular carcinoma in a large-scale population. Furthermore, *miR-499* genetic variation has also been reported as a crucial indicator of diabetic neuropathy susceptibility and could indicate patients with a higher risk of developing cardiovascular autonomic neuropathy [76]. Recently, a link between *miR-499* and NAFLD was also discovered, as it was demonstrated by Hanyun et al. [77] that *miR-499* inhibition improves NAFLD which, as was previously mentioned, is often associated with insulin resistance. The experimental model involved specific pathogen-free (SPF) male C57BL/6 mice fed with a high-fat diet and injected with an *miR-499* inhibitor. Subsequently, it was observed that the degree of steatosis in the liver tissue of the mice treated with the *miR-499* inhibitor was significantly lower that of the untreated control group. Furthermore, compelling evidence that *miR-499* is in fact involved in hepatic glucose metabolism was presented by Wang et al. [26], who observed that *miR-499* levels were significantly lower in the livers of db/db and HFD-fed mice. Interestingly, Akt/GSK activation impairment was observed alongside *miR-499-5p* downregulation. By applying bioinformatics tools followed by in vitro testing, it was revealed that *miR-499* affects the insulin signaling cascade

and glycogen synthesis by suppressing *PTEN*. This observation was further confirmed, because introducing *miR-499* into HFD mice resulted in *PTEN* suppression and the improvement of Akt/GSK activation. The association between *miR-499* levels and T2D was also described in the erythrocytes of African-American adults [27], as it was observed that *miR-499* levels were reduced in pre-diabetic patients. Furthermore, a strong correlation between *miR-499* and HbA1c criterion was visible, which is especially promising in terms of its possible use in diagnostics.

### 3.3. miR 122-5p

*miR-122* is located on human chromosome 18 and was one of the first identified factors of so-called tissue-specific miRNA [78], as it is highly abundant in the liver, in which it is recognized as the dominant miRNA that accounts for 70% of hepatic miRNA content in mice and about 52% of human hepatic miRNome [79]. Moreover, *miR-122* is strongly conserved among vertebrate species, which indicates its crucial function. *miR-122* is highly expressed, particularly in adult livers, as it expression increases during embryonic development. Consequently, *miR-122*, under the regulation of *HNF6* (hepatocyte nuclear factor 6) and *OC2* (one cut homeobox 2), partakes in the terminal differentiation of hepatocytes, as demonstrated by Laudadio et al. [80]. Additionally, the inhibition of *miR-122* activity in BMEL (bipotent murine embryonic liver) cells cultured as floating aggregates led to a repressed expression of 26 genes, 24 of which were coding hepatocyte-specific proteins, while the expression of 22 hepatic non-specific genes was increased. Therefore, it is clear that *miR-122* participates in the differentiation of hepatoblasts towards hepatocytes; however, its activity also extends towards other processes. As was previously described, *miR-122* plays a pivotal role in regulating hepatic gene expression, affecting various aspects of cellular activity such as response to oxidative stress [81], viral infection [82], inflammation [83], and even tumorigenesis, as its dysregulation has notoriously been reported as an viable marker of HCC onset and development [84,85]. *miR-122* has also been described to orchestrate lipid metabolism; as Cheung et al. [28] demonstrated in their study, *miR-122* inhibition in healthy mice resulted in a decreased expression of hepatic *de novo* lipogenesis genes such as *FASN* (fatty acid synthase) and *ACC1* (acetyl-CoA carboxylase), which encode two rate-limiting enzymes. Furthermore, the inhibition of *miR-122* diminished hepatic lipogenesis and served as a protection against liver steatosis in HFD obese mice. Additionally, it was described by Iliopoulos et al. [29] that *miR-122* overexpression in HepG2 cells also resulted in increased *SREB-1c* expression along with the aforementioned *ACC-1* and *FASN*. The culminating evidence indicated a critical role of *miR-122* in hepatic *de novo* lipogenesis regulation and, in consequence, NAFLD development, a common condition among patients affected by either MetS or T2D. Additionally, *miR-122* dysregulation has been shown to accompany hepatic insulin resistance. Dong et al. [30] described how—in both a T2D rat model and an IR HepG2 cell culture—*miR-122* expression was significantly increased. Furthermore, *miR-122* overexpression resulted in a decreased expression of its target: insulin-like growth factor (*IGF-1R*), which, in turn is a part of the IGF-1R/PI3K/Akt signaling pathway. This negative effect could be reversed by *miR-122* inhibition. miR-122 has also been shown to affect the hepatic gluconeogenesis process. In a study published by Wei et al. [31], it was demonstrated that *HNF-4α* (hepatocyte nuclear factor 4 alpha) acts as a regulator of *miR-122* activity, which, in turn, regulates a plethora of targets associated with gluconeogenesis (*G6PC* and *PCK1*) and *de novo* lipogenesis (*SREBP-1c* and *FAS*). Furthermore, compelling evidence regarding the *miR-122* level's prognostic value was demonstrated by Willeit et al. [32]. Specifically, it was showcased that *miR-122's* circulating levels were significantly higher among subjects with insulin resistance. Patients suffering from MetS were characterized by 160% higher circulating *miR-122* levels, while T2D subjects displayed 240% higher levels when compared to a control group; *miR-122* was also recognized as an indicator of future MetS and T2D onset. Taking all supporting evidence into account, *miR-122* seems as a very promising and well established marker of hepatic insulin resistance with a great prognostic value.

### 3.4. Long-Non-Coding RNA MALAT1(Metastasis Associated in Lung Adenocarcinoma Transcript 1)

As one of the first long non-coding RNAs to be discovered, *MALAT1* is very well-conserved among different mammal species. Additionally known as noncoding nuclear-enriched abundant transcript 2 (*NEAT2*), it is located on the short arm of human chromosome 11q13.1 and was has been shown to take a part in regulation of cells proliferation and motility. *MALAT1* is also highly abundant in various organs. Furthermore, the bioinformatics analyses performed by Chen *et al* [86] indicated that *MALAT1* regulates the expression of many distinct genes by affecting various stages of their transcription and elongation. Repeatedly, its dysregulation has been observed in a wide range of human cancers such as osteosarcoma, breast cancer, uterine endometrial stromal sarcoma, cervical cancer, hepatocellular carcinoma, and colorectal cancer [87]. However, bearing in mind the diverse ramifications of *MALAT1* action, it has also been linked with viral infections, stimulating cytokine production and even alcohol abuse. Recent evidence has indicated *MALAT1's* effect on glucose and lipid metabolism [88]. Several studies have discussed its involvement in diabetes and diabetic-complications. In 2019, Puthanveetil et al. [88] observed that maintaining human umbilical vein endothelial cells (HUVECs) in high-glucose conditions led to the overexpression of *MALAT1*, which, in turn, induced *IL-6* (interleukin-6) and *TNF-α* (tumor necrosis factor α) up-regulation via *SAA3* (serum amyloid A3), one of *MALAT1*'s targets. Subsequently, this *MALAT1* activity was confirmed in renal tissue obtained from diabetic mice. Yan et al. [89] provided evidence that *MALAT1* overexpression in the endothelial cells of mice was associated with diabetes. Following this thread, Yan et al. investigated the expression of *MALAT1* in two models of diabetes: the livers of ob/ob mice and hepatocytes exposed to palmitate, and they were able to prove that *MALAT1* expression was significantly up-regulated in both models. Furthermore, it was confirmed that *MALAT1* overexpression led to the up-regulation of mRNA and nuclear *SREBP-1c. SREBP-1* is a major regulator of *de novo* lipogenesis, so its up-regulation is strongly correlated with liver steatosis—a main symptom of NAFLD. Non-alcoholic fatty liver disease is highly prevalent among patients affected by metabolic syndrome—it is often referred to as the manifestation of metabolic syndrome with a prevalence reaching up to 79% [11]. NAFLD is also highly abundant in type 2 diabetes patients (40–60%) [12], as clearly visible insulin resistance affects not only the glucose metabolism but also lipids. Furthermore, it has been repeatedly reported that oxidative stress is highly correlated with insulin resistance and can also be the cause of poor insulin sensitivity. Chen et al. [86] provided evidence of how *MALAT1* is involved in oxidative stress-mediated insulin resistance via the up-regulation of the *Jnk* (c-Jun N-terminal kinase)—a stress-sensitive kinase that, upon activation, can suppress insulin signaling by inhibiting the phosphorylation of IRS and Akt—two major regulators in the insulin signaling cascade. The difference in the expression of *MALAT1* was also observed in GDM patients [23]. By measuring the plasma levels of three long non-coding RNAs, Zhang et al. were able to prove that *MALAT1* levels were higher in patients with GDM when compared to healthy controls. The mentioned study is especially promising in terms of the future utilization of lncRNAs as easily-acquired and sensitive markers for insulin resistance detection.

### 3.5. Long-Non-Coding RNA MEG3

The lncRNA *MEG3* is characterized as an imprinted gene that belongs to the imprinted delta like non-canonical notch ligand (*DLK1-MEG3*). In humans, it is located on the 14q32.3 chromosome, while in mice, it is known as gene trap locus 2 (*Gtl2*) and is located at distal chromosome 12. *MEG3* expression if often affected in various types of cancer. Up to this point, the loss of its expression has been described in gastric cancer, gallbladder cancer, non-small cell lung cancer (NSCLC), and cervical cancer [33]. *MEG3* is sometimes referred to as tumor suppressor because its overexpression promotes apoptosis and inhibits the proliferation of tumor cells. In the recent years, mounting evidence has also revealed *MEG3's* connection to type 2 diabetes and insulin resistance in the pancreas [34], diabetic microvascular dysfunction [35], and nephropathy [36]. Additionally, the overexpression of *MEG3* has been observed in the livers of ob/ob mice and HFD-induced insulin-resistant mice, ultimately resulting in the disruption of the insulin signaling cascade through the upregulation of *Foxo1* [37]. As *Foxo1* regulates the activity

of *G6pc* and *Pck1*, this action of *MEG3* promotes hepatic gluconeogenesis. After further exploring the subject, Zhu et al. [90] proposed a possible connection between *FOXO1* and *MEG3* because the later participates in regulating *ATF4* (activating transcription factor 4) expression—one of *FOXO1*'s co-regulators—by acting as a sponge for *miR-214*, which is known as an *ATF4* suppressor. As was shown in recent years, lncRNA may competitively bind miRNA and, by doing so, act as a ceRNA (competing endogenous RNA). A similar mechanism was described by Chen et al. [38], who observed the upregulation of *MEG3* in two experimental models: HFD mice and palmitate-treated hepatocytes serving as models of insulin resistance. In this instance, the expression of *miR-185-5p* and its target mRNA-*EGR2* (early growth response 2) was evaluated. Again, *MEG3* acted as a ceRNA for *miR-185-5p* and, as a consequence, promoted the expression of *EGR2*—which was reported to inhibit IRS and partake in fat cell differentiation. The expression of MEG3 was also studied in PBMCs (peripheral blood mononuclear cells), and, again, its overexpression was showcased in patients affected by type 2 diabetes [39].

### 3.6. Long-Non-Coding RNA H19

Similarly to *MALAT1*, *H19* was one of the first long-non-coding RNAs to be discovered, and it has consequently been thoroughly studied. It is located on human chromosome 11 or on chromosome 7 in mice, and it is comprised of five exons and four introns. The biological functions of *H19* are quite versatile, as it partakes in the regulation of cells proliferation and differentiation processes [91]. It has been reported to act as a tumor suppressor but also as an oncogene. Furthermore, it plays a pivotal role in proper embryonic development because it regulates various important genes such as *IGF2* that belong to the imprinted gene network (*IGN*). However, after embryonic development occurs, its action in limited to only few tissues such as heart and skeletal muscles. In a healthy adult liver, *H19* has been detected on very low yet appreciable levels; however, Nilsson et al. observed that in adults with type 2 diabetes, *H19* hepatic levels were elevated [40]. Zhang et al. [41] provided evidence of a similar *H19* expression pattern in HFD mice. Furthermore, by performing *H19* knock-down in HepG2 cells followed by an RNA-seq analysis and qPCR, Zhang observed a decreased expression of *HNF4A*, as well as *PCK1* and *G6PC*—two major gluconeogenic genes. In turn, the overexpression of *H19* was accompanied by an increased expression of those genes. Therefore, the mechanism by which *H19* is involved in hepatic glucose homeostasis seems to be strongly associated with excessive hepatic glucose production mediated by *H19's* ability to regulate *HNF4A* methylation. Interestingly, by using a fasting mouse model, Zhang et al. were able to demonstrate that the *H19* upregulation might be a physiological response to fasting aimed at restoring glucose homeostasis by elevating hepatic glucose production. However, contradictory evidence has also been published. Goyal et al. [42] demonstrated that in the livers of db/db mice, *H19* was downregulated and that this alteration was correlated with *Foxo1* upregulation and its nuclear retention, which consequently led to the increased expression of *G6pc* and *Pck1*. Furthermore, Goyal et al. confirmed this mechanism of *H19*'s regulatory activity by treating C57BL/6J mice with an H19 inhibitor [42]. These conflicting findings may possibly be explained by the fact that each study was based on a different in vivo model. Liu et al. [43] observed *H19* up-regulation, *Srebp-1c, Acc1, Scd1, Fasn and Pparγ*, increased expression in a model of NAFLD. This study was also conducted based on HFD mice model, so it is possible that *H19* expression varies between different models, especially since lncRNA expression patterns are tissue- and state-specific. Nevertheless, further research might be needed to provide the answer. Conclusively, evidence presented by Liu et al. further underlined the magnitude of *H19*'s input in hepatic insulin resistance development because it affects not only gluconeogenesis but also hepatic lipogenesis. Recently, Fawzy et al. [44] found that *H19* circulating levels were significantly increased among patients with T2D, which indicates that it may serve as a blood-based marker of hepatic insulin resistance, especially since its dysregulation has been described in a variety of metabolic disorders.

## 4. Prognostic Potential

Mounting evidence published in recent years has indicated that ncRNAs could be applied to developing specific and sensitive diagnostic methods. Numerous publications have described both miRNAs and lncRNAs as precise markers of various diseases. Nevertheless, there are some practical issues that need to be addressed in order to maximize ncRNAs' utility as markers applicable for every day clinical use. As pointed out in various studies, monitoring a single ncRNA's expression is often insufficient to grasp a full and precise diagnosis. The majority of literature published in the field has clearly suggested that establishing a panel of ncRNAs is the most efficient approach. The discussed ncRNAs could be potential candidates for such a panel. As Higuchi et al. [24] demonstrated in their study, circulating levels of *miR-802* are visibly correlated with HbA1c, HDL-C (high density lipoprotein cholesterol), and estimated glomerular filtration rate test eGRF values and, as such, could be used as a biomarker of T2D with MetS. Additionally, Church et al. [92] conducted a study in which hepatobiliary injury and biliary hyperplasia were inflicted in rats with alpha-naphthylisothiocyanate (ANIT) and a proprietary compound, FP004BA. Such a treatment resulted in hepatocellular necrosis and the enrichment of various ingenuity pathways, some of which were also altered during hepatic insulin resistance. At 24 h after the oral administration of ANIT, pathways related to cholesterol biosynthesis and oxidative stress response were among the most affected, while 120 h after administration, glycogen and oleate biosynthesis canonical pathways were enriched. Subsequently, Church et al. demonstrated that among 60 assessed miRNAs, *miR-802* was the most elevated during hepatobiliary injury and, as such, was a precise marker of liver injury. Additionally, even in case of slight hepatocellular injury, a trend towards an *miR-802* level increase was observed. Bearing in mind the aforementioned findings, *mir-802* should be considered as a valuable marker of hepatic distress and insulin resistance. Up to this point, *miR-499-5p* has been strongly associated with cardiovascular pathologies [93–96]; however, evidence regarding its diagnostic potential in diabetic and prediabetic patients has also been published. As Fluitt et al. [27] demonstrated, *miR-499-5p* was significantly reduced in erythrocytes of African American pre-diabetic patients. Additionally, it could be applied to effectively distinguish prediabetic patients from those with T2D (area under the curve (AUC) = 0.7866; $p = 0.02$). A similar expression pattern of *miR-499-5p* was also observed among diabetic ESRD (end-stage renal disease patients) undergoing dialysis [97] and in patients affected by diabetic neuropathy or diabetic polyneuropathy [76]. These findings clearly indicate that *miR-499-5p* is a precise marker in the course of T2D and its complications. Collectively, with its role in hepatic insulin resistance development, *miR-499-5p* could be a potential candidate for developing a diagnostic panel of ncRNAs that are applicable in clinical use. As the most abundant hepatic miRNA, *miR-122* is a well-established marker of liver distress. As Jampoka et al. [98] demonstrated in their study, *mir-122* was an efficient, serum-derived marker of NAFLD, with 75% sensitivity and 82.35% specificity(the AUC was 0.831; $p < 0.0001$). What is more, there was a significant difference in *miR-122* serum levels between patients without steatohepatitis and those affected by it. Therefore, *miR-122* was not only a precise marker of NAFLD but could also be applied to assess the severity of hepatic damage. Furthermore, *miR-122* plasma levels could precisely indicate T2D patients who develop NAFLD [99]. As similar observations have been published by different authors [79,100], the collective body of evidence clearly highlights *miR-122's* hepatic specificity and diagnostic potential. Since lncRNAs are not as well studied as miRNAs, the number of potential lncRNA markers is still growing. However, some lncRNAs have already been established as good indicators of insulin resistance and/or hepatic distress. *MALAT1* has been reported as an important agent in hepatic insulin resistance development. As Konishi et al. [101] described in their study, *MALAT1* plasma levels were associated with liver damage and HCC development. Similar evidence has been published by other authors [102]. Additionally, *MALAT1* has been proven as an acceptable marker of GDM, as Zhang et al. demonstrated in their study that the AUC was 0.654 (95% confidence interval 0.543–0.768), with a 50% sensitivity and an 83% specificity [23]. Unfortunately, up to this point, no data concerning the specificity and sensitivity of circulating *MEG3* in cases of hepatic insulin resistance have been published. However,

such analyses have been performed for other hepatic disorders such as fibrosis, hepatitis B virus (HBV) infection [103], and HCC [104]. It is important to point out that in all the aforementioned disorders, the expression pattern was different from the one observed in T2D patients, i.e., the hepatic expression and/or circulating levels were decreased. Bearing in mind that *MEG3* was overexpressed in the PBMC of T2D patients [39] and was proven as an valuable marker in other hepatic disorders, it is clear that is should be considered a candidate for a diagnostic panel. Similarly, *H19* was described as a significant prognostic marker in chronic liver disease patients with or without cirrhosis and HCC [105]. Consequently, as Fawzy et al. [44] demonstrated in their study, *H19* levels are significantly higher in plasma of T2D patients, though a receiver operating characteristics (ROC) analysis was not performed. Additionally, Tello-Flores et al. [106] demonstrated in their study that the relative expression of serum *H19* was two-fold higher among patients with impaired glycemic control when compared to diabetics with proper glucose control. Furthermore, an association between T2D susceptibility and genetic variants of both *H19* and *MEG3* was demonstrated [107]. Nevertheless, further investigation is needed to fully elucidate the prognostic potential of *H19*. In summary, even though in some cases there have been no data describing the prognostic value of the described ncRNAs in the course of hepatic insulin resistance, due to the existing body of evidence indicating their strong affiliation with hepatic disorders and T2D, they are worth considering for diagnostic purposes.

## 5. Summary and Future Perspectives

According to the International Diabetes Federation and WHO reports, diseases associated with insulin resistance such as T2D, MetS, and NAFLD are become progressively more prevalent among the global population and will become a significant challenge in the coming years. Such a situation calls for immediate, reliable solutions and early, precise diagnosis is undoubtedly one of them. Endocrinology, as a constantly evolving field of medicine, has abandoned the idea of single-causal disease mechanisms, and this perspective has since been replaced by a more comprehensive, broader approach. Therefore, the importance of cellular metabolism alterations in the development of diseases is recognized as equal to other components such as diet or environment. The extensive research of insulin signaling pathways and the occurring pathologies accompanying metabolic diseases has indicated the significance of hepatic insulin resistance. It is recognized as one of the major agents in the development of hyperglycemia and dyslipidemia in the course of T2D and MetS. This statement is supported by evidence showcasing how targeting hepatic metabolism leads to overall clinical improvement in insulin-resistant subjects [108–110]. Along with the discovery of non-coding RNAs, a whole new layer of molecular mechanisms and gene expression regulation has unraveled. RNAs' unique regulatory function links them with virtually all cellular processes, and the dysregulation of miRNAs and lncRNAs has also been described alongside various liver metabolism alterations, insulin resistance among them. Their tissue-specific expression patterns give a valuable insight into particular tissue metabolisms, which further adds to their diagnostic potential. Consequently, both lncRNAs and miRNAs have emerged as precise and versatile potential biomarkers of numerous disorders such as Alzheimer's disease [111], breast cancer [112,113], gastric cancer [114], cardiovascular disease [95], and postmenopausal osteoporosis [115]. Additionally, due to their unique regulatory functions, the analysis of lncRNAs' and miRNAs' dysregulation has expanded our understanding of various conditions and their pathomechanisms. Therefore, miRNAs and lncRNAs are now being considered as potential therapeutic targets [116], not only due to the aforementioned regulatory function but also because of their mutual interactions that allow for precise intervention in cellular processes. Furthermore, the abundant presence of ncRNAs in body fluids allows for their use as precise blood based biomarkers, which indicates their potential in everyday clinical practice. In summary, both miRNAs and lncRNAs exhibit features that make them promising tools in early hepatic insulin resistance diagnosis, so their incorporation into clinical practice would be step forward towards holistic and personalized medicine.

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## Abbreviations

| | |
|---|---|
| MetS | Metabolic Syndrome |
| T2D | Type 2 Diabetes |
| NAFLD | Nonalcoholic Fatty Liver Disease |
| HCC | Hepatocellular Carcinoma |
| WHO | World Health Organization |
| FPG | Fasting Plasma Glucose |
| 2-h PH | 2-h Plasma Glucose |
| OGTT | Oral Glucose Tolerance Test |
| HbA1C | Hemoglobin A1c |
| FSIVGTT | Frequently-Sampled Intravenous Glucose Tolerance Test |
| HOMA-IR | Homeostatic Model Assessment of Insulin Resistance |
| HOMA2 | Homeostatic Model Assessment 2 |
| QUICKI | Quantitative Insulin Sensitivity Check Index |
| PCOS | Polycystic Ovary Syndrome |
| ncRNA | Non-Coding RNA |
| lncRNA | Long Non-Coding RNA |
| pre-miRNA | Precursor miRNA |
| miRNA | microRNA |
| (ce)RNA | Competing Endogenous RNA |
| Nts | Nucleotides |
| mRNA | Messenger RNA |
| MALAT1 | Metastasis Associated Lung Adenocarcinoma Transcript 1 |
| MEG3 | Maternally Expressed Gene 3 |
| H19 | H19 Imprinted Maternally Expressed Transcript |
| HFD-mice | High Fat Diet Mice |
| Lepr db/db | Homozygous for the Diabetes db Mutation of the Leptin Receptor |
| GDM | Gestational Diabetes Mellitus |
| INSR | Insulin Receptor |
| IRS | Insulin Receptor Substrate |
| IRS1 | Insulin Receptor Substrate 1 |
| IRS2 | Insulin Receptor Substrate 2 |
| Pi3K | Phosphatidylinositol 3-kinase |
| PIP3 | Prolactin Induced Protein 3 |
| PIP2 | Prolactin Induced Protein 2 |
| Akt | Protein Kinase B |
| PTEN | Phosphatase and Tensin Homolog |
| FOXO | Forkhead Box |
| FOXO1 | Forkhead Box O1 |
| GSK3 | Glycogen Synthase Kinase 3 |
| mTOR | Mechanistic Target of Rapamycin Kinase |
| G6PC | Glucose-6-Phosphatase Catalytic Subunit |
| PCK1 | Phosphoenolpyruvate Carboxykinase 1 |
| SREBP-1c | Sterol Regulatory Element-Binding Proteins |

| | |
|---|---|
| ROS | Reactive Oxygen Species |
| SOD | Superoxide Dismutase |
| CAT | Catalase |
| GSH-Px | Glutathione Peroxidase |
| Hnf1b | Hepatocyte Nuclear Factor 1-beta |
| PPARγ | Peroxisome Proliferator-Activated Receptor Gamma Coactivator |
| Myh7b | Beta-Myosin Heavy Chain |
| MMP-7 | Matrix Metallopeptidase 7 |
| SPF | Specific Pathogen-Free |
| HNF6 | Hepatocyte Nuclear Factor 6 |
| OC2 | One Cut Homeobox 2 |
| BMEL | Bipotent Murine Embryonic Liver Cells |
| FASN | Fatty Acid Synthase |
| IR | Insulin-Resistant |
| Acc1 | Acetyl-CoA Carboxylase |
| HNF-4α | Hepatocyte Nuclear Factor 4 α |
| NEAT2 | Noncoding Nuclear-Enriched Abundant Transcript 2 |
| HUVECs | Human Umbilical Vein Endothelial Cells |
| IL-6 | Interleukin-6 |
| TNF-α | Tumor Necrosis Factor α |
| SAA3 | Serum Amyloid A3 |
| JNK | c-Jun N-Terminal Kinase |
| DLK1-MEG3 | Imprinted Delta Like Non-Canonical Notch Ligand |
| GTL2 | Gene Trap Locus 2 |
| NSCLC | Non-Small Cell Lung Cancer |
| ATF4 | Activating Transcription Factor 4 |
| EGR2 | Early Growth Response 2 |
| PBMC | Peripheral Blood Mononuclear Cells |
| IGF2 | Insulin-Like Growth Factor 2 |
| IGN | Imprinted Gene Network |
| RNA-seq | RNA Sequencing |
| SCD1 | Stearoyl-CoA Desaturase |
| HDL-C | High-density Lipoprotein Cholesterol |
| eGRF | Estimated Glomerular Filtration Rate Test |
| ANIT | Alpha-Naphthylisothiocyanate |
| HBV | Hepatitis B Virus |
| AUC | Area Under Curve |
| ROC | Receiver Operating Characteristics |

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# Equine Hoof Stem Progenitor Cells (HPC) CD29 + /Nestin + / K15 + – a Novel Dermal/epidermal Stem Cell Population With a Potential Critical Role for Laminitis Treatment

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**Abstract**
Laminitis is a life threating, extremely painful and frequently recurrent disease of horses which affects hoof structure. It results from the disruption of blood flow to the laminae, contributing to laminitis and in severe separation of bone from the hoof capsule. Still, the pathophysiology of the disease remains unclear, mainly due to its complexity. In the light of the presented data, in the extremally difficult process of tissue structure restoration after disruption, a novel type of progenitor cells may be involved. Herein, we isolated and performed the initial characterization of stem progenitor cells isolated from the coronary corium of the equine feet (HPC). Phenotype of the cells was investigated with flow cytometry and RT-qPCR revealing the presence of nestin, CD29, and expression of progenitor cell markers including SOX2, OCT4, NANOG and K14. Morphology of HPC was investigated with light, confocal and SEM microscopes. Cultured cells were characterised by spindle shaped morphology, eccentric nuclei, elongated mitochondria, and high proliferation rate. Plasticity and multilineage differentiation potential was confirmed by specific staining and gene expression analysis. We conclude that HPC exhibit in vitro expansion and plasticity similar to mesenchymal stem cells, which can be isolated from the equine foot, and may be directly involved in the pathogenesis and recovery of laminitis. Obtained results are of importance to the field of laminitis treatment as determining the repairing cell populations could contribute to the discovery of novel therapeutic targets and agents including and cell-based therapies for affected animals.

**Keywords** Stem cells · Hoof · Horse · Laminitis

## Introduction

One of the most debilitating diseases in horses since many years is laminitis – multifactorial disorder that is still poorly understood. During laminitis, serious pathological changes contribute to disruption of the distal phalangeal suspensory apparatus at the dermal–epidermal junction, which as a consequence destroys the lamellar structures [1]. The pathophysiology of laminitis includes sepsis related conditions which might be caused by overload with carbohydrates, abdominal surgery, gastrointestinal disease as well as non-septic endotoxemia that includes insulin resistance, Cushing syndrome or corticosteroid dysmetabolism. During laminitis, acute and chronic phases could be distinguished, that represents two clinical scenarios [2]. In acute phases, severe pain, lameness, and inflammation occur without radiological changes, while in chronic phase rotation of the third phalanx occurs causing detachment of lamina components leading to substantial damage of the basement membrane of the derma so that the structure collapses. Both acute and chronic phases of laminitis could have a poor prognosis and become the reason of equine disability and in consequence euthanasia. Thus, laminitis represents a serious medical event that has a strong socio-economic impact in the equine industry.

Laminitis is a complex disease that induces pathological changes at the meeting point of vascular system, inflammation, oxidative stress, and endocrinological system. Although many efforts has been paid to the development of effective therapeutic solutions for laminitis treatment,

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still no effective therapy is available [3–5]. Since laminitis includes three main molecular events including: inflammation, micromorphological and structural changes, stem cell based therapies seems to be a reasonable solution.

Mesenchymal stromal stem cells (MSCs), exhibit particular molecular and physiological features that allow to use them in clinical practice in many medical fields [6]. MSCs are plastic adherent cells that express the following surface markers CD105, CD44, CD29, CD73, CD90, and no expression of CD45, CD34, CD14, CD11b, or CD19, as well as multilineage differentiation potential [7]. MSC could give rise to multiple types of cell populations and therefore become a promising therapeutic agent [8]. However, the immunomodulatory and antiapoptotic effect of MSCs makes them even more promising when inflammatory-related injury and tissue regeneration are considered. Moreover, recent findings clearly indicate paracrine and autocrine activity of MSCs during various regenerative processes, which underlines their fundamental role in the initiation of so-called "regenerative process" [9–11]. Various sources of MSCs were in recent years were described including bone marrow-derived stem cells (BMSCs), adipose tissue-derived mesenchymal stem cells (ASCs), nail stem cells (NSCc) and many others [12, 13]. It seems that various stem cell pools possess unique properties which potentially could target specific injured tissue.

Therefore, bearing in mind the physiological nature of laminitis, here we identified novel MSCs subpopulation-CD29 + /Nestin + /K15 + HPC that were isolated from the equine coronary corium. These cells exhibited typical MSCs surface markers additionally extended to keratin 15 (K15), keratin 14 (K14), keratin 19 (K19), CD29, CD34, CD200, angiopoietin 1 (Ang1) and leucine-rich repeat-containing G protein-coupled receptor 6 (Lgr6) similarly to nail stem cells [12]. They multipotent nature as well as expressing markers of dermis and epidermis could in the near future become effectively applied during laminitis treatment. Here, for the first time we identified a novel stem cell pool of equine hooves identifying their surface markers as well as differentiation potential. Presented data shed a promising light on clinical application of HPC in the treatment of laminitis in the near future.

## Materials and Methods

### Tissue Harvest and Cell Culture

Samples of coronary corium tissue were collected post-mortem from 6 foals, at a local slaughterhouse. The animals were euthanized for reasons unrelated to this study. The tissue was dissected in sterile conditions with a scalpel blade and placed in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Sigma Aldrich/Merck, Poznan, Poland) supplemented with 1% penicillin/streptomycin mix (P/S, Sigma Aldrich, Munich, Germany).Within 1,5 h of harvest, EHSPC cells were isolated and cultured, following the protocol previously described by Yang et al. [14]. The osteogenic and chondrogenic differentiation were induced using StemPro™ Osteogenesis Differentiation Kit and StemPro™ Choondrogenesis Differentiation Kit(Thermo Fisher Scientific, Warsaw, Poland). Additionally, extracellular mineralization was visualized with Alizarin-Red staining, after 7 days of osteogenic differentiation. Safranin O stain was applied to confirm the formation of proteoglycans at day 7 and 14 of the chondrogenic differentiation. Images from cell culture were obtained using an inverted microscope Leica DMi1 integrated with camera MC170 (Leica Microsystems, KAWA.SKA Sp. z o.o., Zalesie Gorne, Poland). The proliferation rate of EHSPC cells was analysed using the MTS Assay Kit (ab197010, Abcam,Cambridge, UK), accordingly to the manufacturers protocol. The population doubling time was assessed using the reazurin-based assay kit (TOX -8) (Sigma Aldrich, Munich, Germany). All of the assays in this study were performed using EHSPC cells at maximum 3$^{rd}$ passage.

### Cells Morphology Assessment

The morphology of EHSPC cells was visualized using a epifluorescent microscope (Zeiss, Axio Observer A.1) as well as confocal microscope (Leica TCSSPE, Leica Microsystems, KAWA.SKA Sp. z o.o., ZalesieGorne, Poland) and analyzed with Fiji is just ImageJ software (ImageJ 1.52n, Wayne Rasband, National Institute of Health, USA). The nuclei were stained with Hoechst stain(Thermo Fisher Scientific, Warsaw, Poland), accordingly to the manufacturers protocol. Additionally, MitoRed and Phalloidin-Atto 488 (Sigma Aldrich/Merck, Poznan, Poland) staining was performed, following established protocols [15]. Furthermore, cells were observed with a scanning electron microscope (SEM)( Evo LS 15 Zeiss). For the SEM imagining EHSPC cells were prepared as it was previously described [16].

### HE Staining

Fragments of fresh tissue were fixed in 4% Paraformaldehyde (PFA) solution for minimum 24 h. H&E staining was performed, using well established protocols. Briefly, after the incubation in the 4% PFA solution, tissue fragments were washed 3 times in Hank's Balanced Salt Solution (HBSS) and dehydrated in a graded series of ethanol dilutions (from 50 to 96%, every 10%). Next, samples were first incubated in xylene (POCH SA—Avantor Performance Materials Poland SA) for 30 min and then moved into a xylen-parrafin solution(50% xylen, 50% paraffin) for 60 min

at 37 °C. Subsequently, tissue fragments were placed in pure paraffin(Chempur, Poland) for 24 h at 65 °C. The paraffin was changed after 24 h, and the samples were incubated in pure paraffin at 65 °C for another hour. The tissue fragments were then embedded into paraffin blocks. Paraffin blocks were trimmed and cut into 6 µm slices, which were mounted onto glass slides. The hematoxylin and eosin staining (H&E) was conducted using Hematoxylin Solution (Harris Modified) and Eosin Y-solution 0.5% aqueous (Sigma Aldrich, Munich, Germany), respectively, accordingly to the manufacturers protocol. Lastly, the slides were sealed with DPX medium (Aqua-Med ZPAM – KOLASA sp.j, Łodź, Poland).

## Flow Cytometry

Flow Cytometry analyses were performed using S3e Cell Sorter (Bio-Rad, Hercules, CA, USA). In order to evaluate the expression of Nestin and CD29, cells were detached from the culture flask and washed 3 times, one time with HBSS supplemented with 2% FBS (Fetal Bovine Serum)

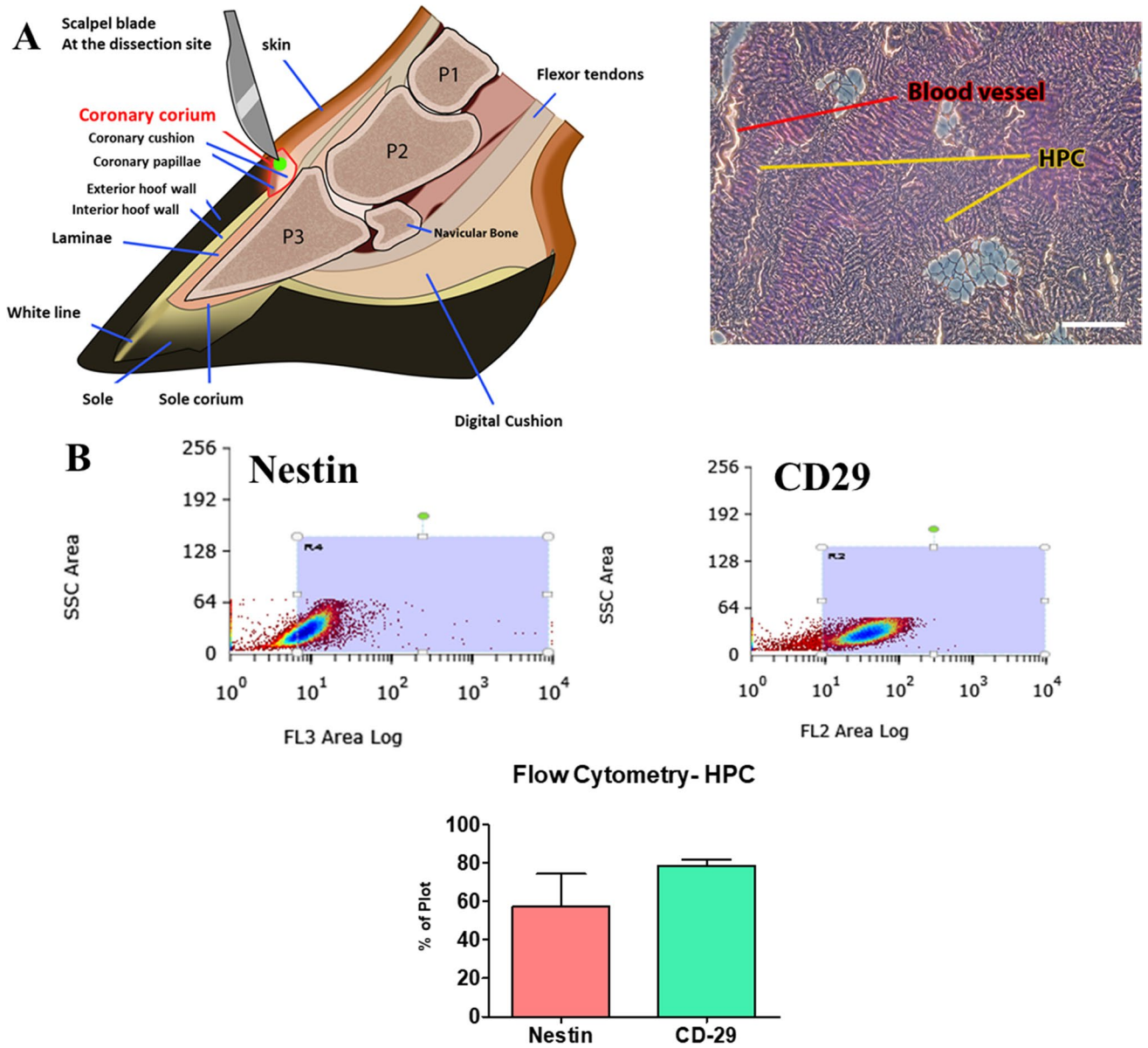


**Fig. 1** Cells isolation and phenotyping. Cells were isolated from coronary corium of horse feet (schematic drawing shows the cells niche). Tissue sections was harvested, fixed and subjected to H&E staining **A**. Isolated cells were cultured in vitro and subjected to flow cytometry analysis. Histograms and quantitative data show the results from nestin and CD29 analysis **B**. Furthermore, phenotype of cells was investigated with RT-qPCR analysis of Nestin **C**, K14 **D**, K15 **E**, VEGFA **F**, CD200 **G**, ANG1 **H**, OCT4 **I**, SOX2 **J** and NANOG **K** expression. Results expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

and twice with pure HBSS. After each wash cells were centrifuged at 1200 g for 5 min. Subsequently, EHSPC cells were suspended in 300 µl of HBSS and incubated with PE Mouse anti-Nestin Clone 25/NESTIN antibody (BD Biosciences) and Alexa Fluor® 488 anti-human CD29 Antibody (BioLegend) for 30 min in dark, at the room temperature. Following the incubation, cells were centrifuged at 1200 g for 5 min, the supernatant was discarded and the cell pellet was suspended in 300 µl of HBSS for the flow cytometry analysis.

### RT and qPCR

Total RNA was isolated using the phenol–chloroform method [17]. The experimental cultures of EHSPC cells were homogenised with Extrazol® (Blirt DNA, Gdansk, Poland). The elimination of the genomic DNA (gDNA) from the samples was carried out using the DNase I from PrecisionDNAse kit (Primerdesign, BLIRT S.A, Gdansk, Poland). Following the purification, total RNA (500 ng) was transcribed into cDNA using Tetro cDNA Synthesis Kit (Bioline Reagents Limited, London, UK), according to the manufacturers protocol. The digestion of gDNA and the reverse transcription reaction (RT) were performed in the T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Following the RT reaction, samples were used for qPCR analyses in order to analyse the expression of targeted genes. The specific primers used in the qPCR reaction are listed in Supplementary Table 1. All qPCR analyses were performed using the SensiFast SYBR & Fluorescein Kit (Bioline Reagents Ltd., London, United Kingdom) and the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Reactions were performed in a 10-µL final volume. The analyses were performed at the following cycling conditions: 95 °C for 2 min, followed by 39 cycles at 95 °C for 15 s, annealing for 30 s, and elongation at 72 °C for 15 s. The obtained results were normalized to a reference gene expression- glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the relative expression was calculated with the 2-ΔΔCQ method [18].

### Statistical Analysis

The data obtained in this study was analyzed with GraphPadPrism 5 software (La Jolla, CA, USA). Each time the mean was calculated from a minimum of three measurements. Differences between various groups were determined with one-way analysis of variance (ANOVA),parametric assays or the unpaired Student's t–test. Differences with a probability of $p < 0.05$ were considered as significant.

## Results

### Cells Isolation and Phenotyping

Light photomicrographs of H&E stainings of coronary corium from where cells were isolated (Fig. 1A). Passage 2

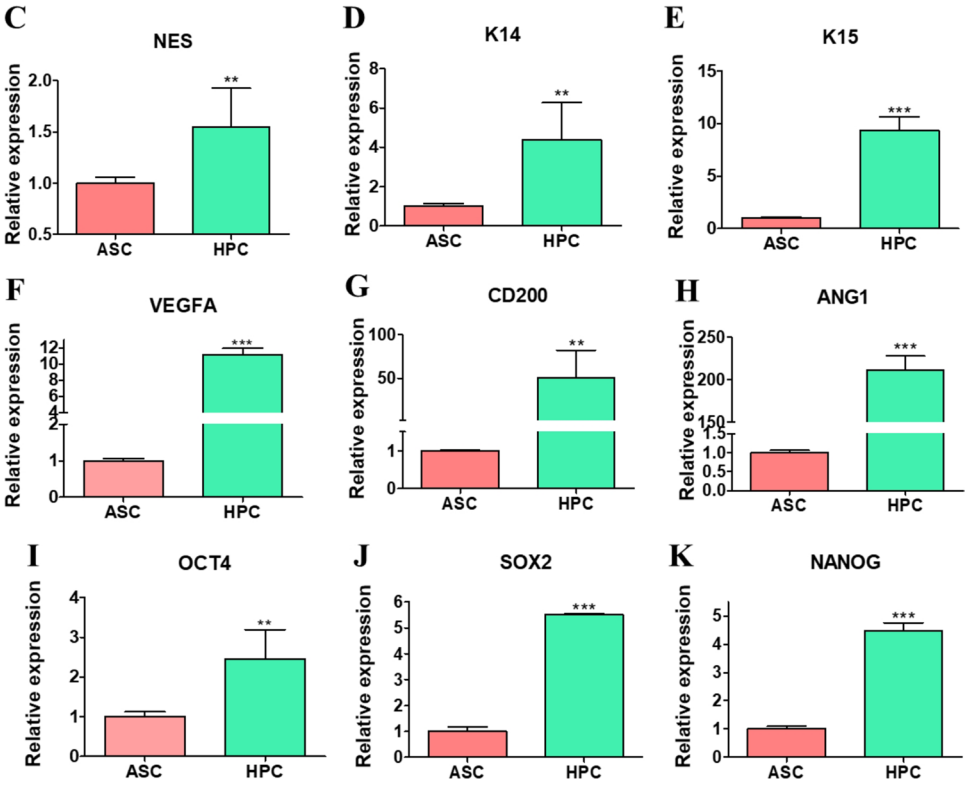


**Fig. 2** Morphology and proliferation. Fluorescent photomicrographs of cultured HPC visualised in epifluorescent **A** and confocal microscope **B**. Proliferation of cells was established with MTS in relation to ASC growth kinetics **C**. Based on obtained data, PDT was calculated **D**. Results expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. Epifluorescent microscope magnification × 100, scale bar: 250 µm, confocal × 600, scale bar 10 µm

HPC cells were subjected to flow cytometry analysis which confirmed the expression of Nestin and CD29 (Fig. 2B). RT-qPCR analysis of isolated cells, confirmed the expression of Nestin (Fig. 1C), K14 (Fig. 1D), K15 (Fig. 1E), VEGFA (Fig. 1F), CD200 (Fig. 1G), ANG1 (Fig. 1H), OCT4 (Fig. 1I), SOX2 (Fig. 1J) and NANOG (Fig. 1K).

## Morphology and Proliferation

In order to visualise the morphology of isolated cells, they were subjected to fluorescent stainings. Nuclei were stained with DAPI while f-actin with phalloidin. Images were acquired with epifluorescent (Fig. 2A) and confocal microscope (Fig. 2B). Large, eccentric nuclei and elongated mitochondria evenly distributed within cytoplasm were observed. Proliferation of HPC of similar to ASC, however after 72 h, cells of hoof origin displayed increased growth kinetic (Fig. 2C). PDT of HPC was reduced while in comparison to ASC (Fig. 2D) which indicates on greatest proliferative potential of these cells.

## Multilineage Differentiation Potential

Passage 1 HPC were subjected for osteogenic and chondrogenic differentiation and their morphology was visualised with light, phase contrast microscope after 3rd, 7th and 14th day of culture (Fig. 3A). Cell cultured in control medium displayed spindle shaped morphology similar to progenitor cells including ASC. Cells cultured in differentiation medium underwent morphological change, become more cuboidal in shape, formed aggregates and extracellular matrix (ECM). Detailed morphology was visualised with SEM (Fig. 3B) and formation of ECM and bone modules was noted. Differentiation was further confirmed with specific stainings after 7th day (Fig. 3C). Safranin O revealed accumulation of proteoglycans during chondrogenesis while Alizarin Red stained mineralised matrix formed during osteogenic differentiation. What is more, the expression of marker genes after 14 day of differentiation was investigated with RT-qPCR in cells cultured in control (CTRL), osteogenic (O) and chondrogenic

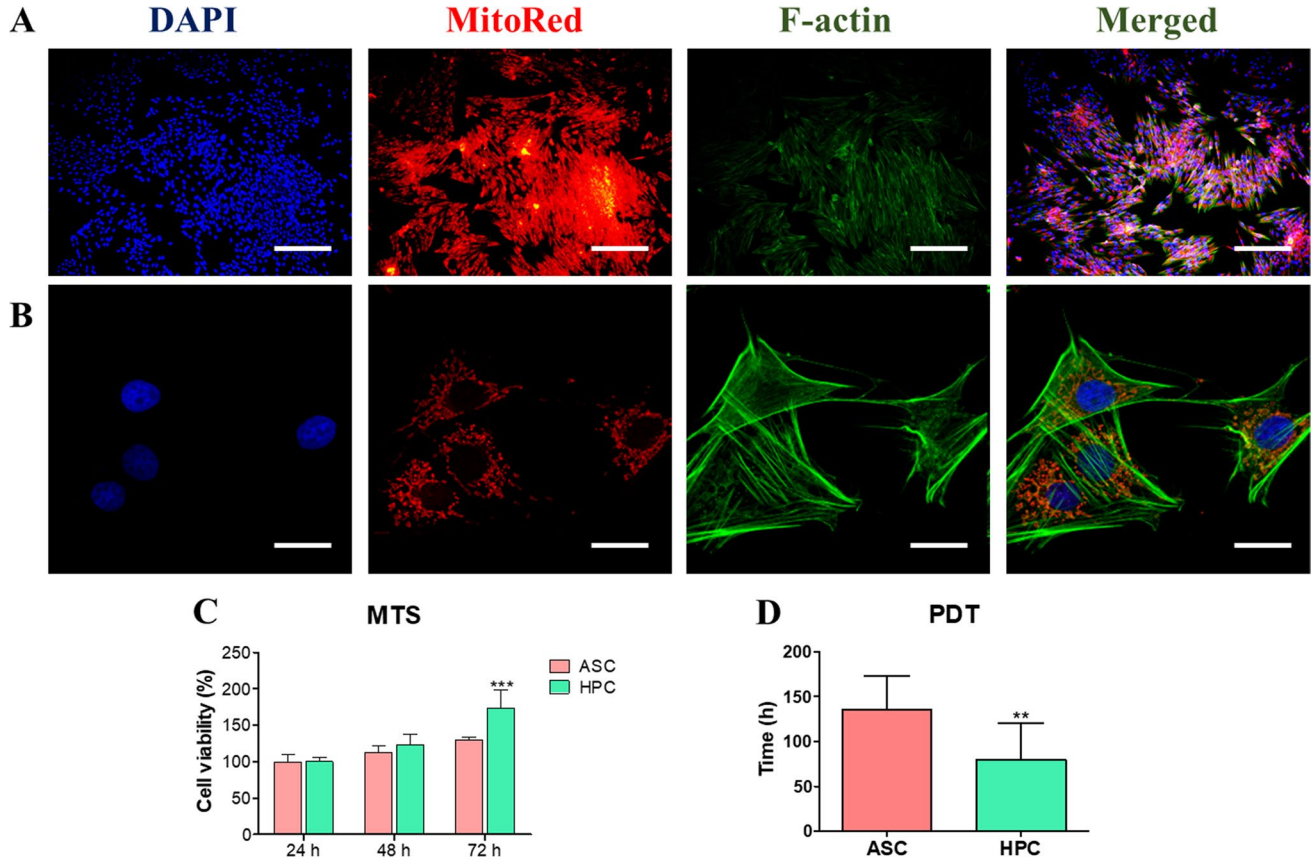


**Fig. 3** Multilineage differentiation potential of HPC. Photomicrographs of passage 1 cells cultured in control, chondrogenic and osteogenic medium **A**. Detailed morphology of cells after 14$^{th}$ day of differentiation visualised with SEM **B**. Differentiation of cells after 7th day of culture was confirmed with Safranin O and Alizarin Red **C**. Expression of genes involved with differentiation process- COMP **D**, RUNX2 **E**, ACAN **F**, OPN **G**, RUNX3 (H), DCN **I**, COLL1 **J** and COLL2 **K** was investigated with RT-qPCR (CTRL- cells cultured in control medium, O- cells cultured in osteogenic and CH- cells cultured in chondrogenic differentiation media). Results expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Magnification (light microscope) × 100, scale bar: 250 µm

medium (CH). Expression of COMP (Fig. 3D), RUNX2 (Fig. 3E), ACAN (Fig. 3F), OPN (Fig. 3G), RUNX3 (Fig. 3H), DCN (Fig. 3I), COLL1 (Fig. 3J) and COLL2 (Fig. 3K) was investigated in relation to culture condition.

## Discussion

Equine adult stem progenitor cells of various origins are recently extensively used for the treatment of different disorders including musculoskeletal or endocrine systems [8, 19, 20]. The mesenchymal stem progenitor cells represent a unique population of stem cells that can be used for the treatment of particular disorders in horses, however, the efficacy of injured tissue treatment might strongly depend on their molecular phenotype and the place from which the stem cells were isolated. The tissue-specific microenvironment modulates stem cell fate and regenerative potential after their transplantation, which can suggest the clinical crosstalk between their place of origin and final tissue regeneration [21, 22].

Here, for the first time we identified a novel stem progenitor cell population that resides in the equine hoofs, particularly in coronary corium that could give rise to both: coronary dermis/epidermis as well as lamellar epidermis and finally the hoof wall. We speculate that this stem cell pool- HPC could play a protective function within the coronary corium, being capable of responding to coronary band injury, digital extensor tendons as well as hoof lamellae. For the first time, we identified octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2), $CD105^+$, $CD200^+$, $K15^+$ and $Ang1^+$ expressing HPC. Isolated cells are highly proliferative, plastic adherent and display fibroblast like morphology – all fundamental for confirmation of stemness. They also possess to multilineage differentiation potential as they were shown to give rise to mature and functional osteoblasts and chondroblasts.

In this study, we have identified that HPC express high levels of CD29, Nestin, K15, CD200, VEGF, and Ang1, which indicates on their potential role in the regeneration of dermal/epidermal and vascular injury during laminitis. CD200 has been shown to have immunomodulatory and immunosuppressive functions via apoptosis protecting the follicular stem cell niche from autoimmune attacks as well as modulating the local immune system [23]. In turn, both: vascular endothelial growth factor (VEGFA) and angiopoietin-1 (Ang1) have been proposed in horses to be a critical player in angiogenesis initiation by triggering endothelial cell proliferation, migration and neovascularization – all critical to regenerate the injured vascular system during laminitis [24]. Recent showed that Ang1 is critically involved in vascular and hematopoietic development as well as embryonic stem cell differentiation, mainly through its cognate receptor Tie2, which indicates on HPC involvement in the modulation of the new vascularization process [25]. Interestingly, when compared to ASCs, HPC were characterized by increased Ang1 expression which could suggest they advantage over ASCs during the vascularization process.

Here, we have found that HPC, when compared to widely investigated adipose derived stem progenitor cells (ASCs), possess significantly higher expression of keratin 15 (K15), suggesting their high potential for regeneration dermis/epidermis injure – that are a common events during laminitis. Cytokeratin 15 (clone C8/144B) has been shown to be a stem cell marker for hair follicle regeneration, since $K15^+$ cells could be recruited to assist in epidermal re-epithelialization following injury [26]. Numerous adult epidermal stem cells have been shown to express K15 [12, 26–28] which suggests that identified by the us stem cell population could give rise a dermis/epidermis structure similar to human nail stem cells. Although previous excellent studies of Yang and Lopez [29] demonstrated and investigated the hoof progenitor stem cells isolated from the stratum lamellatum, that have both ectodermal (neurogenic) and mesodermal (osteogenic, adipogenic) differentiation abilities, we put our attention on HPC, since we hypothesize their localization as well as specific markers expression could be a beneficial for laminitis treatment.

Limitation of our research is that due to COVID-19 we did not have enough samples for performing more detailed cytometric analyses, immunomodulatory assays, or western blot which we could be the focus of the next research.

To summarize, here we identified a novel stem cell pool – OCT4, SOX2, $CD105^+$, $CD200^+$, K15 +, and $Ang1^+$ HPC which meet the criteria of International Society of Cellular Therapy to be entitled stem cells and applied as a therapeutic agent. The HPC exhibit high pro-vasculogenic and immunosuppressive character, which shed a promising light for their future clinical application during laminitis.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s12015-021-10187-x.

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**Authors' Contributions** KM, AP performed the animal experiments, AP performed cell culture and molecular biology assay, KM, KKG, AP drafted the manuscript, revised the manuscript and discussed the results. KM and KKG designed and coordinated the experiments, interpreted the data, revised and confirmed the paper.

**Data Availability** All datasets generated and/or analyzed during the current study are presented in the article, the accompanying Source Data or Supplementary Information files, or are available from the corresponding author upon reasonable request.

## Declarations

**Consent for Publication** All authors have given their consent for publication and have reviewed and approved the submission.

**Conflicts of Interest** Authors declare that there is no conflict of interest.



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# Equine Hoof Progenitor Cells Display Increased Mitochondrial Metabolism and Adaptive Potential to a Highly Pro-Inflammatory Microenvironment

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**Abstract:** Medicinal signaling cells (MSC) exhibit distinct molecular signatures and biological abilities, depending on the type of tissue they originate from. Recently, we isolated and described a new population of stem cells residing in the coronary corium, equine hoof progenitor cells (HPCs), which could be a new promising cell pool for the treatment of laminitis. Therefore, this study aimed to compare native populations of HPCs to well-established adipose-derived stem cells (ASCs) in standard culture conditions and in a pro-inflammatory milieu to mimic a laminitis condition. ASCs and HPCs were either cultured in standard conditions or subjected to priming with a cytokines cocktail mixture. The cells were harvested and analyzed for expression of key markers for phenotype, mitochondrial metabolism, oxidative stress, apoptosis, and immunomodulation using RT-qPCR. The morphology and migration were assessed based on fluorescent staining. Microcapillary cytometry analyses were performed to assess the distribution in the cell cycle, mitochondrial membrane potential, and oxidative stress. Native HPCs exhibited a similar morphology to ASCs, but a different phenotype. The HPCs possessed lower migration capacity and distinct distribution across cell cycle phases. Native HPCs were characterized by different mitochondrial dynamics and oxidative stress levels. Under standard culture conditions, HPCs displayed different expression patterns of apoptotic and immunomodulatory markers than ASCs, as well as distinct miRNA expression. Interestingly, after priming with the cytokines cocktail mixture, HPCs exhibited different mitochondrial dynamics than ASCs; however, the apoptosis and immunomodulatory marker expression was similar in both populations. Native ASCs and HPCs exhibited different baseline expressions of markers involved in mitochondrial dynamics, the oxidative stress response, apoptosis and inflammation. When exposed to a pro-inflammatory microenvironment, ASCs and HPCs differed in the expression of mitochondrial condition markers and chosen miRNAs.

**Keywords:** equine; hoof; progenitor cells; stem cells; laminitis; adipose stem cells; ASC



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## 1. Introduction

The rise of stem cell-based therapy in regenerative medicine is a direct answer to the need for personalized and efficient treatment strategies in various human and animal conditions [1,2]. With the application of various types of stem cells, the main objective of stem cell-based therapy is to enhance the repair of the targeted cells, tissues, or organs by restoring homeostasis and promoting regeneration [2]. Thus far, several stem cell types have been studied and utilized in clinical trials, including induced pluripotent stem cells (IPSCs) and multipotent mesenchymal stem cells (MSCs). Due to a lack of ethical concerns as well as a relatively simple isolation and culture process, MSCs are being widely applied in different stem cell-based therapies in human and veterinary medicine [1,3].

MSCs can be isolated from several different tissue sources, primarily including bone marrow, adipose tissue, placenta and umbilical cord. Various studies have previously identified distinct molecular signatures and biological abilities for each MSC population depending on the type of tissue they originate from. Therefore, the expected outcomes of MSCs-based therapies may strictly depend on their intrinsic properties and defined secretome, and can provide better insights into the selection of appropriate MSC pools in the context of disease-tailored therapies [4].

Importantly, the origin of stem cells and the condition of the donor play a crucial role in their therapeutic potential [5]. It is apparent that the residing stem cell populations are affected in the course of many diseases, and that their impairment contributes to the evolvement of symptoms [6–9]. Furthermore, the inflammatory microenvironment negatively affects the residing population of stem cells. Since the state of the donor plays such a vital role in the MSC condition, in general, allogenic MSC therapy seems to have more advantages, as it provides biologically fit cells in an "off-shelf" manner [10].

Recently, we isolated and described a new population of stem cells residing in the coronary corium: hoof progenitor cells (HPCs) [11,12]. HPCs are plastic adherent, and their capacity for multilineage differentiation was confirmed based on chondrogenesis and osteogenesis results. The expression of two surface markers: Nestin and CD29, was confirmed in HPCs using flow cytometry. Additionally, HPCs were compared to a well-established MSC subset, ASCs. Based on this comparison, we observed that HPCs were characterized by high gene expression of *Nestin*, *K14*, *K15*, *Vegfa*, *CD200*, *Ang1*, *Oct4*, *Sox2* and *Nanog*. We hypothesized that, similar to other stem cell populations, HPCs might also be affected in the course of inflammation, just as ASCs are affected in the course of EMS, a disorder in which inflammation is one of the crucial components for the development of insulin resistance [13,14]. In the case of ASCs isolated from horses with EMS, lower viability, reduced clonogenic potential, senescent phenotype and increased accumulation of oxidative stress factors were observed [13]. Therefore, further research is imperative to fully understand HPCs' reaction to the inflammatory environment.

Furthermore, due to the location of HPCs in the coronary corium, we hypothesized that they could play a crucial role in the course of laminitis, which is a severe equine foot disorder defined as an inflammation of the laminae—the structure responsible for the attachment of the hoof capsule to the third phalanx (coffin bone) [15,16]. Most often, the cause of laminitis is endocrinopathic [17,18]. The local cellular and humoral milieu of laminitis is characterized by endothelial cell swelling, leukocyte infiltration, erythrocyte accumulation, and the secretion of proinflammatory cytokines such as Il-6, Il-8, Il1β and matrix metalloproteinases [15,19–21]. In recent years, more focus has been directed towards understanding the pathophysiology of EMS and laminitis, especially regarding the ramifications for stem cells residing in both adipose tissue and hoofs.

Importantly, attempts to utilize mesenchymal stem cells in laminitis treatment have been proposed. Angelone et al. [21] used ASCs' platelet-rich plasma, and such treatment was proven to be effective. Whether HPCs could also be utilized in laminitis therapy is currently unknown. Possibly, due to their unique secretome, the result of such intervention could be favorable. It is apparent that further research into the underlying mechanism of the disease and a search for new therapeutic strategies and targets, including MSCs, is necessary.

The aim of this study was to compare the native populations of ASCs and HPCs in standard culture conditions and their response to the inflammatory milieu that occurs during EMS and laminitis. As the inflammatory microenvironment negatively affects ASCs, we sought to determine how such conditions will affect HPCs. Therefore, we performed priming using a cytokine cocktail mixture (TNFα + IL1β + IFNγ) on these two distinct cell populations. Cellular morphology and metabolism were assessed using methods such as cytometry, qPCR, Western blot and immunofluorescent staining. The presented results could provide insight into HPC metabolism and their regenerative potential in clinical applications.

## 2. Results

### *2.1. ASCs and HPCs Have Similar Morphologies but Exhibit Different Phenotypes*

The general morphology of ASCs and HPCs cells was assessed using confocal microscopy (Figure 1A). Both ASCs and HPCs displayed characteristic, fibroblast-like morphology, with oval-shaped nuclei. In both populations, the cells exhibited a highly developed cytoskeleton with well-defined F-actin tubules and a low nuclear–cytoplasm ratio. The mitochondrial network was evenly distributed in both populations, and the mitochondria appeared in the form of short, separated tubules and round spheres. We sought to identify the differences in the phenotypes of ASC and HPC native populations; therefore, we compared the relative gene expression of surface markers (Figure 1B). The expression of *Nestin* was significantly higher in the HPC population, while the *CD105* transcript was more abundant in the ASC population. There was no difference in the expression of *CD29* between ASCs and HPCs. Furthermore, we observed that ASC and HPC native populations were characterized by disparate expression of angiogenesis markers (Figure 1B). Transcripts such as *Vegfa* (**) and *Hif1a* (*) were more abundant among HPCs, while there was no statistical difference in the expression of *Ang1*, and the expression of *Igf1* (**) was higher in the ASC population. The expression of matrix metalloproteinases (Figure 1B) was significantly higher in the ASC population (*Mmp14****, *Mmp2****), with the exception of *Mmp9* (**), as the levels of this transcript were higher in HPCs.

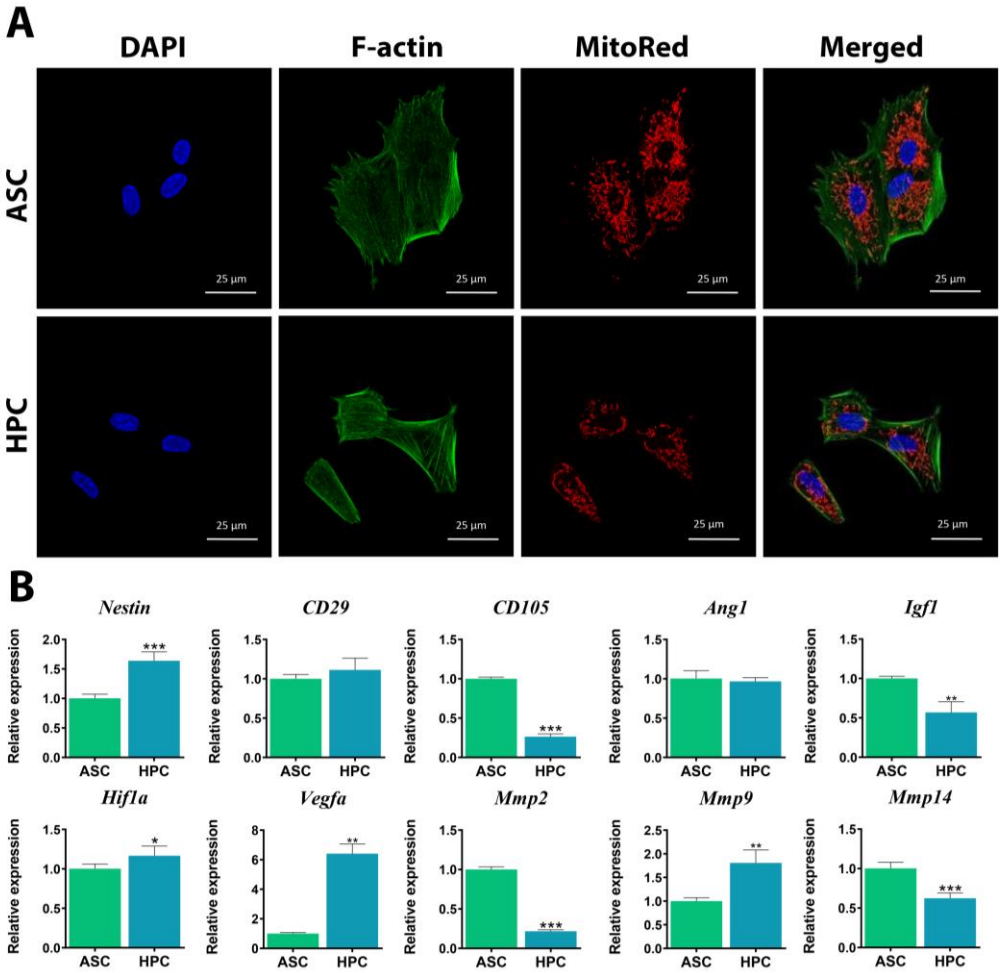


**Figure 1.** Characterization of ASC and HPC morphology and gene expression of surface markers. (**A**) Representative confocal photographs of ASCs and HPCs labelled with DAPI (blue), phalloidin (green) and MitoRed (red) showcase differences in cellular morphology. (**B**) The phenotypes of ASC and HPC native populations were assessed using RT-qPCR to determine the relative expression

analysis of surface markers (*CD105*, *Nestin*, *CD29*), angiogenesis markers (*Ang1*, *Igf1*, *Hif1a*, *Vegfa*) and matrix metalloproteinases (*Mmp2*, *Mmp9*, *Mmp14*). Results are expressed as mean ± SD. Statistically significant differences are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

### 2.2. HPCs Have Lower Migration Capacity and Different Cell Cycle Dynamics Compared to ASCs

The capacity for migration of both native cell populations was assessed using a scratch wound healing assay (Figure 2A,B). The difference between ASCs and HPCs was noticeable at the first time point at 0 h after the scratch wound was performed, with ASCs exhibiting better migration properties. The difference was significant throughout the entire assay, as the wound in the ASC wells was visibly less prominent when compared to HPCs at all time points (Figure 2A,B). Furthermore, native ASCs displayed significantly (**) higher colony-forming efficiency when compared to HPCs (Figure 2C). Differences between ASCs and HPCs were also apparent in the cell cycle dynamic; the HPCs population had a significantly higher percentage of cells in the G0/G1 phase and a lower percentage of cells in the G2/M phase compared to ASCs (Figure 2D).

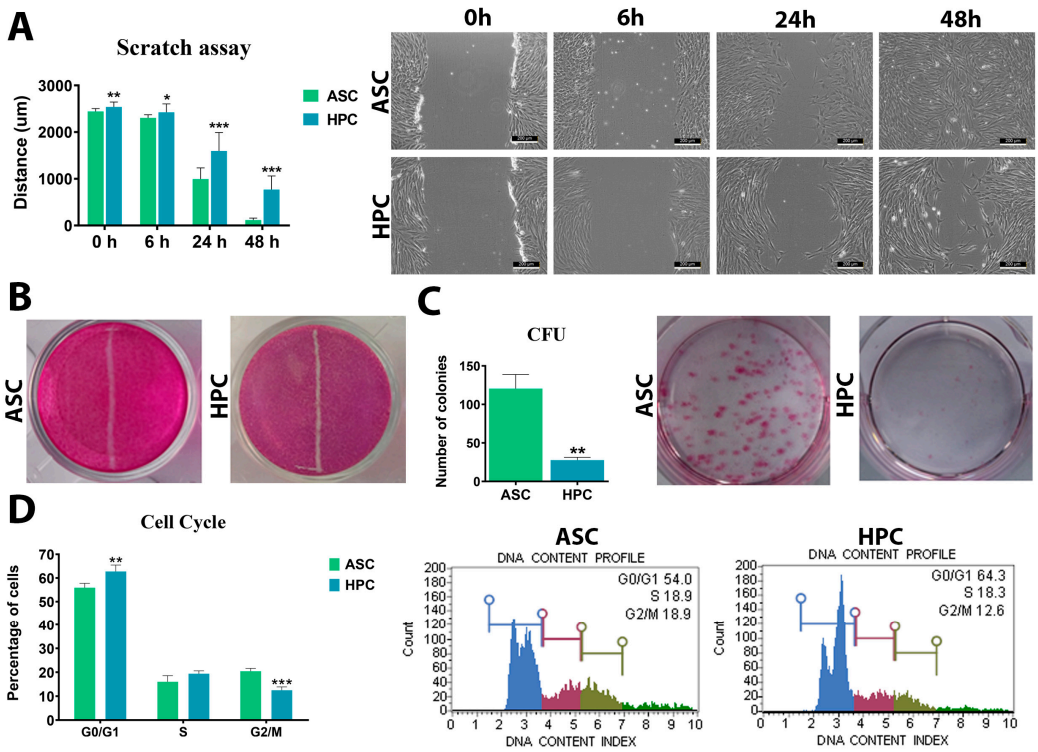


**Figure 2.** (**A**) Scratch wound healing assay of ASC and HPC native populations. Scale bar = 200 µm. (**B**) Pararosaline staining of the scratch wound healing assay performed 48 h after the scratch wound. (**C**) Analysis of colony-forming efficiency in ASC and HPC native populations. (**D**) The distribution of ASCs and HPCs in the cell cycle (blue-G0/G1; pink-S; olive-G2/M, green-ungated/debris). Results are expressed as mean ± SD. Statistically significant differences are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

### 2.3. Native HPCs Possess a Distinct Mitochondrial Metabolism Compared to ASCs

The assessment of mitochondrial metabolism in ASC and HPC native populations was based on the analysis of mitochondrial membrane polarization and the gene expression of markers involved in mitochondrial dynamics. The HPC population displayed a significantly (**) higher content of live cells with polarized mitochondrial membranes (Figure 3A). The number of cells with depolarized mitochondrial membranes, whether dead or alive, was higher in the ASC population. Overall, the HPC population was characterized by a lower percentage of dead cells. Furthermore, the gene expression of fission-related *Fis1*

(***) and *Mief2* (***) was lower in the HPC population, while the expression of *Dnm1l* was notably higher (Figure 3B). There was no significant difference in the *Mief1* gene expression. At the mRNA level, the expression of *Pink1*, which is involved in mitophagy, was lower in native HPCs, and a similar pattern was observed for the *Mfn1* (***) transcript. Finally, the expression of *Opa1* (**) and *Rhot1* (***) transcripts was significantly higher in HPCs compared to ASCs.

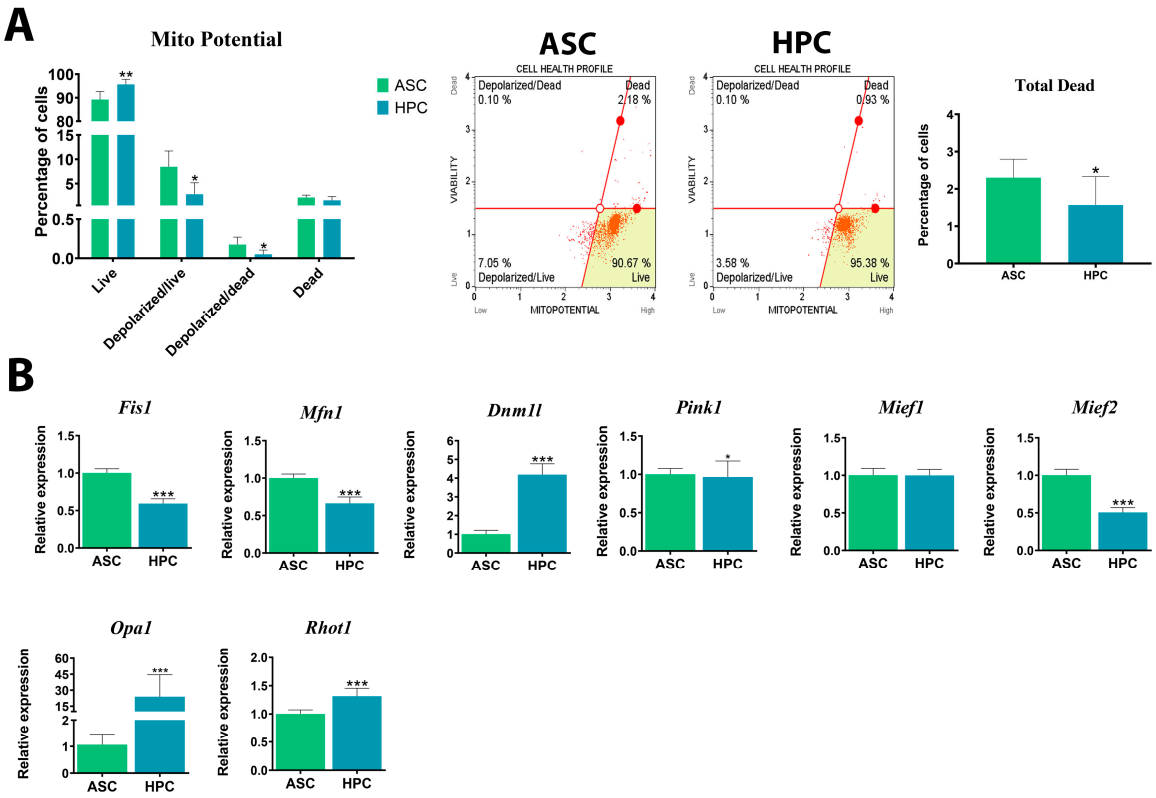


**Figure 3.** (**A**) Mitochondrial membrane polarization analysis of HPC and ASC native populations. The analysis was performed with the Muse® MitoPotential Kit. (**B**) Gene expression of key mitochondrial dynamics markers (*Fis1*, *Mfn1*, *Dnm1l*, *Pink1*, *Mief2*, *Mief1*, *Opa1*, *Rhot1*). The mitochondrial dynamics in HPC and ASC native populations were tested using a RT-qPCR assay. Results are expressed as mean ± SD. Statistically significant differences are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

### *2.4. HPCs Exhibit Lower Oxidative Stress Levels Compared to ASCs*

The HPC native population exhibited minimal levels of oxidative stress, as evidenced by the reduced percentage of ROS-positive cells (***) (Figure 4B) and a lower expression of key antioxidant enzymes (*Sod1****, *Sod2****, *Cat1***) (Figure 4A). The content of total nitric oxide (**) was also significantly lower in the HPCs (Figure 4C).

### *2.5. HPCs Display Different Gene Expression Patterns of Apoptotic and Immunomodulatory Mediators Compared to ASCs*

Overall, the expression of key apoptosis markers (Figure 5) was significantly lower in the HPC native population (*p21****, *p53****, *Bax****, *Bcl2****, *Casp3****, *Casp9****), and the *Bax:Bcl2* ratio was also significantly lower (**). The immunomodulatory marker expression varied between the two cell populations. The gene expression of proinflammatory cytokines was significantly higher in the HPCs (*Il6***, *Il8***); however, the expression of anti-inflammatory cytokines was lower (*Il10****, *Il13****). Yet, the gene expression of *Tnfα* (***) was higher in the ASC population. Additionally, the expression of *Il1β* (***) was higher in the ASCs. Furthermore, *Tgfβ1* (**) and *Mcp1* (**) mRNA transcripts were more

abundant in the HPC population. The expression of genetic markers associated with the *Nfkb* pathway was higher in the ASCs (*Ikbkb* **, *Nfkbia****, *Nfkb****).

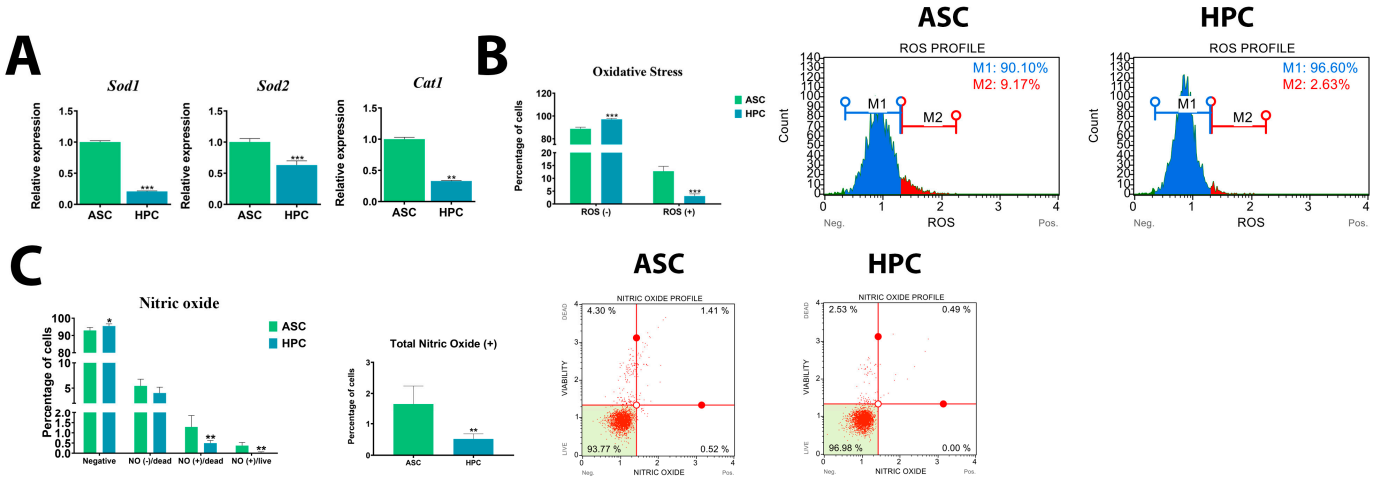


**Figure 4.** (**A**) Gene expression of key oxidative stress markers (*Sod1*, *Sod2*, *Cat1*) assessed using RT-PCR. (**B**) Characterization of oxidative stress in HPC and ASC native populations. The analysis was performed using the Muse® Oxidative Stress Kit. (**C**) Nitric oxide activity analysis in ASC and HPC native populations. The analysis was performed using the Muse® Nitric Oxide Kit. Results are expressed as mean ± SD. Statistically significant differences are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

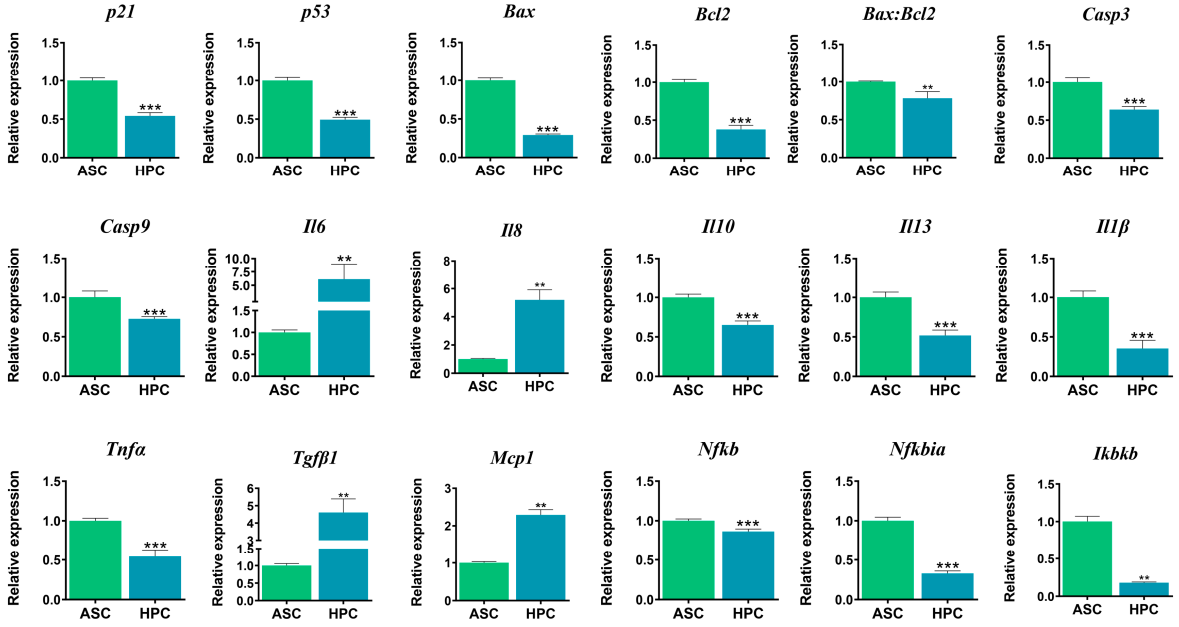


**Figure 5.** Gene expression of apoptosis markers (*p21*, *p53*, *Bax*, *Bcl2*, *Casp3*, *Casp9*) and immunomodulatory mediators (*Il6*, *Il8*, *Il10*, *Il13*, *Il1b*, *Tnfa*, *Tgfb1*, *Mcp1*, *Nfkb*, *Nfkbia*, *Ikbkb*) in native ASCs and HPCs, assessed using RT-qPCR. Results are expressed as mean ± SD.Statistically significant differences are marked with an asterisk (** $p < 0.01$, *** $p < 0.001$).

### 2.6. Priming with a Cytokine Cocktail Exerts a Different Effect on ASC and HPCs' Mitochondrial Metabolisms

In order to assess the effect of priming on the ASC and HPCs' mitochondrial networks, microphotographs of untreated cells (CTRL) and cells treated with a TNFα + IL1β + IFNγ (CC) mixture were captured (Figure 6B). Interestingly, for both populations, in the cells subjected to priming, the staining intensity of the mitochondria was higher; however, the difference was more visible in the ASCs (Table S2, Supplementary Materials). Furthermore, we analyzed the gene expression of mitochondrial metabolism markers (Figure 6A,C). Generally, the gene expression of fission, fusion, mitophagy and mitochondrial trafficking-related markers (Figure 6A) either decreased or did not change in both ASCs and HPCs as a result of priming. The expression of *Fis1* and *Mfn1* was significantly downregulated as a result of the cytokine cocktail treatment in HPCs (*Fis1****, *Mfn1***), while in the ASCs, only *Mfn1* was downregulated (*Mfn1****). *Pink1* was downregulated in primed ASCs (*), but no difference in its' expression was detected in primed HPCs. Both *Dnm1l* (*) and *Opa1* (**) transcripts were less abundant in the HPCs after the incubation with the cytokine cocktail, but there was no statistical difference in the expression of these markers in primed ASCs. *Rhot1* was significantly downregulated in HPCs (***) as a result of priming, but not in ASCs. As for the mitochondrial metabolism markers (Figure 6C), most were downregulated in the primed ASCs, while in HPCs, the gene expression of most markers did not change upon priming. In ASCs treated with the cytokine cocktail mixture, the expression of *Mrlp24*(***) and *Ppargc1b* (***) was significantly downregulated, but there was no significant difference in the expression of *Mterf4*. None of the abovementioned markers showcased any expression changes in the primed HPCs. *Uqcrc2* was significantly downregulated in ASCs (**) and HPCs (*) after incubation in the inflammatory conditions. The expression of *Oxa1l* (***) and *Cox4i1* (***) was only significantly downregulated in primed ASCs. The cytokine cocktail treatment resulted in significant downregulation of *Ndufa9*, but only in ASCs (*), and there was no significant difference in its' expression in HPCs. *Pusl1* was significantly downregulated in primed ASCs (**), but it was upregulated in primed HPCs (***). *Mief1* expression was significantly downregulated in ASCs (**) after the cytokine cocktail treatment; however, in HPCs (**), it was significantly upregulated. *Mief2* was downregulated in primed ASCs (*), but no change in expression was detected in HPCs.

### 2.7. Apoptosis Dynamics and Inflammatory Response to Priming Are Similar in HPC and ASC Populations

To assess how ASCs and HPCs respond to a highly pro-inflammatory microenvironment, the gene expression of apoptosis, immunomodulatory mediators, and oxidative stress markers was analyzed (Figure 7). Overall, the gene expression of apoptosis markers (Figure 7A) either decreased or did not change in ASCs and HPCs alike. The expression of *p21*(*) and *p53* (***) was significantly lower in ASCs after priming; yet, in HPCs, no significant difference was observed for *p21*, while *p53*(*) was significantly downregulated. *Casp9* expression significantly decreased in ASCs (**) and HPCs (**) after incubation with the cytokine cocktail mixture. There was no difference in the expression of *Bax* in either population, and the *Bcl2* transcript was only significantly less abundant in primed HPCs (***). Furthermore, the *Bax:Bcl2* ratio was significantly higher in HPCs (***) after priming, but no difference was noted for ASCs. Proinflammatory cytokine (Figure 7B) mRNA transcripts were significantly upregulated in ASCs (*Il6* **, *Il8* **) and HPCs (*Il6* **, *Il8* **) after the cytokine cocktail treatment. Anti-inflammatory cytokine (Figure 7B) gene expression was downregulated after priming in both ASCs (*Il10**, *Il13****) and HPCs (*Il10**, *Il13****). Additionally, *Il1β* and *Tgf β1* were also significantly downregulated in both ASC (*Il1β***, *Tgf β1**) and HPC (*Il1β****, *Tgf β1***) primed experimental groups. As for the markers of oxidative stress (Figure 7C), *Sod1* was significantly downregulated in both ASCs (***) and HPCs (**) as a result of priming, contrary to *Sod2*, which was significantly upregulated in both populations (ASCs **, HPCs ***) after the cytokine cocktail treatment. Finally, *Cat1*

was significantly downregulated in primed HPCs (*), but no significant difference was noted for the ASCs.

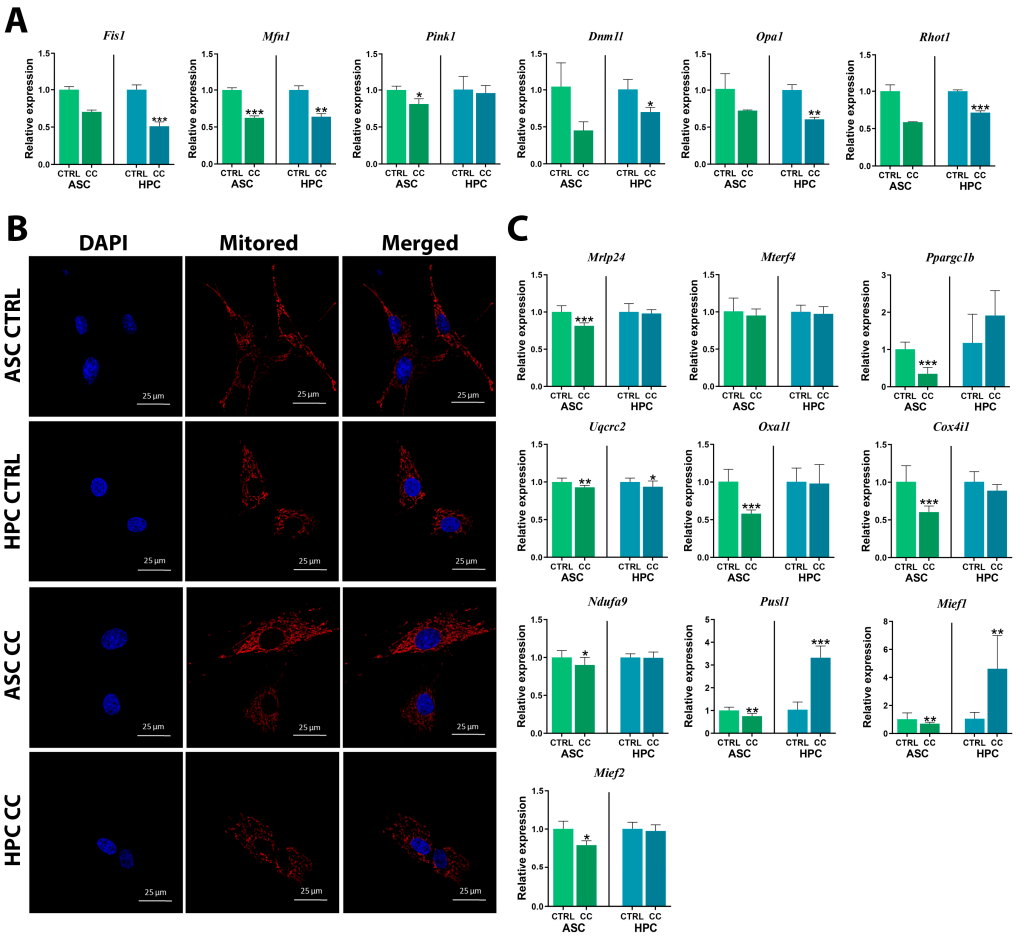


**Figure 6.** (**A**) Gene expression of key mitochondrial dynamics markers (*Fis1*, *Mfn1*, *Pink1*, *Dnm1l*, *Opa1*, *Rhot1*) in control (CTRL) and cytokine cocktail-treated (CC) ASCs and HPCs, assessed with RT-qPCR. (**B**) Confocal photographs of the control (CTRL) and cytokine cocktail-treated (CC) ASCs and HPCs labelled with DAPI (blue) and MitoRed (red). (**C**) Gene expression of key mitochondrial metabolism markers (*Mrpl24*, *Mterf4*, *Ppargc1b*, *Uqcrc2*, *Oxa1l*, *Coxhi1*, *Ndufa9*, *Pusl1*, *Mief1*, *Mief2*) in control (CTRL) and cytokine cocktail-treated (CC) ASCs and HPCs, assessed using RT-qPCR. Results are expressed as mean ± SD. Statistically significant differences are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

### *2.8. HPC and ASC Native and Primed Populations Display Different miRNA Expression Patterns*

Due to the crucial regulatory role of miRNAs in various cellular processes, their expression was assessed in HPC and ASC native populations and after priming with the cytokine cocktail mixture (Figure 8A,B). *miR-21-5p* (**), widely known as an onco-miR, was more abundant in the native HPC population, similarly to *miR-27-3p* (***) and *miR-96-5p* (**). The expression of *miR-30c-5p* (*) and *miR-34a-5p* (**) was significantly higher in the native ASCs, while *miR-34c* (**) was expressed at a lower level compared to HPCs. Native ASCs exhibited a higher expression of *miR-125a* (**) and *miR-218* (**), and lower levels of *miR-125b-5p* (***) and *miR-451* (**) transcripts.

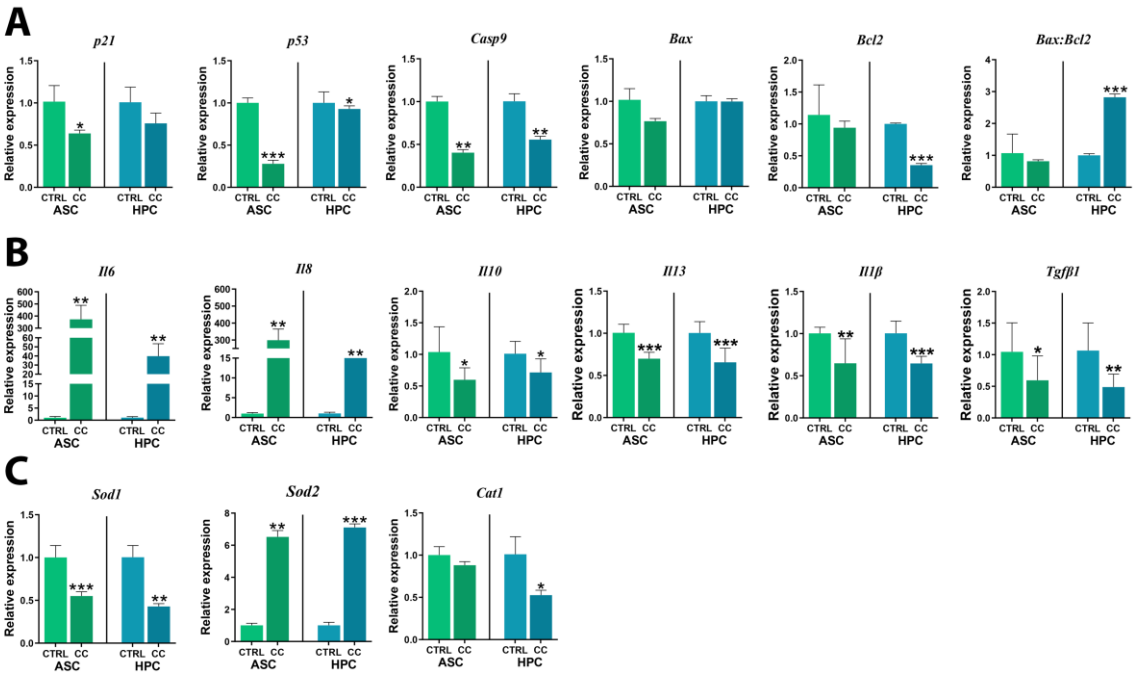


**Figure 7.** Gene expression of (**A**) apoptosis markers (*p21*, *p53*, *Casp9*, *Bax*, *Bcl2*), (**B**) immunomodulatory mediators (*Il6*, *Il8*, *Il10*, *Il13*, *Il1β*, *Tgfβ*) and (**C**) oxidative stress markers (*Sod1*, *Sod2*, *Cat1*) in control (CTRL) and cytokine cocktail-treated (CC) ASCs and HPCs, assessed with RT-qPCR. Results are expressed as mean ± SD. Statistically significant differences are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

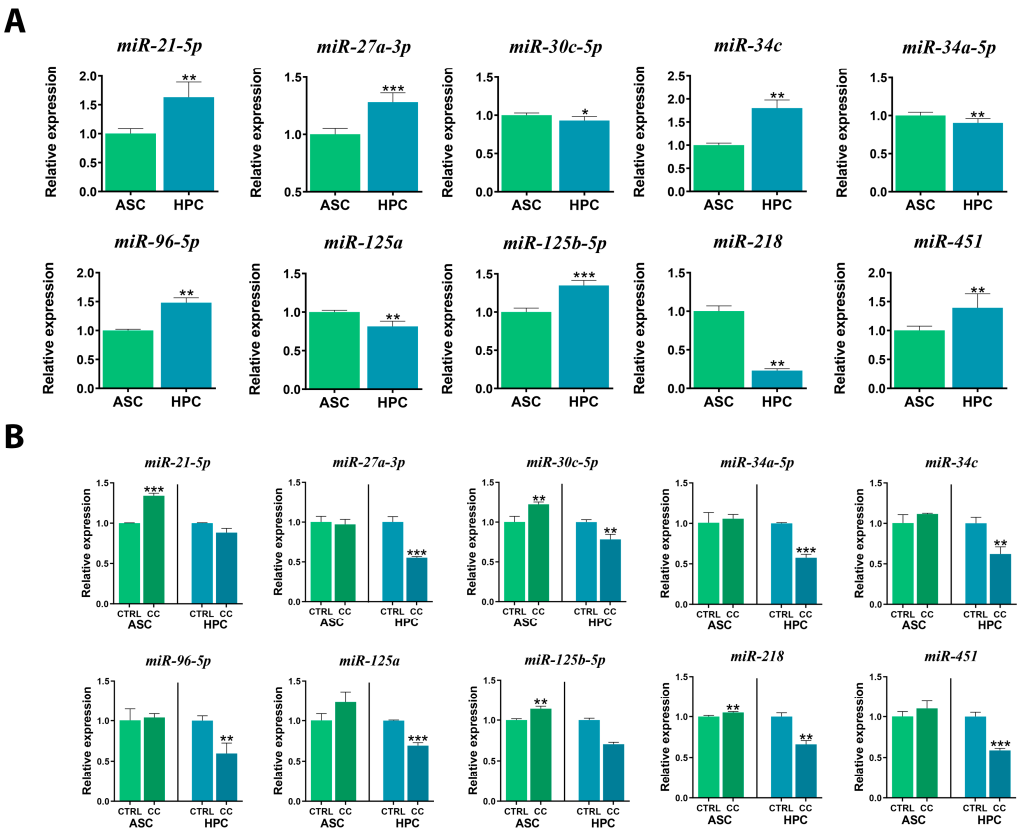


**Figure 8.** The expression of miRNAs (*miR-21-5p*, *miR-27a*, *miR-30c-5p*, *miR-34a-5p*, *miR34c*, *miR-96-5p*, *miR-125a*, *miR-125b-5p*, *miR-218*, *miR-451*) in (**A**) native ASCs and HPCs, (**B**) cytokine cocktail-treated

(CC) ASCs and HPCs, assessed using RT-qPCR. Results are expressed as mean ± SD. Statistically significant differences are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The expression patterns for miRNAs changed visibly in both populations as a result of priming (Figure 8B). Generally, the expression of miRNAs either did not change or increased after priming in ASCs, while in HPCs, the expression of all studied miRNAs decreased. The expression of *miR-21-5p* was significantly higher (***) in ASCs after priming, while in HPCs, no difference in the expression of the mentioned miRNAs was detected. A similar pattern was observed in *miR-125b-5p* expression (ASCs**). As for *miR-30-5p* (ASCs**, HPCs**) and *miR-218* (ASCs**, HPCs**), their expression was increased in ASCs after priming and decreased in HPCs. Interestingly, there was no significant difference in the expression of *miR-27a-3p*, *miR-34a-5p*, *miR-34c*, *miR-96-5p*, *miR-125a* and *miR-451* in ASCs after incubation with the cytokine cocktail. Yet, in HPCs, all of the above-mentioned markers' expression was significantly lower after priming. Overall, the expression of all the analyzed miRNAs decreased in HPC cultures after incubation with the cytokine cocktail mixture, and all the differences were statistically significant at (***) for *miR-27a-3p*, (***) for *miR-34a-5p*, (**) for *miR-34c*, (**) *miR-96-5p* and (***) *miR-125a* and *miR-451*(***).

## 3. Discussion

In the past decades, MSC clinical applications have been the focus of numerous studies and clinical trials. The development of stem cell-based therapies is ongoing, and MSCs have been considered for various novel clinical applications, mainly related to their immunomodulatory potential [22–25]. However, the influence of the environment in which MSCs are expected to exert their immunomodulatory activity plays a huge role in their function [26]. Inflammation is the common link between EMS and laminitis, which strongly modifies the molecular signalling of stromal stem cells [15,19,27,28]. Therefore, it is crucial to establish how inflammatory conditions affect HPCs and ASCs and how this correlates with their clinical potency.

In this study, we investigated the morphology, expansion, apoptosis, and gene expression patterns in ASCs that are commonly used in equine veterinary regenerative medicine practice [29,30], as well as in recently identified HPCs [11,31]. We found that both ASCs and HPCs, although they are of mesenchymal origin, exhibited different expansion, apoptosis, and mitochondrial metabolism, especially when exposed to an in vitro pro-inflammatory microenvironment.

The morphology of both ASCs and HPCs was similar, consistent with previous observations of mesenchymal cells of various origins isolated from human patients, which typically display a fibroblast-like shape with a prominent nucleus [28,32]. MSC populations are also known to express a panel of cell surface and intracellular markers that define their mesenchymal origin and drive their specific functions. We found that native HPCs displayed higher *Hif1a*, *Vegfa* and *Nestin* transcription capacity when compared to ASCs. *Vegfa* is universally considered to be a marker of angiogenesis and vascular remodelling [33]. However, it has also been reported to partake in energy metabolism regulation [34], especially in adipose tissue. Native HPCs exhibited higher levels of *Vegfa* transcript, which may indicate their potential towards angiogenesis modulation. Numerous studies have addressed the role of angiogenesis and endothelial cells in laminitis [15,35,36]. HPCs expressed higher levels of *Hif1a* transcript, which may suggest their possible innate resistance towards hypoxia. As described by Bingke et al. [37], *Hif1a* can improve MSC survival under hypoxic conditions by improving their viability and inhibiting apoptosis. *Nestin*+ MSCs were previously shown to be associated with angiogenesis [38]. Furthermore, expression of *Nestin* was reported as a requirement for the latter astrocytic or neuronal differentiation of MSCs [39]. This could indicate an advantage of HPCs over ASCs in the context of new nerve formation and neuroprotective function. It is especially important in the scope of laminitis, which is associated with neuropathic changes. As Jones et al. reported [40], laminitic horses exhibited abnormal nerve morphology, accompanied by a reduced number of unmyelinated and myelinated fibres. Moreover, both studied populations expressed

the *CD105* surface marker, which was interestingly more abundant in the ASC population. However as Maleki et al. [41] demonstrated, depending on the origin, stem cells vary in the expression of endoglin. No difference was observed in the expression of *Ang1* and *CD29*, contrary to our previous results [11]. This discrepancy might be attributed to genetic variation between samples obtained from different animals. HPCs exhibited higher expression of *Mmp9*, but lower expression of *Mmp2* when compared to ASCs. The latter might be associated with HPCs lower migratory capacity [42], as evidenced by the scratch wound healing assay. Gelatinases are associated, among others, with tissue remodeling, angiogenesis [43] and MSC invasion and migration capacity [42]. Furthermore, their involvement in laminitis has also been described [19,44]. *Mmp14* can facilitate the activation of *Mmp2* and *Mmp9*, which are crucial for the degradation of the extracellular matrix and the subsequent migration of MSCs [45]. The higher expression of *Mmp14* in the ASC native population might further suggest their higher capacity for migration. However, lower expression of metalloproteinases involved in connective tissue degradation, which include *Mmp2* and *Mmp14*, potentially indicates that HPCs might exert a beneficial effect on connective tissue regeneration. Although various MSCs have been proposed as good potential candidates for palliating laminitis-induced ECM excessive degradation, other lines of evidence suggest that some stem cells, including ASCs and BM-MSCs, can further participate in proteolytic ECM remodelling as a part of their angiogenic and anti-fibrotic potential. This is accomplished by locally releasing various MMPs such as MMP-2 and MMP-9, which would limit their applicability for laminitis treatment [46]. Here, we found that HPCs express lower levels of MMPs and higher proangiogenic *Vegfa* marker levels, suggesting that HPCs may represent a better alternative in the treatment of laminitis than other MSCs due to their unique paracrine profile. Yet, at the same time, HPCs limited clonogenic potential and expansion in vitro, which might also hinder their long-term clinical applicability and require additional investigation to establish their efficacy using various experimental models and conditions.

MSC biological and therapeutic properties that include proliferation, migration, expansion and differentiation have been closely associated with organelle dynamics and functions. Thus, mitochondrial biogenesis and metabolism have been highlighted as master maintainers of MSC homeostasis by regulating self-renewal, apoptosis, immunomodulation and multi-directional differentiation, and by collectively determining stem cells fate within the organism [47].

In this study, the observed differential gene expression of selected cell surface markers, angiogenic regulators and matrix turnover regulators might be associated with the distinct mitochondrial dynamics noted in native and primed ASCs and HPCs. The processes of mitochondrial fission and fusion are linked to constant energetic shifts within the cell. While fission is overall related to apoptosis, fusion is associated with its' inhibition [48,49]. We observed that native HPCs exhibit a dichotomy of fission-related markers, with a lower baseline expression of *Mief2* and *Fis1* and a significantly higher expression of *Dnm1l* compared to ASCs. Notably, such results were previously published in the case of diabetic retinopathy [50] by Zhong and Kowluru, which might be related to the cytosolic location of *Dnm1l*, which is recruited during fission initiation. A similar discrepancy was observed with fusion markers, with *Mfn1* being responsible for inner membrane fusion and *Opa1* being responsible for outer membrane fusion [51]. In general, native ASCs exhibited intensified baseline mitophagy and mitochondria biogenesis compared to HPCs, as evidenced by a higher expression of *Parkin*, *Pgc1a* and *Rhot1* [52,53]. Notably, most mitochondrial fission and fusion-related marker expression decreased in both ASCs and HPCs as a result of the pro-inflammatory environment. Indeed, this deterioration of mitochondrial dynamics has previously been described in ASCs exhibiting impaired immunomodulatory properties that were isolated from patients with type 2 diabetes [54] and older horses [55]. However, HPCs exhibited markedly higher resistance towards inflammation in cases of general mitochondrial condition, metabolism, transcription and mito-ribosomal biogenesis, as evidenced by the unchanged expression of *Mrlp24*, *Oxa1l*, *Cox4il*, *Ndufa9*, *Ppargc1b* and

*Mief2* [56–58] and the upregulation of *Pusl1* and *Mief1*. Furthermore, the mitophagy dynamics seemed to be retained under a pro-inflammatory milieu in HPCs, indicating their advantage over ASCs in maintaining optimal mitochondrial functions under unfavourable conditions.

A comparison of the basal immunomodulatory mediator gene expression profiles of both MSCs populations showed that HPCs exhibited a distinct immune reactive molecules transcriptome and were characterized by lower expression of *Il-1β*, *Tnf-α*, *NF-κB* and *Il-10*, as well as higher *Il-6*, *Il-8*, *Mcp-1* and *Tgf-β* transcript levels compared to ASCs. MSC immunomodulatory properties that are essentially mediated by their secretome can be strongly modulated by various extrinsic stimuli, including other tissue sources or the pro-/anti-inflammatory milieu, which further determines the fate of MSCs and their polarization towards either a pro-inflammatory or an immunosuppressive phenotype [59]. Therefore, the ASC and HPC response to pro-inflammatory stimulation was also studied.

Here we have shown that in a high pro-inflammatory milieu, both ASCs and HPCs displayed considerably reduced gene expression levels of the key pro-inflammatory cytokine *Il-1β* and the pro-fibrotic mediator *Tgf-β1*, as well as significant upregulation of *Il-6* and *Il-8* cytokines. Interestingly, the exposure of ASCs and HPCs to the priming cocktail did not enhance the gene expression levels of the anti-inflammatory cytokines *Il-10* and *Il-13*. Several previous studies have examined the impact of MSC preconditioning with various inflammatory cytokines, alone or in combination, and reported differential responses. Wang et al. [60] demonstrated that IFN-γ-primed MCSs from various origins expressed higher levels of immunomodulatory factors including *Ido*, *Pge2*, *Hgf*, *Il-6* and *Il-10*, as well as decreased production of *Ifn-γ* and *Tnf-α*. Sivanathan and colleagues [61] investigated the influence of IL-17 priming and found that treated MSCs presented considerably higher immunosuppressive abilities that were mainly mediated by increased expression of *Il-6*. They concluded that IL-17-stimulated MSCs presented superior immunoregulatory properties over IFN-γ-activated MSCs. Conversely, a functional study showed that TNF-α induction promoted the expression of immunoregulatory factors such as IL-2, PGE2, IDO, and HGF, but with much less intensity compared with IFN-γ priming [62]. As reported by Najar and collaborators, MSCs from different origins can respond differently to cytokines elicitation, and consistent with our model, the exposure of MSCs to a cocktail of cytokines composed of IL-1β, TNF-α, IFN-α and IFN-γ triggered increased synthesis of PGE2 and IL-6. This subsequently mediated the switch of monocyte from the pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype [63]. Moreover, the presence of IL-1β in the priming cocktail has been shown to potentiate the transcription of *Cxcr4*, *Cox-2*, *Il-6* and *Il-8* genes, which all participate in the polarization of peritoneal M2 macrophages [64]. In this study, stimulated ASCs and HPCs were characterized by substantially increased *Il-6* and *Il-8* gene expression. This is of particular interest, as both cytokines have been previously reported to play a critical role in boosting the regenerative capacity of MSCs. IL-8 stimulates the release of regenerative signalling molecules that promote MSC survival, angiogenesis and migration through the activation of the PI3K/AKT/MAPK axis [65], while IL-6 has been shown to maintain MSC stemness and stimulate proliferation and wound healing capacity in an ERK1/2-dependent manner [66]. More importantly, the mechanisms driving the MSC phenotype switch are complex and multi-sequential and requires a highly pro-inflammatory milieu. Recently, it has been proposed that under pro-inflammatory conditions, MSCs tend to polarize into an MSC1 phenotype, which enhances the inflammatory response and induces the release of reactive cytokines such as IL-6, IL-8 and GM-CSF. These mediators recruit more neutrophils and macrophages to the inflamed site to further promote inflammation. As a consequence, MSC1 evolves in an environment deprived of anti-inflammatory mediators and is exposed to adequate pro-inflammatory signals, which facilitates the subsequent transition from the MSC1 phenotype to a highly immunosuppressive MSC2 population, promoting tissue repair and regeneration [67]. Therefore, the obtained data is in good agreement with the literature. The increased expression of *Il-6* and *Il-8* and the concomitant *Il-10*, *Il-13* and *Tgf-β* depletion

suggest that both ASCs and HPCs may promote the cytokines storm for a more efficient phenotype transition to an immunosuppressive pool of cells with an enhanced migratory and regenerative capacity at early inflammation stages.

Inflammation is often accompanied by oxidative stress [68–70]. In fact, horses with active laminitis have been characterized by increased ROS and RNS generation, excessive lipids peroxidation and overall impaired endogenous antioxidant defences within the injured hoof laminar tissue, causing severe lesions that exacerbate the inflammatory response [71]. Here, we observed that native HPCs displayed lower levels of oxidative stress than ASCs, which correlated with *Sod1*, *Sod2* and *Cat1* expression. However, under inflammatory conditions, both ASCs and HPCs reacted similarly, displaying a protective antioxidant mechanism [72].

Increased apoptosis is another serious laminitis and inflammation hallmark, which is a direct consequence of aberrant cellular lesions and the accumulation of malfunctions. In this study, we observed that native ASCs were characterized by intensified apoptosis compared to HPCs with higher baseline gene expression of proapoptotic markers and a higher *Bax:Bcl2* ratio. In inflammatory conditions, their response was generally similar. However, reduced expression of anti-apoptotic *Bcl2* and an increased *Bax:Bcl2* ratio could indicate more intensive apoptosis in HPCs. Finally, although native HPCs and ASCs exhibited different baseline gene expression patterns of immunomodulatory mediators, their response was similar under inflammatory conditions. Interestingly, at the baseline level, HPCs displayed lower expression of NF-κB signalling pathway markers. Several studies have demonstrated that overexpression of NF-κB and its downstream mediators, IKKs, impairs MSCs stemness and differentiation capacity. Shakibaei and colleagues [73] found that lower activation of the NF-κB pathway increased the production of type II collagen and cartilage-specific proteoglycans during chondrogenesis, further protecting MSCs from apoptosis. Likewise, NF-κB depletion has been shown to potentiate myogenic differentiation [74], while others have reported that NF-κB similarly hampers osteogenic differentiation through β-catenin ubiquitination and Smurf1/Smurf2 activation [75]. The observed lower NF-κB transcripts in HPCs may thus facilitate multilineage differentiation and tissue regeneration. However, additional studies are required to evaluate whether HPCs exhibit higher multipotency compared to other types of MSCs.

In addition to the involvement of cellular organelles and environmental stimuli in MSC behaviour under particular conditions, microRNAs have been largely studied for their role as crucial regulators of stemness, survival, immunomodulation and stem cell differentiation [76–80]. In this context, our data demonstrated a distinct expression pattern of selected miRNAs in native ASCs and HPCs prior to treatment with the cytokine cocktail mixture and after treatment. In the past few decades, a large number of microRNAs have been identified in MSCs from different sources, which have been implicated in the regulation of various cellular processes [81]. For example, Baglio et al. [82] found that human ASCs abundantly secrete *miR-486-5p*, *miR-10a-5p*, *miR-10b-5p*, *miR-191-5p* and *miR-222-3p*. This is in contrast to bone marrow-derived MSCs, in which *miR-143-3p*, *miR-10b-5p*, *miR-486-5p*, *miR-22-3p* and *miR-21-5p* were found at higher levels. Moreover, they reported that *miR-21-5p*, *miR-22-3p*, *miR-10b-5p* and *miR-222-3p* represented the common microRNAs produced by both cell types. Here, we found that equine ASCs only presented higher expression of *miR-218*, *miR-30c-5p* and *miR-125a* over HPCs under basal unstimulated conditions. Interestingly, HPCs displayed higher expression levels of *miR-21-5p*, *miR-27a*, *miR-34c*, *miR-125b-5p*, *miR-451* and *miR-96-5p*, suggesting a distinct microRNA signature in HPCs with a possible impact on cellular biogenesis. To the best of our knowledge, this is the first attempt at profiling equine HPC microRNAs expression, and whether these cells express additional sets of miRNAs, including the *let-7* family members, remains to be determined. However, as described in various investigations, most miRNAs discovered so far, such as *let-7* family subsets, the *miR-23–24-27* clusters, *miR-10*, *miR-29*, *miR-30* and *miR-125*, exert similar effects on various MSCs and modulate their survival, proliferation, stemness, differentiation capacity and immunomodulatory potential. This further suggests

that, under physiological conditions, HPCs may present higher cellular flexibility in relation to increased miRNA types and abundances [81]. The MSC microRNA transcriptome profile is also governed by cell milieu and status. In this study, the inflammatory cytokine cocktail strongly affected the HPC miRNome but not ASC miRNome, which seemed to be mostly unaffected. The ASC miRNome reacted with upregulation of only three markers, *miR-21*, *miR-30-5p* and *miR-125b-5p*, which suggest its potential implication in the immunosuppressive mechanisms of ASCs under pro-inflammatory conditions. Decreased levels of virtually all the analyzed miRNAs were observed in HPCs under similar conditions.

*miR-21* has been described as an important regulator of stem cell differentiation. Furthermore, its expression and secretion via exosomes have been linked to the therapeutic activity MSCs and their immunomodulatory function [83–87]. Notably, even though ASCs exhibited lower baseline expression of *miR-21*, under the inflammatory conditions, the expression increased, while in HPCs, it decreased significantly. Due to the importance of *miR-21* in stem cell regulation, this may suggest an advantage of ASCs, or their tendency towards differentiation in inflammatory environments. It has been previously described that inflammation may mediate signals in the competition between adipocyte and endothelial differentiation of ASC [88]. *miR-30c-5p* loss has been described in mitochondria dysfunction and oxidative stress in hMSCs. Therefore, it is interesting that under inflammatory conditions, HPCs maintained mitophagy, mitoribosome biogenesis and a proper oxidative stress response despite *miR-30c-5p* downregulation [89]. This suggests a possible establishment of miRNA-independent regulation mechanisms in HPCs. Primed HPCs also exhibited lower expression levels of *miR-96*, *miR-125a* and *miR-125b*. Both the *miR-96* and *miR-125* family could be crucial in laminitis treatment. miR-96 has previously been shown to be associated with wound healing, keratinocyte proliferation, migration and the NF-κB signaling pathway [90–94]. The miR-125 family has diverse functions and is involved in neuronal differentiation and injury, as well as self-renewal and differentiation of skin stem cells, among other functions [95–97]. *miR-218* which was downregulated in primed HPCs is crucial for MSC osteogenic and chondrogenic differentiation. Furthermore, *miR-218* takes part in regulating skin and hair follicle development [98,99]. Finally, *miR-451* was also downregulated in HPCs under inflammatory conditions. Importantly, its' immunomodulatory and inflammation-suppressing activity has been described in microglia-mediated neuroinflammation and diabetic retinopathy [100,101]. Collectively, these data indicate a modified HPC microRNAs profile under pro-inflammatory conditions, which questions whether these changes may impact their immunosuppressive and regenerative properties and reduce their clinical potency. Hence, further analysis is required to profile additional microRNAs and perform a comparative study of HPC efficiency under various environmental conditions. The main limitation of this study is the small sample size, as tissue was collected only from three horses. Furthermore, the information regarding age, sex and breed of the horses from the slaughterhouse was limited.

## 4. Materials and Methods

### *4.1. Study Design*

Coronary corium tissue samples were collected from 2 young horses, post-mortem, at a local slaughterhouse (Figure 9). Adipose tissue was collected from an 8-year-old warmblood mare from the site surrounding the base of the tail. Following the sample acquisition, ASCs and HPCs were isolated and cultured in both standard conditions (native populations) and in an inflammatory microenvironment created by priming the cells with a cytokine cocktail mixture. The native populations were subjected to immunofluorescent staining in order to visualize their morphology, while their phenotype was assessed based on RT-qPCR expression analysis of chosen surface markers, angiogenesis regulators and matrix metalloproteinases. Furthermore, the migratory capacity of native cells was assessed based on the scratch wound healing assay. The native populations were subjected to a colony forming unit assay in order to determine their clonogenic potential, and their proliferation was assessed based on a cell cycle microcapillary cytometry analysis. Micro-

capillary cytometry was also used to analyze the mitochondrial membrane potential and oxidative stress in native populations. Additionally, mitochondrial dynamics and oxidative stress in native populations were also validated with RT-qPCR. Autophagy, apoptosis and immunomodulation in the native populations were assessed using RT-qPCR and Western blot techniques. The chosen miRNA expression in native populations was also determined using RT-qPCR. To determine how the inflammatory microenvironment affects HPCs, both ASCs and HPCs were subjected to priming with a cytokine cocktail mixture (TNFα + IL1β + IFNγ) for 18 h. The mitochondrial network in primed ASCs and HPCs was assessed using immunofluorescent staining. Additionally, the mitochondrial dynamics and general mitochondria conditions were determined in primed ASCs and HPCs using RT-qPCR. The expression of key markers for apoptosis, autophagy, oxidative stress and immunomodulation was assessed in primed cells using RT-qPCR. Finally, the expression of the chosen miRNAs was analyzed in primed cells using RT-qPCR.

### 4.2. Ethical Approval

The Local Ethical Committee in Wroclaw for animal experiments approved the study protocol (permit no. 84/2018).

### 4.3. Tissue Harvest and Cell Culture

Samples of coronary corium and adipose tissue were acquired post-mortem from 6 young horses of random sexes, aged between 1–2 years, at the local slaughterhouse. The reasons for the animals' euthanasia were unrelated to this study. Before the slaughter, the good health and condition of the animals was ensured based on a mandatory clinical examination performed by a veterinarian. The tissue was isolated with a scalpel blade in sterile conditions and subsequently placed in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Sigma Aldrich/Merck, Poznan, Poland) supplemented with a 1% penicillin/streptomycin mix (P/S, Sigma Aldrich, Poznan, Poland). The samples were transported to the lab, and within 1.5 h, HPCs were isolated and cultured according to the protocol described by Yang et al. [12], and ASCs were isolated and cultured as previously described by Marędziak et al. [102]. Briefly, coronary corium and adipose tissue samples were washed 3 times with Hank's balanced salt solution (HBSS), then minced in a sterile petri dish into 1 × 1 mm squares with a #10 scalpel blade. The minced tissue was transferred into 50 mL sterile falcon tubes containing collagenase type I (1 mg/mL) solution in DMEM/F12 (Sigma Aldrich/Merck, Poznan, Poland) and incubated for 2 h at 37 °C with 2-dimensional agitation for coronary corium, and 40 min at 37 °C for adipose tissue. After digestion, the solution containing coronary corium tissue was filtered, first through a 100 µm filter and then through a 70 µm filter. The solution containing the adipose tissue was not filtered. Next, both samples were centrifuged at 1200× *g* for 10 min, then the supernatant was discarded, and the obtained cell pellet was washed with PBS and centrifuged again at 300× *g* for 4 min. Following the isolation, the obtained HPCs and ASCs from 6 animals were pooled to create one HPC culture and one ASC culture. The HPCs were characterized as previously described [11]. Briefly, at the second passage, the HPCs were analyzed via flow cytometry, which confirmed the expression of Nestin and CD29.The HPCs were cultured in DMEM/F12 (Sigma Aldrich/Merck, Poznan, Poland) supplemented with 10% FBS (fetal bovine serum, heat inactivated, Sigma Aldrich/Merck, Poznan, Poland) and a 1% penicillin/streptomycin mix (P/S, Sigma Aldrich, Poznan, Poland). The ASCs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1000 mg/L glucose, supplemented with 5% fetal bovine serum (FBS) and a 1% penicillin/streptomycin mix (P/S, Sigma Aldrich, Poznan, Poland). The medium was changed every other day, and the HPCs and ASCs were passaged at 80–90% confluency using a Trypsin/EDTA solution (Gibco Carlsbad, CA, USA). Prior to the experiment, the HPCs and ASCs were cryopreserved in a freezing medium (89% FBS, 10% DMSO, 1% penicillin/streptomycin mix) and thawed before use. Both the ASCs and HPCs used in the experiments were at their 4th passage.

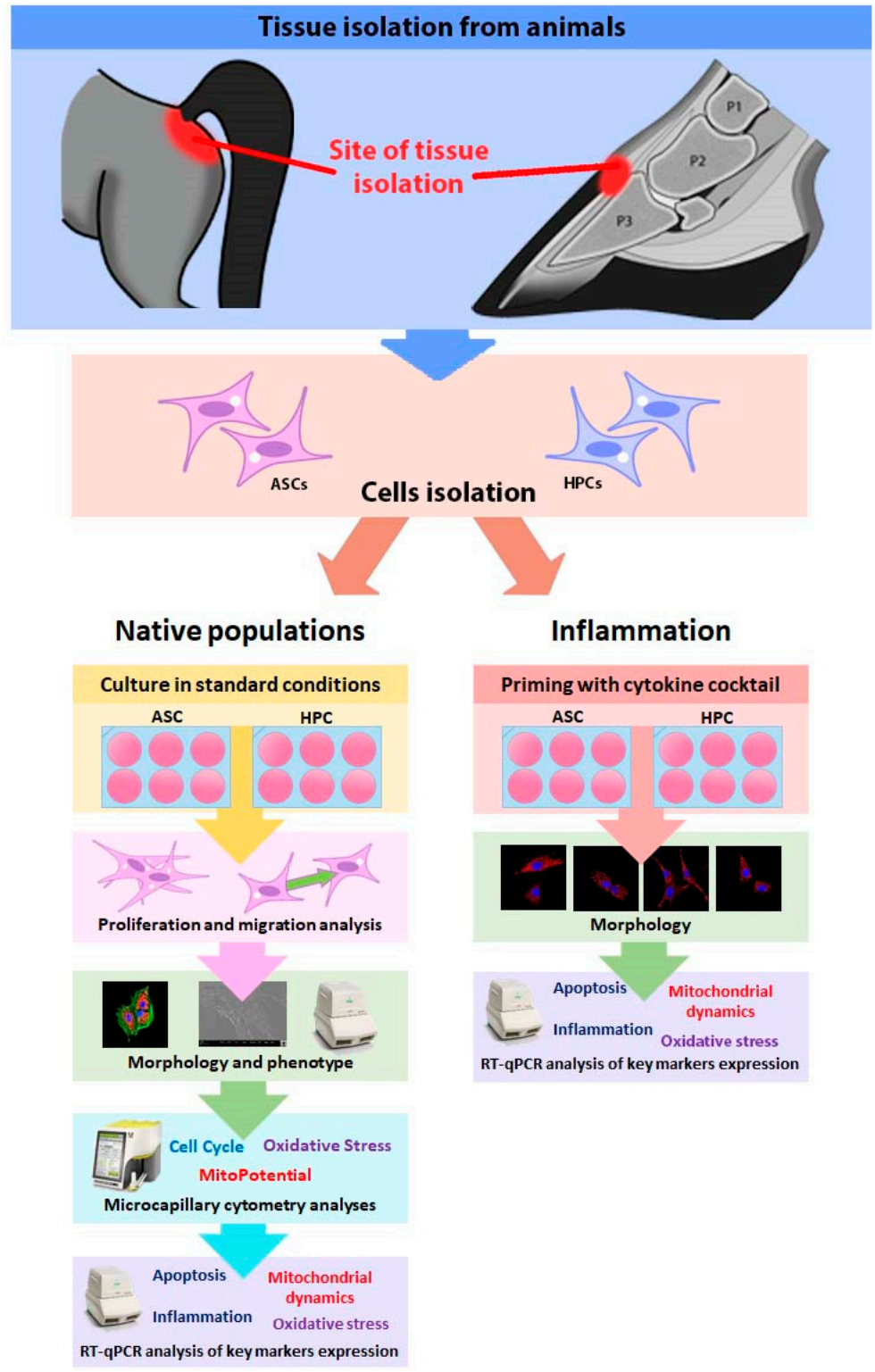


**Figure 9.** Graphical representation of the study design. P1 (Proximal phalanx bone), P2 (Middle phalanx bone), P3 (Distal phalanx/coffin bone).

### *4.4. Priming with Cytokine Cocktail*

For priming, ASCs and HPCs were incubated with a cytokine cocktail mixture containing TNFα (1000 U/mL), IL1β (50U/mL) and IFNγ (1000 U/mL) for 18 h, then harvested for further analysis.

### 4.5. Cell Morphology Assessment

The morphology of native ASCs and HPCs was assessed using fluorescent staining. For the mitochondrial network visualization, the mitochondria were stained with MitoRed dye (Sigma-Aldrich/Merck, Poznan, Poland) prepared in a culture medium (1:1000). Phalloidin-Atto 488 (Sigma-Aldrich/Merck, Poznan, Poland) was used to visualize the actin cytoskeleton. The nuclei were stained with 4′,6-Diamidine-2′-phenylindole dihydrochloride, and the coverslips were mounted onto glass slides with ProLong™ Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, Warsaw, Poland). The cells were observed and photographed using a confocal microscope under 630× magnification (Leica TCSSPE, Leica Microsystems, KAWA.SKA Sp. z o. o., Zalesie Górne, Poland). The obtained images were processed using Fiji software (ImageJ 1.52n, Wayne Rasband, National Institute of Health, Bethesda, MD, USA).

### 4.6. Scratch Wound Healing Assay and Colony-Forming Units

The migration of native HPCs and ASCs was assessed using the scratch wound healing assay. The cells were seeded onto a 12-well plate and cultured until fully confluent, then a 10 µL pipette tip was used to create the scratch wound. The cells were then cultured for 48 h, and microphotographs of the wells were taken at 4 time points: 0 h, 6 h, 24 h and 48 h using an inverted Leica DMi1 microscope equipped with a MC170 camera (Leica Microsystems, KAWA.SKA Sp. z o. o., Zalesie Gorne, Poland). After 48 h, the cells were fixed with 4% PFA and stained with a pararosaline solution for the purpose of photographic documentation. The scratch closure was calculated based on the Leica software scale bar (Leica Application Suite- LAS EZ, version 3.4.0), using photographs from 4 wells. To assess the clonogenic potential of native ASCs and HPCs, a colony-forming unit assay was performed as described previously [102,103]. Briefly, 250 cells were seeded onto a 6-well plate and cultured with medium changes every 3 days. After 8 days, the cells were fixed with a 4% PFA solution and stained with a 2% pararosaline solution (Sigma Aldrich/Merck, Poznan, Poland). The number of colonies was counted, and any cluster of 50 cells or more was regarded as a colony.

### 4.7. Microcapillary Cytometry Analyses

ASCs and HPCs were analyzed using commercially available Muse™ reagent kits and the Muse™ Cell Analyzer (Merck, Darmstadt, Germany). Cell cycle analysis was performed using a Muse™ Cell Cycle Assay Kit (Merck, Warsaw, Poland), according to the manufacturers' protocol. The potential of the mitochondrial membrane in ASCs and HPCs was assessed using the Muse® MitoPotential Assay Kit (Luminex, Austin, TX, USA). A Muse® Oxidative Stress kit (Luminex) was used for the assessment of intracellular oxidative stress factors according to the instructions provided with the kit. In order to detect nitric oxide activity within ASCs and HPCs, a Muse® Nitric Oxide Kit (Luminex) was used in accordance with the manufacturer's protocol.

### 4.8. RNA Isolation and qPCR

In order to assess the expression of key markers for apoptosis, immunomodulation, mitochondrial metabolism and miRNAs, the total RNA was isolated from the ASC and HPC cultures using the phenol–chloroform method described previously by Chomczynski et al. [104]. TRIZOL reagent was used according to the instructions provided by the manufacturer. The concentration and purity of the obtained RNA were assessed using a nanospectrophotometer (Epoch, Biotek, Bad Friedrichshall, Germany) at a 260/280 wavelength. A total of 150ng of total RNA was taken for further analysis. The gDNA was digested with RNAse-free DNAse I (Sigma-Aldrich/Merck, Poznan, Poland) and used for cDNA synthesis with a PrimeScript RT Reagent Kit (Takara Bio Europe, Saint-Germaine, Laye, France). For the digestion and synthesis, a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) was used according to the manufacturer's protocol. The obtained cDNA was then diluted with nuclease-free water in a 1:3 ratio and used for the RT-qPCR analysis. The expression of

mRNA and miRNA was detected using specific primers (Table S1, Supplementary Materials) and a SensiFAST SYBR and Fluorescein Kit (Meridian Bioscience, London, UK). All the reactions were performed using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The exact cycling conditions were previously described [17]. The results obtained from the RT-qPCR analysis were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression for mRNA and U6 for miRNA. The relative expression of each marker was calculated using the $2-\Delta\Delta CQ$ method [18]. For the native populations, ASCs were used as a reference sample for the calculations, while for the primed cells, the unprimed controls were used as a reference sample.

### 4.9. Mitochondria and mtRNA Isolation

To further examine the status of mitochondria in ASC and HPC cultures in standard and inflammatory conditions, intact mitochondria were isolated from both populations. The isolation was performed using a mitochondria isolation kit for cultured cells (ThermoFisher Scientific, Warsaw, Poland) accordingly to the manufacturer's protocol. Subsequently, mtRNA was isolated from the extracted mitochondria using the phenol–chloroform method with a TRIZOL reagent, as described above.

### 4.10. Statistical Analysis

At least two biological and three technical replicates of each experiment were performed, and the obtained results are presented as a means ± SD. The normality was assessed using either the Kolmogorov–Smirnov or Shapiro–Wilk test, and the variance was tested with the Fisher test. The statistical differences between the experimental groups were calculated using unpaired Student's *t*-test or the Mann–Whitney U test. The obtained data were analyzed with GraphPad Prism 8 software (La Jolla, CA, USA). Differences with a probability of $p < 0.05$ were considered significant, and the statistical significance was indicated with an asterisk (*). Furthermore, differences with a probability of $p < 0.01$ were marked with two asterisks (**), and differences with a probability of $p < 0.001$ were marked with three asterisks (***).

## 5. Conclusions

Overall, our findings demonstrated that native HPCs display a similar morphology to ASCs, but possess a distinct phenotype with higher expression levels of *Nestin* stemness-related marker and a lower migration capacity, indicating that HPCs may present a reduced ability to migrate across injured tissues and could potentially exert their effects at a local level rather than systemically. Moreover, HPCs typically express lower levels of MMPs, conferring them better control of defective EMC remodelling. In standard culture conditions, native ASCs and HPCs exhibit different baseline expression of markers commonly involved in mitochondrial dynamics, mitophagy, oxidative stress response, apoptosis and inflammation, whereas HPCs were characterized by higher expression levels of genes involved in mitochondrial metabolism modulation and a higher capacity to express antioxidant enzymes. This suggests a more active cellular machinery and potentially higher metabolic capacities. Importantly, native HPCs and ASCs showcased dissimilar microRNAs expression patterns, and increased expression of *miR-21-5p*, *miR-27-3p*, *miR-34c* and *miR-125b-5p* was observed in HPCs compared to the ASCs population, evoking the ability of HPCs to produce a wide range of reactive microRNAs that are able to modulate various cellular processes. However, under physiological conditions, pro-inflammatory signals induced a drop in the expression of the mentioned miRNAs, which indicates that they may not regulate HPC immunomodulatory properties. Importantly, HPCs appeared to exhibit a distinct immune phenotype under basal and pro-inflammatory conditions compared to ASCs and seemed to specifically express high levels of *Il-6*, *Il-8* and *Tgf-β*. These markers are involved in CD4+ T cell modulation, highlighting the probable implication of HPCs in the regulation of CD4+ T cell fate in response to inflammation. The obtained results do not unequivocally indicate which population might be more suitable for clinical use,

as both have certain advantages. However, our results indicated that HPCs may implement different molecular mechanisms for the regulation of inflammatory responses, and could potentially be considered as more suitable for the management of laminitis due to their lower ability to release MMPs, which could worsen ECM degradation. Based on our obtained data, we hypothesize that the combination of ASCs and HPCs or their EVs could be utilized in clinical applications such as laminitis treatment. However, this hypothesis should be further confirmed with ex vivo studies. Additionally, further research is necessary to fully elucidate how the HPC secretome and mitochondrial dynamics might affect their clinical potency.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms241411446/s1.

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## Abbreviations

| | |
|---|---|
| *Ang1* | Angiopoietin 1 |
| ASCs | Adipose-derived stem cells |
| *Bax* | BCL-2-associated X protein |
| BCA | Bicinchoninic acid |
| *Bcl2* | B cell lymphoma 2 |
| *Casp3* | Caspase 3 |
| *Casp9* | Caspase 9 |
| *Cat1* | Catalase 1 |
| CC | Cytokine cocktail |
| *Ccl2* | C-C Motif Chemokine Ligand 2 |
| *CD105* | Endoglin |
| *CD29* | Integrin subunit beta 1 |
| CFU | Colony-forming units |
| *Cox4il* | Cytochrome c oxidase subunit IV |
| CTRL | Control |
| DAMPs | Damage-associated molecular patterns |
| DMEM/F12 | Dulbecco's modified Eagle's medium/F12 |
| *Dnm1l* | Dynamin-related protein 1 |
| EAE | Experimental autoimmune encephalomyelitis |
| EMS | Equine metabolic syndrome |
| EVs | Extracellular vesicles |
| *Fis1* | Mitochondrial fission molecule |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |

| | |
|---|---|
| *Hif1a* | Hypoxia-inducible factor 1 subunit alpha |
| hMSC | Human mesenchymal stem cells |
| HPCs | Hoof progenitor cells |
| HRP | Horseradish peroxidase |
| IFNγ | Interferon gamma |
| *Igf1* | Insulin-like growth factor 1 |
| *Ikbkb* | Inhibitor of nuclear factor kappa B kinase subunit beta |
| *Il6* | Interleukin 6 |
| *Il8* | Interleukin 8 |
| *Il10* | Interleukin 10 |
| *Il13* | Interleukin 13 |
| IL1β | Interleukin 1 beta |
| IPSCs | Induced pluripotent stem cells |
| *Mcp1* | Monocyte chemotactic protein 1 |
| *Mfn1* | Mitofusin 1 |
| *Mief1* | Mitochondrial elongation factor 1 |
| *Mief2* | Mitochondrial elongation factor 2 |
| miRNA | microRNA |
| *Mmp14* | Matrix metallopeptidase 14 |
| *Mmp2* | Matrix metallopeptidase 2 |
| *Mmp9* | Matrix metallopeptidase 9 |
| *Mrlp24* | Mitochondrial ribosomal protein large 24 |
| mRNA | messenger RNA |
| MSCs | mesenchymal stem cells |
| *Mterf4* | Mitochondrial transcription termination factor 4 |
| *Ndufa9* | NADH:ubiquinone oxidoreductase subunit A9 |
| *Nfkb* | Nuclear factor kB |
| *Nfkbia* | NFKB inhibitor alpha |
| NO | Nitric oxide |
| *Opa1* | OPA1 mitochondrial dynamin-like GTPase |
| *Oxa1l* | OXA1L mitochondrial inner membrane protein |
| P/S | Penicillin/streptomycin mix |
| *p21* | Cyclin-dependent kinase inhibitor 1A |
| *p53* | Tumor protein P53 |
| PFA | Paraformaldehyde |
| *Pink1* | PTEN-induced kinase 1 |
| Ppargc1b | PPARG coactivator 1 beta |
| *Pusl1* | Pseudouridine synthase-like 1 |
| *Rhot1* | Ras homolog family member T1 |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RT-qPCR | Quantitative reverse transcription polymerase chain reaction |
| *Sod1* | Superoxide dismutase 1 |
| *Sod2* | Superoxide dismutase 2 |
| *Tgfβ1* | Transforming growth factor beta 1 |
| TNFα | Tumor necrosis factor alpha |
| *Uqcrc2* | Ubiquinol–cytochrome c reductase core protein 2 |
| *Vegfa* | Vascular endothelial growth factor A |

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# A Comparative Study of Equine Hoof Progenitor Cells and Adipose-Derived Stem Cells in Hyperinsulinemia.

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**Abstract:** Recently, we isolated and characterized a new pool of mesenchymal stem cells- HPC (Hoof Progenitor Cells), which reside in the coronary corium of equine hooves. We hypothesize, that due to their location, HPC may be involved in the pathogenesis of laminitis, and could possibly be utilized in its treatment. The aim of this study was to compare the newly described HPC to a well-established cell pool-ASC (Adipose Stem Cells). The two cell populations were maintained either in standard culture conditions or under a hyperinsulinemic milieu as hyperinsulinemia is often observed alongside laminitis. Cell cycle dynamics, mitochondrial membrane potential and oxidative stress were analyzed with microcapillary cytometry. Furthermore, the expression of key markers of mitochondrial metabolism, oxidative stress, apoptosis, immunomodulation and insulin signalling were analyzed with the RT-qPCR method. Additionally, a selected panel of miRNA was analyzed with the RT-qPCR method. In standard culture conditions, ASC exhibit higher proliferation than HPC while in hyperinsulinemia a shift towards S phase was observed in both populations. As for the mitochondrial membrane potential, in standard culture conditions, HPC was characterized by a higher percent of live cells. Hyperinsulinemia did not strongly affect the mitochondrial membrane potential of the analyzed populations. Our findings indicate that HPC are characterized by higher activity in terms of mitochondrial dynamics, mitophagy, and mitochondrial biogenesis under standard culture conditions. Yet in both populations, a diminished mitochondrial dynamic and metabolism was observed in hyperinsulinemia. The analysis of oxidative stress revealed, that HPC might possess somewhat higher resistance towards oxidative stress than ASC. Minimal change was observed in the expression of key markers of apoptosis in ASC and HPC under hyperinsulinemia. The analysis of key immunomodulatory markers indicated that HPC may help the cytokine storm for a more effective phenotype change to an immunosuppressing pool of cells with better migratory and healing abilities under hyperinsulinemia than ASC. As for insulin signalling, the obtained data suggests that HPC might be more resistant to hyperinsulinemia and may have greater therapeutic potential in reducing laminitis. Obtained results demonstrated that HPC possesses better potential to modulate the PTEN-AKT pathway by miR-21 and reverse high glucose and high insulin-induced insulin resistance. Taken together, the obtained results indicate that HPC may be a very promising cell pool with therapeutic potential in laminitis treatment, and could be more effective than ASC.

**Keywords:** equine metabolic syndrome; hyperinsulinemia; miRNA; mitochondrial metabolism; laminitis; stem cells; HPC; ASC; hoof

## 1. Introduction

A direct response to the demand for effective and individualised treatment options for various human and animal conditions is the growth of stem cell-based therapy in regenerative medicine[1–3]. The main goal of stem cell-based therapy is to improve the repair of the targeted cells, tissues, or organs by restoring homeostasis and encouraging regeneration [3]. This objective is achieved by increasing the population of stem cells or influencing the activity of locally present endogenous stem cells [4]. In the past, various types of stem cells, such as multipotent mesenchymal stem cells (MSC) and induced pluripotent

stem cells (IPSCs), have been investigated and used in clinical trials [5]. Furthermore, modern treatment strategies are willing to use MSCs derived from various sources. Most of which are muscle, bone marrow, adipose tissue, placenta, liver, and umbilical cord, and are widely used in various stem cell-based therapies in both human and veterinary medicine due to a lack of ethical concerns and a relatively simple isolation and culture process [5]. During the last decade, various studies have identified different molecular signatures and biological abilities for each MSC population, depending on the type of tissue from which they originate [6–8]. Therefore, the expected results of MSC-based therapies may strictly depend on their intrinsic properties [6]. Furthermore, the origin of donor stem cells and the condition play a crucial role in their therapeutic potential [6,9]. It is evident that resident stem cell populations are affected during the progression of numerous diseases and that their impairment contributes to the development of symptoms, as they influence the function of other cell types within the tissue [10–12]. Adipose-derived stem cells (ASC) are one of the most promising subsets of MSC due to their isolation, which is easily accessible in large amounts and has been used in a variety of clinical applications [13]. Furthermore, numerous soluble mediators, inflammatory cytokines, and trophic and growth factors, including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), transforming growth factor beta 1 (TGF-1) and interleukins, as well as paracrine factors that aid tissue regeneration, wound healing, and organ regeneration, are secreted by ASC [14]. Recently, we isolated and described new stem progenitor cells (HPC) that reside in the coronary corium of the equine hoof [15]. These cells have shown high levels of gene expression of surface markers such as cell adhesion molecules CD29 and an immunoglobulin superfamily membrane protein CD200, as well as nestin, a class VI intermediate filament protein, keratin 15 (K15), vascular endothelial growth factor (VEGF), and angiopoietin-1 (Ang1), indicating their high potential role in regeneration of the dermis and epidermis during laminitis [15]. Furthermore, as with other stem cell populations, HPC may be affected by inflammation in the same way that ASC are affected by equine metabolic syndrome (EMS), a disorder in which inflammation is one of the critical components for the development of insulin resistance [16,17]. Furthermore, laminitis, associated with conditions such as EMS, can develop as a result of endocrinopathy in association with dysregulation of insulin homeostasis, often in obese horses and foals. It is also worse in animals that graze in lush pastures (pasture laminitis) [18]. Therefore, it is believed that hyperinsulinemia with or without IR, which can occur as transient or persistent postprandial hyperinsulinemia, is the most common and important pathophysiologic factor of laminitis and has profound consequences for horses [19–22]. The common explanation is that chronically high doses of insulin in the bloodstream could cross-react with IGF-1 receptors, which are abundant in lamellar tissue. According to this notion, insulin-mediated activation of the IGF-1 receptor can cause lamellar structural damage by stimulating cell proliferation [23,24]. However, this theory has since been called into question [23]. Recent research has revealed that lamellar epithelial cells exposed to insulin proliferate, which can be blocked by an IGF-1 receptor-blocking antibody. Abnormal cell signalling may occur in conjunction with growth factor-activated cell proliferation, altering the cellular cytoskeleton and/or cell adhesion dynamics. Furthermore, during the acute stage of laminitis, there is a decrease in mitochondrial respiration. A decrease in the activity of the mitochondrial oxidative phosphorylation system (OXYPHOS) may lead to a decrease in the levels of ATP in the cells. This is particularly true if the glycolytic pathway is unable to compensate for inadequate ATP. A decrease in the availability of nutrients and/or oxygen may result in an energy deficit, as may mitochondrial dysfunction itself [25]. Therefore, targeting mitochondria during EMS and Laminitis should be considered essential to a better understanding of the physiopathology and new treatment strategies that support mitochondria, preventing their dysfunction and main-

taining their homeostasis. Persistent hyperinsulinemia also causes stress in the endoplasmic reticulum, disrupting the complex lamellar architecture. Furthermore, there is limited evidence to support a specific therapeutic method that directly inhibits the progression of laminitis in active episodes. Endocrinopathic laminitis therapy is very difficult and focuses mainly on prevention interventions and analgesia in active patients. Therefore, we hypothesise that Equine Hoof Progenitor Cells (HPC) could be a new promising cell pool for the treatment of laminitis. Therefore, this study aimed to compare native HPC populations with ASC under both standard culture and hyperinsulinemic conditions.

## 2. Results

### *2.1. Hyperinsulinemia exerts a similar effect on the cell cycle dynamics in ASC and HPC*

To assess the cell cycle dynamics in ASC and HPC under standard and hyperinsulinemia conditions, a microcapillary cell cycle analysis was performed. Under standard conditions, HPC were characterised by a significantly higher percentage of cells in the G0/G1 phase (****) and a lower percentage of cells in the S phase (****) and the G2/M phase (***). In both cell populations, as a result of the insulin treatment, the percentage of cells in the G0/G1 phase decreased while the percentage of cells in the S phase increased significantly. Interestingly, the hyperinsulinemia conditions did not affect the percentage of cells in the G2/M phase.

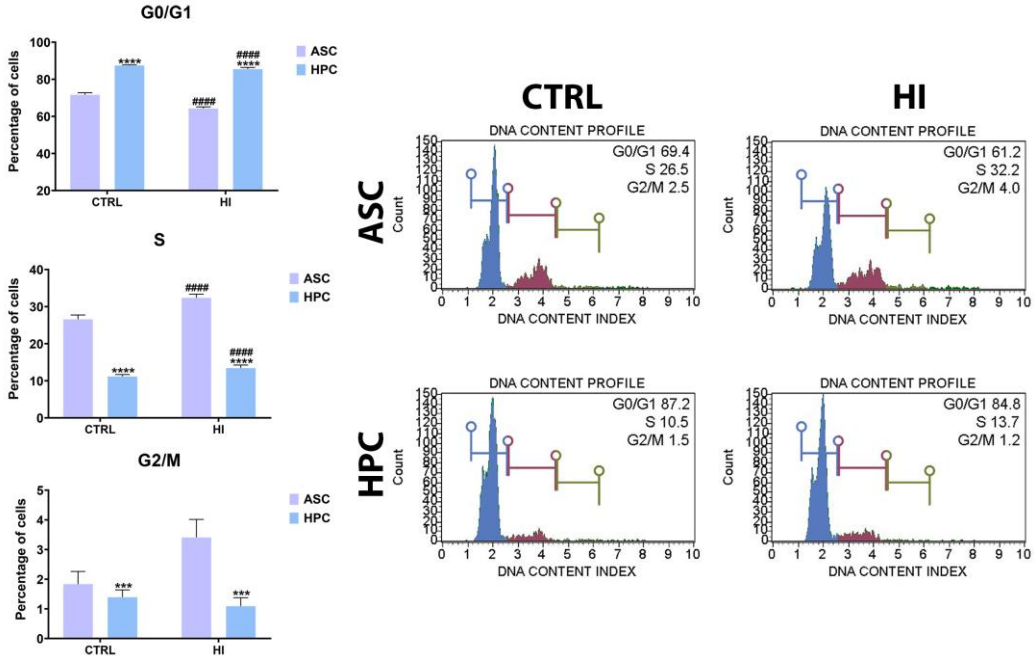


**Figure 1.** Cell cycle analysis was performed using a Muse™ Cell Cycle Assay Kit and the Muse™ Cell Analyzer. Results are expressed as mean ± SD. The statistically significant difference between the ASC and HPC is marked with an asterisk (*p < 0.05, **p < 0.01, ***p < 0.001,***p < 0.0001), while the statistically significant difference between the control group (CTRL) and the hyperinsulinemia group (HI) is marked with a hash sign (#p < 0.05, ##p < 0.01, ###p < 0.001,####p < 0.0001).

### *2.2. Mitochondrial membrane potential is not strongly influenced by hyperinsulinemia in ASC and HPC.*

To elucidate the effect of hyperinsulinemia on metabolic activity, a mitochondrial membrane evaluation was performed using a Muse® MitoPotential Assay Kit (Luminex). In standard culture conditions, HPC were characterised by a higher percentage of alive cells (**) and a lower percentage of dead(**) and total dead cells (****), compared to ASC.

Furthermore, a higher percentage of alive cells with depolarised membranes (**) and total depolarized cells(*) was observed in HPC in standard culture conditions. There was no significant difference in the number of dead cells with depolarised membranes and the total depolarized cell count between all experimental groups. Interestingly, the percentage of dead cells (##) decreased in ASC after hyperinsulinemia induction. Generally, hyperinsulinemia did not strongly affect the mitochondrial membrane potential of both populations.

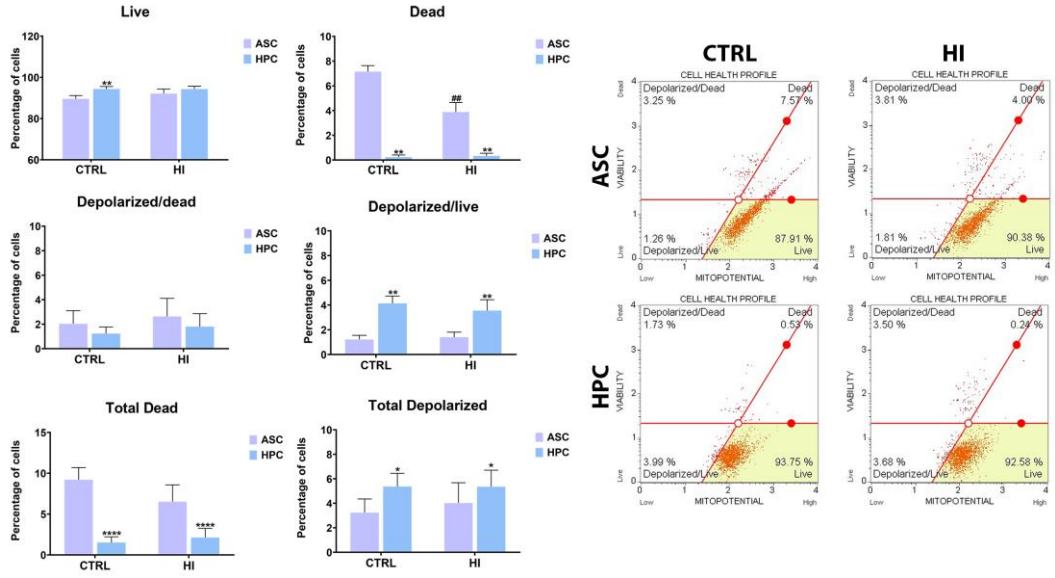


**Figure 2.** Mitochondrial membrane potential was assessed using the Muse® MitoPotential Assay Kit and the Muse™ Cell Analyser. Results are expressed as mean ± SD. The statistically significant difference between ASC and HPC is marked with an asterisk (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$), while the statistically significant difference between the control group (CTRL) and the hyperinsulinemia group (HI) is marked with a hash sign (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, #### $p < 0.0001$).

### *2.3. Hyperinsulinemia exerts a different effect on the mitochondrial metabolism of ASC and HPC*

To analyse the effect of hyperinsulinemia on mitochondrial metabolism in ASC and HPC, the expression of key markers for mitophagy, fission&fussion, mitochondria trafficking and metabolism was evaluated with the RT-qPCR method. The expression of markers related to fission and fusion was generally higher in HPC in standard culture conditions when compared to ASC (*MFN1**, *RHOT1***, *OPA1***, *MIEF1***), except for *FIS1*, *DNM1L* AND *MIEF2*, where there was no significant difference between the groups. Furthermore, the expression of *PINK1*(****), a marker of mitophagy, was also higher in HPC in standard culture conditions. Overall, the expression of fission and fusion-related markers, as well as the mitophagy marker *PINK1*, decreased as a result of the hyperinsulinemia induction in both groups, except for *DNML1*, in which case, no significant difference was observed when comparing standard culture groups to hyperinsulinemia groups. Interestingly, the expression of *MFN1(**), PINK1(****), RHOT1(**)* AND *OPA1(**)* was higher in the HPC HI group, compared to the ASC HI group.

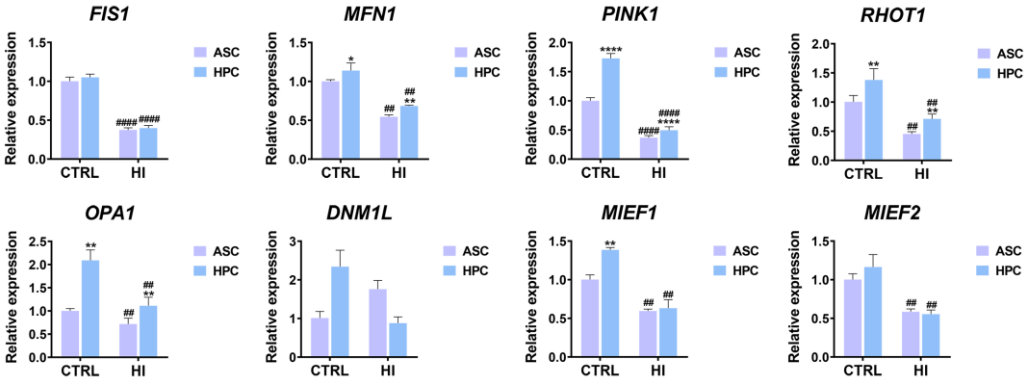


**Figure 3.** The expression of key markers for mitophagy and mitochondrial fission&fussion was assessed using the RT-qPCR method. Results are expressed as mean ± SD. The statistically significant difference between ASC and HPC is marked with an asterisk (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), while a statistically significant difference between the control group (CTRL) and the hyperinsulinemia group (HI) is marked with a hash sign (#p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001).

Furthermore, the expression of mitochondrial metabolism markers was generally higher in HPC in standard culture conditions (*MRPL24*****, *MTERF4***, *OXA1L***, *PPARGC1B*****, *TFAM***, *NDUFA9***, *UQCRC2*****, *COX4I1*****) except for *PUSL1* and *PIGBOS1*, for which no statistical significance in expression between the groups was observed. After insulin treatment, the expression of most markers analysed decreased in ASC (*MRPL24*$^{\#\#\#\#}$ *OXA1L*$^{\#\#}$, *PPARGC1B*$^{\#\#\#\#}$, *PIGBOS1*$^{\#\#\#\#}$, *TFAM*$^{\#\#}$, *NDUFA9*$^{\#\#}$, *UQCRC2*$^{\#\#\#\#}$, *COX4I1*$^{\#\#\#\#}$), except for *MTERF4* and *PUSL1*, where no statistical significance has been noted. A similar trend was observed in HPC (*MRPL24*$^{\#}$, *MTERF4*$^{\#\#}$, *OXA1L*$^{\#\#}$, *PPARGC1B*$^{\#\#\#\#}$, *PIGBOS1*$^{\#\#\#\#}$, *TFAM*$^{\#\#}$, *NDUFA9*$^{\#\#}$, *UQCRC2*$^{\#\#\#\#}$, *COX4I1*$^{\#\#\#\#}$) except for *PUSL1,* in which case there was no difference in the expression between the untreated and insulin-treated HPC. Overall, in standard culture conditions and under hyperinsulinemia, the expression of most analyzed markers was significantly higher in HPC when compared to ASC. Finally, the mitochondrial network was visualized with fluorescent staining (Fig 4, A), and the fluorescence intensity was measured with Fiji just ImageJ software (Fig 4, C). There was no difference between ASC and HPC fluorescence intensity in standard culture conditions, however, the fluorescence intensity was significantly higher after the hyperinsulinemia induction in both populations. Additionally, after the insulin treatment, the fluorescence intensity in HPC was significantly lower when compared to ASC.

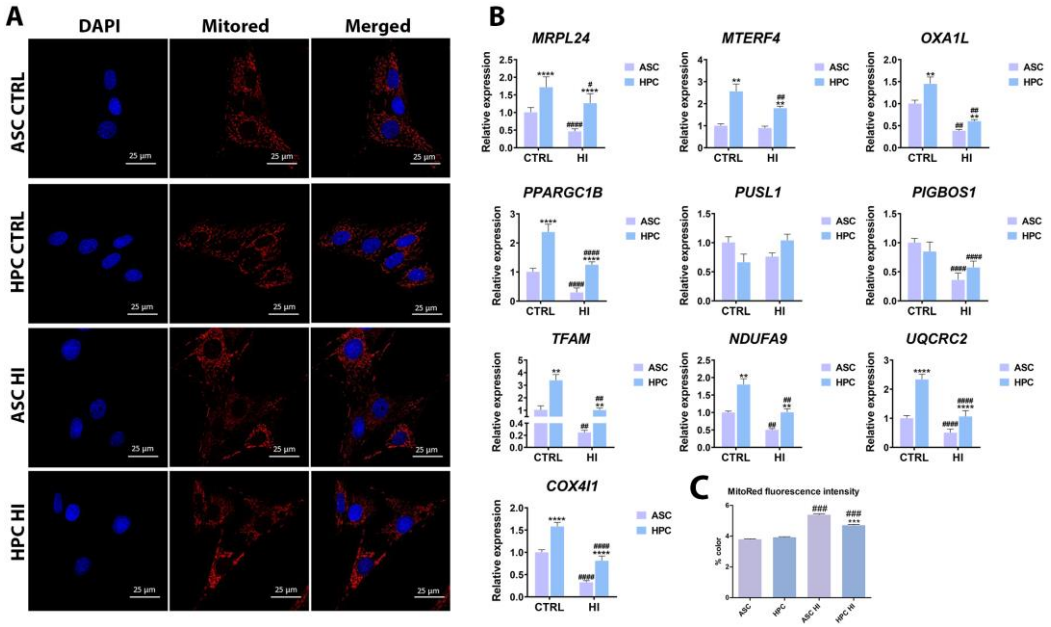


**Figure 4.** (**A**) Mitochondrial network staining performed with MitoRed dye and DAPI. (**B**) Gene expression analysis of key mitochondrial metabolism markers *(MRPL24, MTERF4, PPARGC1B, UQCRC2, OXA1L, COXHI1, NDUFA9, PUSL1, MIEF1, MIEF2)*. (**C**) MitoRed dye fluorescence intensity (% colour) measured with Fiji is just ImageJ software. Results are expressed as mean ± SD. The statistically significant difference between ASC and HPC is marked with an asterisk (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), while the statistically significant difference between the control group (CTRL) and the hyperinsulinemia group (HI) is marked with a hash sign (#p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001).

*2.4 Hyperinsulinemia exerts a different effect on ASC and HPC oxidative stress levels*

The gene expression of key oxidative stress markers (Fig. 5, A) as well as reactive oxygen species (ROS), (Fig. 5, B) and nitric oxide (NO), (Fig. 5, C) content in all experimental groups was assessed to elucidate how hyperinsulinemia affects ASC and HPC oxidative stress levels. In standard culture conditions, the HPC population was characterized by a lower expression of *SOD1(**)*, *SOD2(****)* and a higher expression of *CAT1(***)* than ASC. As a result of hyperinsulinemia, the expression of *SOD2* and *CAT1* decreased in both ASC and HPC, but the expression of *SOD1* only decreased in the HPC and remained unchanged in the ASC population. The amount of ROS(+) cells (was significantly lower in HPC, both in standard culture conditions as well as hyperinsulinemia. As a result of insulin treatment, an increase of ROS(+) cells in the ASC population was observed, but not in the HPC. The percentage of alive and dead, nitric oxide (+) cells as well as the Total Nitric Oxide (+) cells count was higher in the ASC(**) population, before the insulin treatment and after. Simultaneously, the percentage of nitric oxide negative cells was higher in the HPC population, both before and after the hyperinsulinemia induction.

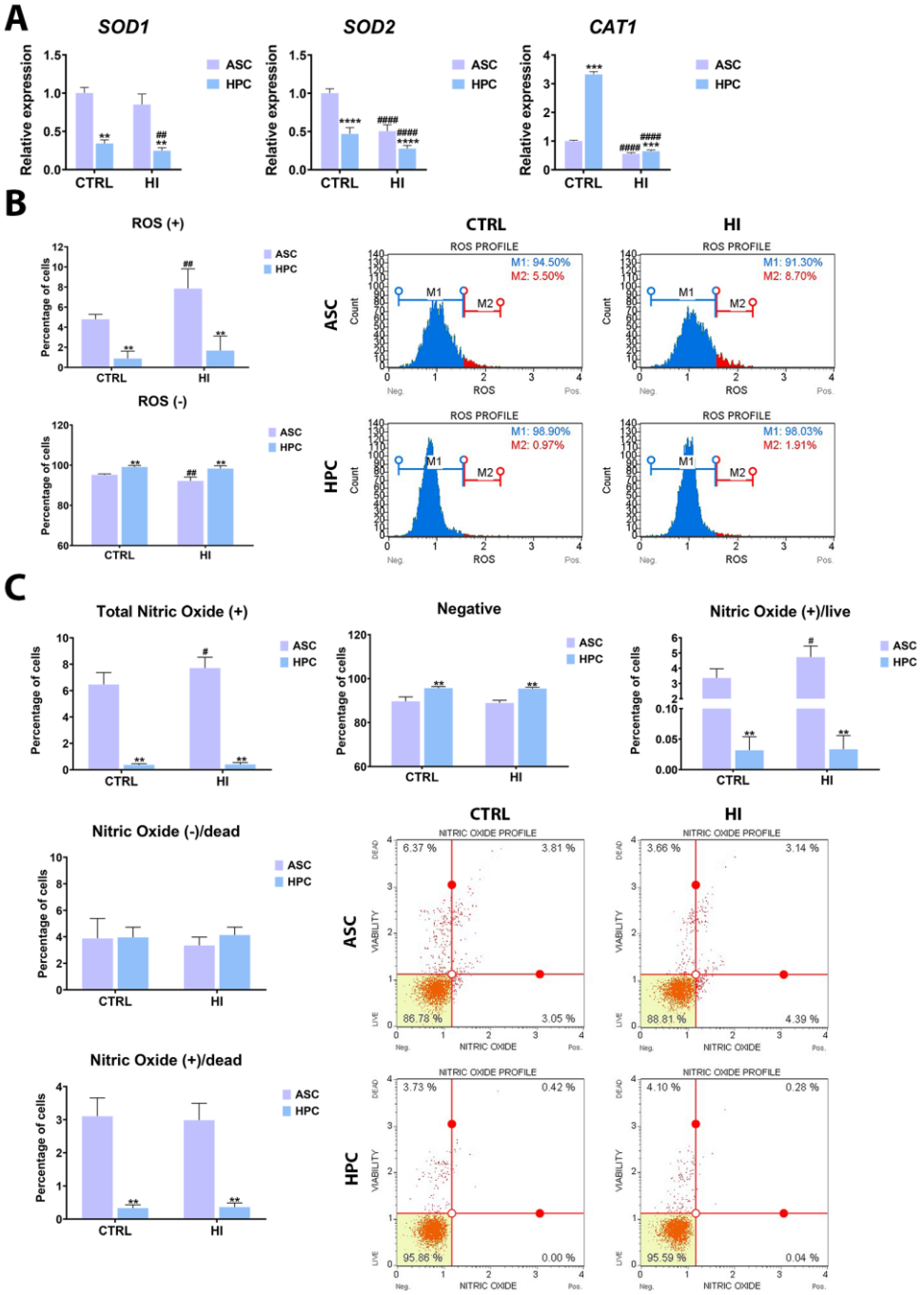


**Figure 5.** (**A**) The gene expression of key oxidative stress markers (*SOD1, SOD2, CAT1*). (**B**) Oxidative stress analysis performed with Muse® Oxidative Stress kit. (**C**) Nitric oxide activity was assessed with Muse® Nitric Oxide Kit. Results are expressed as mean ± SD. The statistically significant difference between ASC and HPC is marked with an asterisk (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), while the statistically significant difference between the control group (CTRL) and the hyperinsulinemia group (HI) is marked with a hash sign (#p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001).

*2.5 Apoptosis, immunomodulation and insulin signalling markers expression differs between ASC and HPC in standard culture conditions and hyperinsulinemia*

The expression of genes associated with apoptosis (Fig.6, A), immunomodulation (Fig.6, B) and insulin signalling (Fig.6, C) was assessed in ASC and HPC in standard culture

conditions and after hyperinsulinemia induction. The expression of *p53* was lower (**) in HPC in standard culture conditions, but there was no significant difference between the two populations in the expression of its target- *P21*. Yet, after the insulin treatment, the expression of *P21*(##) decreased in HPC only, while the expression of *P53* remained unchanged in both groups. As for other markers associated with apoptosis, in both standard culture conditions and after the insulin treatment, HPC exhibited lower expression of *CASP9*(**) and *BLC2*(****) with simultaneous higher expression of pro-apoptotic *BAX*(****), when compared to ASC. Furthermore, the *BAX: BCL2*(**) ratio was significantly higher in the HPC population. In both ASC and HPC, the expression of *CASP9*, *BAX* and *BCL2* decreased as a result of hyperinsulinemia, while the *BAX: BCL2* ratio increased significantly. The expression of pro-inflammatory cytokines, *IL6*(***) and *IL8*(****), was higher in the HPC population and decreased in both populations as a result of hyperinsulinemia. Furthermore, the expression of *IL1β* and *TNFA* was higher in the HPC population, both in standard culture conditions and hyperinsulinemia. Importantly, the expression of *IL1β* and Tnf*α* decreased in both populations after the hyperinsulinemia induction. Finally, the expression of *TGFB* also decreased after the insulin treatment but there was no difference between ASC and HPC in standard culture conditions. As for the markers of the insulin signalling pathway, HPC was characterized by a lower expression in standard culture conditions than ASC. As a result of hyperinsulinemia induction, a decrease in the expression of insulin signalling-related markers was observed, notably the expression of *IR* and *AKT* was higher in the insulin-treated HPC when compared to ASC.

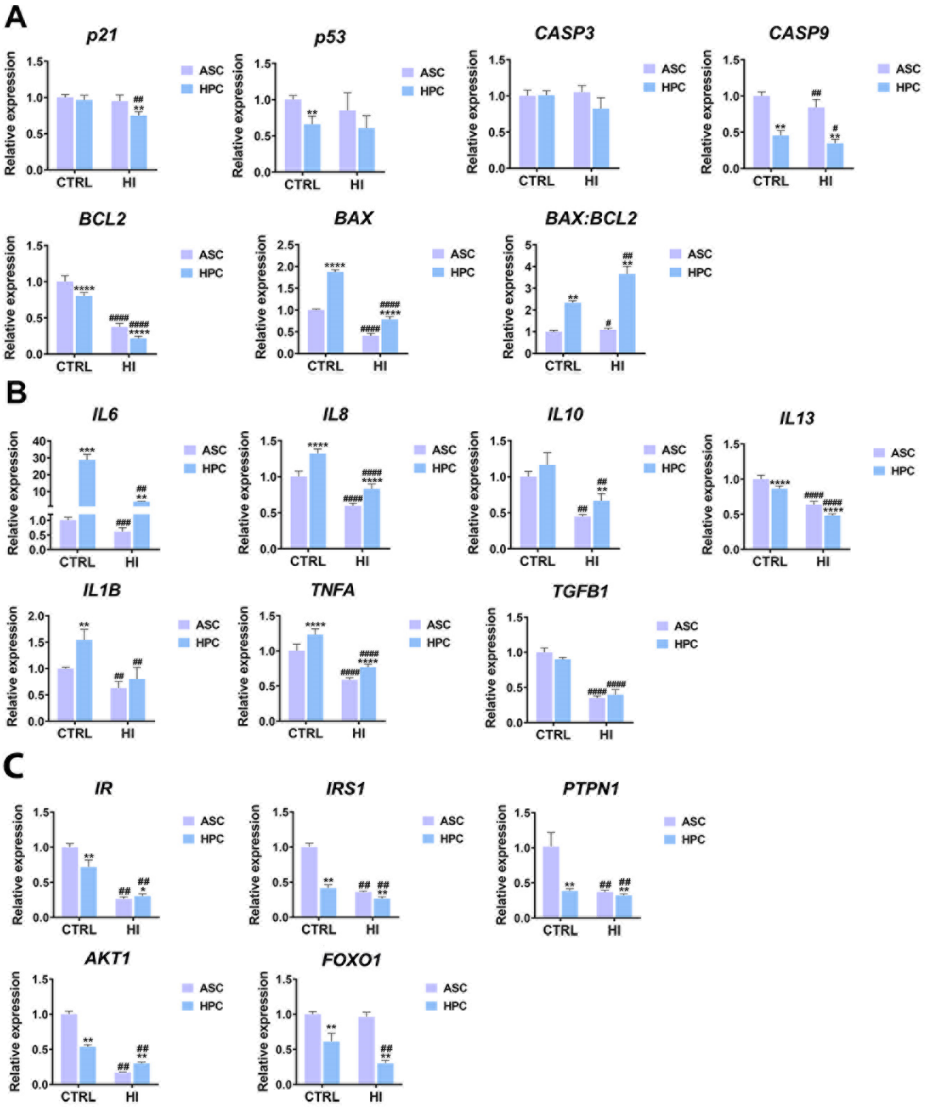

**Figure 6.** Hyperinsulinemia induces changes in gene expression of *P21, P53, CASP3, CASP9, BAX,* and *BCL2* that are associated with apoptosis, *BAX: BCL2* ratio was calculated (**A**), immunomodulation (*IL6, IL8, IL10, IL13, IL18, TNFA,* and *TGFB1*) (**B**), and insulin signalling (*IR, IRS1, PTPN1, AKT,* and *FOXO1*) (**C**). Data represent the mean ± SD**.** The statistically significant difference between ASC and HPC is marked with an asterisk (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), while the statistically significant difference between the control group (CTRL) and the hyperinsulinemia group (HI) is marked with a hash sign (#p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001).

*2.6 HPC and ASC are characterized by a disparate miRNome in standard culture conditions and hyperinsulinemia*

The expression of 9 out of 10 analyzed miRNAs was higher in HPC under standard culture conditions (mir-21**, miR-27a*, miR-30c*, miR-34a**, miR-34c**, miR-96**, miR-125a**, miR-125b**, miR-451**). In hyperinsulinemia, the expression of most miRNAs decreased in both ASC and HPC, except for mir-27a## and mir-96##, whose expression increased in ASC. Overall, after the hyperinsulinemia induction, the expression of 6 analyzed miRNAs was lower in the HPC (miR-27a**, miR-30c, miR-96**, miR-125b**, miR-218****, miR-451**), except for miR-21** and miR34a**, in which case the expression was higher in the HPC when compared to ASC.

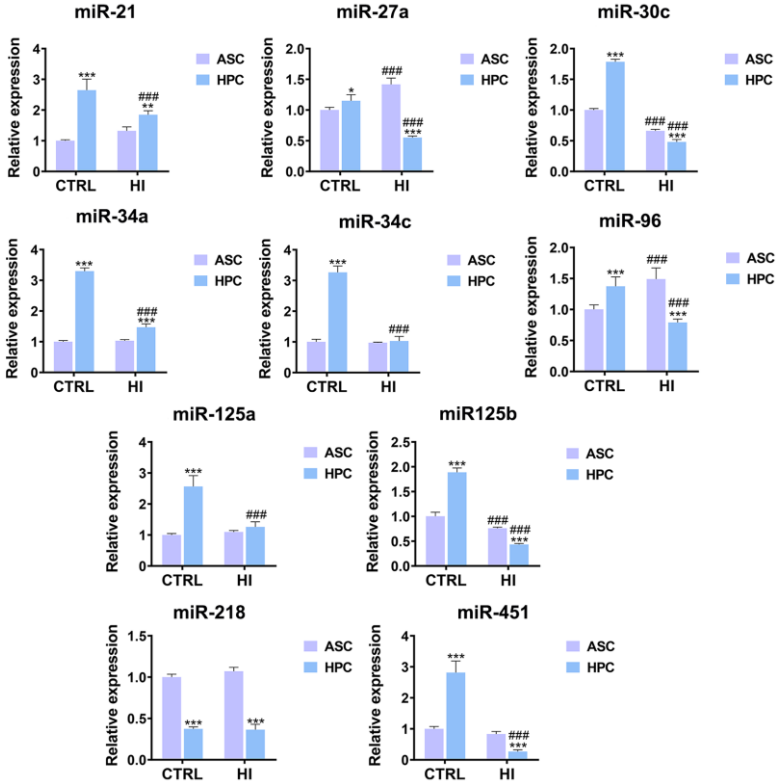


**Figure 7.** Relative expression level of miRNA in ASC and HPC cells in control and hyperinsulinemia conditions. Data represent the mean ± SD**.** The statistically significant difference between ASC and HPC is marked with an asterisk (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), while the statistically significant difference between the control group (CTRL) and the hyperinsulinemia group (HI) is marked with a hash sign (#p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001).

## 3. Discussion

Hyperinsulinemia is a common symptom of various endocrinopathic and metabolic disorders in equids such as laminitis, EMS, Cushing's syndrome, or iatrogenic corticosteroid administration and is often a manifestation of insulin resistance. In general, it has been widely accepted that insulin resistance and hyperinsulinemia are crucial components in the pathology of laminitis. Although high serum insulin concentrations have previously been reported in ponies with reoccurring laminitis, in the absence of clinical signs, this could be attributed to developing some tolerance margin for insulin resistance. Therefore, it is important to note that laminitis is a multifactorial disorder with complicated pathology. However, de Laat et al., [26] demonstrated that a prolonged and consistent elevated serum insulin concentration may cause laminitis in Standardbred horses, in the absence of pre-existing insulin resistance.

In this study, our objective was to assess how hyperinsulinemia will affect two pools of stem cells with potential in the treatment of laminitis, the well-established ASC and the recently described HPC [15,27]. Cell cycle dynamics, mitochondrial metabolism, oxidative stress, apoptosis, and miRNA expression were compared between the two populations, under standard culture conditions and hyperinsulinemia.

The present study indicates that, under standard culture conditions, ASC exhibits a higher proliferation rate than HPC, which might be a negative prediction for possible therapeutic use. Interestingly, a progression from the G0/G1 phase to the S-phase of the cell cycle was observed in both populations, as a result of the insulin treatment [28]. It was reported by Li et al that insulin can, in a dose-dependent manner, promote a transition from the G0/G1 phase to the S-phase, in human Umbilical Cord Matrix-Derived mesenchymal stem cells. However, it is imperative to note, that the insulin concentration used by Li et al (maximum 20 μM) was much lower than the concentration used in this study to induce hyperinsulinemia. Chronic hyperinsulinemia was also found to increase the proliferation of myoblasts in fetal sheep skeletal muscle [29]. Endometrial epithelial cells and colorectal epithelial cell proliferation were also enhanced by hyperinsulinemia [30,31]. On the other hand, hyperinsulinemia has a negative impact on the proliferation of the circulating endothelial progenitor cells, as demonstrated by Tan et al. [32]. In this study, the cells were harvested after 24h incubation with insulin, therefore, the observed shift from the G0/G1 phase towards the S phase might be an initial response, which could change after a longer incubation. Additional research with prolonged incubation time would be necessary to further establish such a hypothesis.

To further elucidate how HPC react in the face of hyperinsulinemia, mitochondrial membrane potential and gene expression of mitochondrial markers were analysed. Interestingly, hyperinsulinemia did not strongly affect the mitochondrial membrane potential of the analysed populations. However, in standard culture conditions, HPC was characterised by a higher percent of live cells; this difference was diminished in hyperinsulinemia, but the percent of alive cells with depolarised mitochondrial membranes was higher in the HPC populations in both sets of experimental conditions. Mitochondrial membrane polarisation is a vital marker of overall cell health, and its loss can be attributed to the early stages of apoptosis [33]. Mitochondrial fission and fusion are processes associated with normal cellular functioning, but also with apoptosis and the cell response to stress. Fusion can mitigate cell stress by mixing contents of partially damaged mitochondria while fission leads to the creation of new mitochondria, but can also facilitate apoptosis by allowing the removal of comprised mitochondria [34]. Generally, there was no difference between ASC and HPC in the expression of fission-related markers, except for *MIEF1*, whose expression was higher in the HPC, however, the expression of *MFN1* and *OPA1* was higher in the HPC. In instances of type 2 diabetes, a shift towards a higher expression of fission-related markers was observed in several studies [35–38]. Further-

more, decreased expression of *MF1* and *MFN2* was reported in HepG2 cells in hyperinsulinemia and hyperglycemia [39]. The results obtained are in keeping with the reported findings, as hyperinsulinemia led to a decrease in the expression of fusion and fission-related markers in both populations, suggesting a diminished mitochondrial dynamic.

Furthermore, the mitophagy marker *PINK1* was expressed at a higher level in HPC compared to ASC under standard culture conditions and after induction of hyperinsulinemia. Nevertheless, its expression also decreased in both populations after hyperinsulinemia induction. The results obtained indicate that under hyperinsulinemic conditions, mitochondrial dynamics are diminished. Extensive research has been published on the role of mitophagy and mitochondrial dynamics in type 2 diabetes and insulin resistance [40–43]. For example, mice depleted of a Fundc1 mitophagy receptor were characterised by impaired mitochondrial quality, more severe obesity, and insulin resistance, due to mitophagy dysfunction [44]. Another study reported that in patients with type 2 diabetes, the expression of mitophagy-related genes such as *NIZ*, *PINK1,* and *PARKIN* was reduced [45]. The detrimental effect of hyperinsulinemia on the mitochondrial condition of ASC and HPC was further indicated by an analysis of the expression of genes related to mitochondrial metabolism and biogenesis. Under standard culture conditions, HPC were characterised by a higher expression of most of the analysed mitochondrial metabolism and biogenesis markers, with the exception of *PUSL1* and *PIGBOS1.* In hyperinsulinemia, downregulation of almost all the markers (except *PUSL1)* occurred in both ASC and HPC. *PPARGC-1B* encodes the peroxisome proliferator–activated receptor-$\gamma$ coactivator 1$\beta$ (*PGC-1β*) [46] that plays a crucial role in mitochondrial activity in tissues with active oxidative metabolism; it is crucial for proper mitochondrial biogenesis and metabolism [47]. In a study published by Sparks et al., a 3-day high-fat diet led to a reduction in *PGC-1* gene expression of *PGC-1β* in healthy men [48]. Down-regulation of *PGC-1 B* has also been observed in CGI-58-deficient peritoneal macrophages from mice fed a high-fat diet [49]. Mammalian mitochondria possess ribosomes that synthesise 13 key subunits of the oxidative phosphorylation system (OXPHOS). *MRPL24* and *MTERF4* are involved in mitoribosome formation and assembly [50]. Bourebaba et al. [39] demonstrated that in the hyperinsulinemia-hyperglycemia environment, HepG2 cells exhibited lower expression of the markers mentioned above. The mitochondrial oxidative phosphorylation system (OXPHOS) is composed of four respiratory chain complexes and the $F_1F_0$-ATP synthase (complex V) and is responsible for the majority of ATP production in aerobic cells. The biogenesis of OXPHOS is complicated and therefore several specific gene products are needed to meet these requirements, among them *OXA1L* is involved in the biogenesis of mitochondrial membrane proteins [51]. Bioinformatic analysis indicated that *OXA1L* may be related to diabetic retinopathy [52]. *OXA1L* was also down-regulated in serum collected from patients with uncontrolled diabetes [53]. The *PIGBOS1* gene encodes PIGBOS which is an integral mitochondrial outer membrane protein. Functional investigations show that the absence of PIGBOS increases UPR and cell death and can, in turn, modulate cellular sensitivity to ER stress [54]. Our work reveals that higher expression levels of *PIGBOS1* in HPC cells suggested that this cell pool could be more resistant to ER stress than ASC. Furthermore, a similar higher expression of *TFAM* was observed in HPC. *TFAM* is crucial for the maintenance of mitochondrial DNA, as it acts as a master regulator for the expression of 13 gene products of mitochondrial DNA as well as a repair protein [55]. In hyperinsulinemia, downregulation of *TFAM* is observed in vascular smooth muscle cells (VSMC) and the liver [56,57]. Finally, our results demonstrated that under hyperinsulinemia both ASC and HPC exhibited a decrease in the expression of the OXPHOS-related genes *NDUFA9, UQCRC2,* and *COX4I1*, similar results were reported in HepG2 cells by Bourebaba et al. [39]. Our findings indicate that HPC are characterised by higher activity in terms of mitochondrial dynamics, mitophagy, and mitochondrial biogenesis under standard culture conditions, yet the effect of hyperinsulinemia on these processes is similar in ASC and HPC.

Oxidative stress is a state in which the accumulation of excessive reactive oxygen species (ROS) leads to cellular disruption and damage in important structures such as proteins, lipids, and nucleic acids. ROS are produced by the mitochondria, as a product of cellular respiration, and homeostasis is maintained through a cellular antioxidant defence system, composed mainly of enzymes such as superoxide dismutase (SOD) or catalase (CAT), among others. Importantly, oxidative stress can be a major risk factor and component of various diseases, including type 2 diabetes, metabolic disorders, or cardiovascular diseases [58]. Under standard culture conditions, ASC exhibited a higher expression of *SOD1* and *SOD2* while HPC exhibited a higher expression of *CAT1*, however, under hyperinsulinemia the expression of the markers decreased in both populations, which may suggest an aggravation of oxidative stress in the cell and a disruption of the cellular antioxidant defence system. A similar dynamic was observed in Fisher rats fed a high-refined sugar diet, where Roberts et al. observed down-regulation of SOD isoforms in the kidney and aorta [59]. Deterioration of the expression of the genes of antioxidant enzymes in hyperglycemia-hyperinsulinemia was also observed in HepG2 cells [39]. Following our previous findings, under standard culture conditions, HPC were characterised by a lower level of ROS and nitric oxide [27]. Interestingly, ROS and nitric oxide levels did not change in HPC after insulin treatment, yet they increased in ASC. Such results may indicate a somewhat higher resistance towards oxidative stress in HPC, however, it is not apparent when weighting in the diminished antioxidant enzymes gene expression. In general, increasing evidence indicates an inverse dependency between insulin sensitivity and ROS levels [60] and the presented results show that hyperinsulinemia has a negative impact on oxidative stress levels in both ASC and HPC.

Apoptosis, also known as programmed cell death, is crucial to maintaining homeostasis, however, intensified apoptosis is often associated with various pathologies, oxidative stress, hyperinsulinemia, and insulin resistance [32,61–63]. Interestingly, the obtained results do not unequivocally support this notion, except for the increase in *BAX: BCL2* ratio under hyperinsulinemia in both populations. Such findings could be explained by the short duration of hyperinsulinemia in the experimental environment, for example., Turina et al. showed that short-term changes in glucose and insulin concentrations do not affect neutrophils' ability to enter the apoptotic program [61]. Furthermore, the relationship between hyperinsulinemia and apoptosis is not universally similar; for example, hyperinsulinemia can also be observed alongside impaired apoptosis, especially in some cancers such as colorectal adenoma [64] or prostate cancer [65].

Inflammation is also often observed alongside insulin resistance, hyperinsulinemia, and type 2 diabetes [66,67]. Acute hyperinsulinemia has been reported to increase plasma interleukin-6 levels in non-diabetic and type 2 diabetes patients [67]. Interleukin-6 expression was also upregulated in digital lamellar tissue collected from horses subjected to the euglycemic-hyperinsulinemic clamp (EHC) model of laminitis [68]. Similarly, plasma IL-8 concentration increases in obese subjects [69] and in diabetic patients [70]. Interestingly, both ASC and HPC exhibited decreased expression of the inflammatory markers mentioned in hyperinsulinemia. The expression of all the markers analysed associated with inflammation was decreased in both cell populations as a result of insulin treatment. Importantly, insulin is well known for its anti-inflammatory properties and its' immunomodulatory activity [71]. For example, in a rat model of severe inflammatory insult, insulin therapy led to significant suppression of circulating proinflammatory TNF-$\alpha$ and IL-6 [72]. Furthermore, the expression of IL-1$\beta$ in human macrophages challenged with LPS was suppressed, following an insulin treatment (insulin dose at 25 IU/mL and higher) [73]. In a porcine model of sepsis, the hyperinsulinemic-euglycemic clamp decreased the release of TNF and IL-6 [74]. Previous results indicated that IL-10 and IL13 decreased in type 2 diabetic patients and are associated with metabolic syndrome [75,76]. The level of

investigated IL10 and IL13 in the present work decreased in both types of cells under hyperinsulinemia. Interestingly, the levels of IL10 were found to be considerably higher in HPC than in ASC. However, opposite results were found for IL13. Camilo et al., [77] demonstrate that serum levels of IL-13 are significantly elevated in insulin-resistant patients without showing a correlation with parameters of low-grade systemic inflammation such as TNF-$\alpha$, and IL-10. Together with higher expression of IL-8 and IL-6, this suggests that HPC may help the cytokine storm for a more effective phenotype change to an immunosuppressing pool of cells with better migratory and healing abilities under hyperinsulinemia than ASC.

We also investigated the effects of hyperinsulinemia on genes involved in insulin action. In HPC, we observed significantly lower expression of *PTPN1* than in ASC. The protein tyrosine phosphatase PTPN1 plays a crucial role in the regulation of insulin signalling and metabolic health through dephosphorylation of the insulin receptor, inhibition of insulin action, and increased glucose absorption through increased transporter expression [78]. Lower PTPN1 activity promotes glucose uptake through increased cell surface expression of its transporter. Furthermore, HPC cells showed a decreased level of *FOXO1*. FoxO1 is a transcription factor; however, it also shows transcription-independent effects by regulating the activities of numerous other transcription factors, including the androgen receptor, the signal transducer, and the transcription-3 activator (STAT3) and regulating *IR* expression. Nakae et al., [79] showed that FoxO1-heterozygous mice are resistant to diet-induced diabetes. Increased FoxO1 activity in animal models contributes to the phenotype of insulin resistance and diabetes, however, it is crucial to stress that FoxO1 does not function primarily as an insulin antagonist with a pro-diabetic role. For example, mouse FoxO1 has been shown to enhance adiponectin receptor expression, which is expected to promote insulin sensitivity [80].

Hyperinsulinemia was found to cause a significant decrease in the expression of the *IR gene* (alias INSR) and, as a result, a decrease in surface INSR and total INSR protein, implying that reduced IR expression by hyperinsulinemia may be a key independent factor in INSR down-regulation and insulin resistance [81,82] . Moreover, hyperinsulinemia has a large impact on AKT and ERK signalling, INSR abundance, localisation, and transcriptional activities [82]. Previous studies have shown that exposing the neuronal cell line to insulin induced bluted AKT signalling and lysosomal degradation of INSR. However, it did not influence the *IR* mRNA level [83]. Here, we show that HPC as opposed to ASC show significantly increased expression of *IR* and *AKT* genes. In addition to them, the opposite result was shown for the *IRS1* gene which encodes IRS1- a major mediator between the insulin receptor and phosphatidylinositol 3-kinase (PI3K) in the insulin signalling pathway. However, the fold change of *IRS1* expression was lower in HPC than in ASC. This leads us to conclude that HPC are more resistant to hyperinsulinemia and may have greater therapeutic potential in reducing EMS and/or Laminitis.

Obesity is the primary risk factor for the development of EMS (Equine Metabolic Syndrome), covering risk factors for endocrinopathic laminitis in which metabolic alterations promote injury to the laminae of the hoof material and disturb the balanced interrelationship between plasma concentrations of insulin, glucose, and lipids [84]. High insulin concentrations may restrict the veins, resulting in inadequate blood flow to the foot [85] and may have a direct influence on hoof cells through insulin-like growth factor (IGF-1), causing a delay in hoof keratinization [86]. Moreover, insulin dysregulation can manifest as one or more of the following: basal hyperinsulinemia; an excessive or prolonged hyperinsulinemic response to carbohydrates, with or without excessive or prolonged hyperglycemia, and tissue insulin resistance [84]**.** MicroRNAs are endogenously produced short noncoding RNAs that can regulate gene expression. Dysregulated miRNA expressions, either directly or indirectly, can have a large impact on a wide range of cellular functions, including proliferation, apoptosis, and cell fate [87]. Furthermore, microRNAs affect the expression and/or activity of molecules involved in antioxidative signalling pathways

(e.g., FOXOs), as well as effector enzymes (for example, CAT, SOD1 / 2) and genes involved in many signalling pathways related to insulin sensitivity and lipid metabolism that contribute to metabolic imbalance[88]]. Additionally, miRNAs have emerged as important players in both adipogenesis and osteogenesis, targeting and degrading critical adipogenic and osteogenic genes [89–91]. Furthermore, stem cells have specific miRNA expression profiles that modulate stem cell fate during tissue regeneration due to their fundamental role in the control of stem cell destiny [92]. To determine the effects of hyperinsulinemia on miRNA strand selection, ASC and HPC were subjected to hyperinsulinemic conditions. The miRNA strand selection was then analysed and compared between ASC and HPC. We showed that miR-125b and miR-125a were down-regulated in both cell types investigated under hyperinsulinemia. Overexpression of both miRNAs has been reported to inhibit proliferation and osteogenic differentiation [93]. Furthermore, miR-125 family is involved in neuronal differentiation as well as self-renewal and differentiation of skin stem cells [94–96]. We also observed lower expression of miR-96, miR-218, and miR-451 in HPC, which are involved in the improvement of new bone formation, the acceleration of fracture healing, and osteoblast differentiation [97–99]. miR-96 has been reported to partake in wound healing, keratinocyte proliferation and migration [100–103] , while miR-218 was described to regulate skin and hair follicle development [99,104]. As for miR-451, it has been demonstrated that it exhibits immunomodulatory and inflammation-supressing activity in diabetic retinopathy and microgila-mediated neuroinflammation [105,106]**.** However, during hyperinsulinemia, miR-96 and miR-218 increased in ASC cells, suggesting a more promising therapeutic target for bone repair in future clinical applications. Here we also demonstrated the decrease in the expression of miR-34a and miR-34c which are responsible for repressed reprogramming [107]. miR-34 miRNAs and p21 are the main downstream targets that work together to suppress iPSC production. A significant downstream target of p53 is p21, which inhibits cell growth. Changes in cell proliferation, immortalization, apoptosis, and DNA damage response are all influenced by p53 [107]. What is interesting is that the miR-34 family has a higher expression level in HPC in control and experimental conditions. In the current study, we also investigate the expression of miR-21, miR-27a and miR-30c. Previously, it was discovered that patients with metabolic syndrome and type 2 diabetes had higher circulating levels of miR-27ainvolved in the PPAR-γ-PI3K/AKT-GLUT4 signalling axis, thus leading to increased glucose uptake and decreased insulin resistance [108–110]. Compared to ASC, mir-27a expression significantly decreased in HPC during hyperinsulinemia. It could be consistent with the findings of Yao et al. who discovered that miR-27a is important for obesity through modulating insulin resistance in adipocytes and serving as an adipocyte differentiation repressor [110]. In our study, we observed two times higher relative expression of miR-30c in equine hoof progenitors than in adipose-derived stem cells. However, during hyperinsulinemia miR-30c was downregulated in both cells. The miR-30 family is highly conserved and known to target the Notch1 ligand DLL4, which is a ligand that contributes to metabolic disease and macrophage inflammation [111,112]. These data suggest that miR-30 induction holds therapeutic potential for regulating macrophage-driven inflammatory and metabolic disorders. On the other hand, miR-30c was induced during adipogenesis but was downregulated in obese adipocytes [113]. Furthermore, diabetes mellitus significantly downregulated miR-30c-5p associated with stem cell survival, proliferation, and differentiation in cardiac progenitor cells [114]. The last of the analysed miRNAs was miR-21,s which was significantly downregulated in insulin-resistant adipocytes [115]. Moreover, Kim et al., reported that miR-21 regulated adipogenic differentiation through the modulation of TGF-β signaling [116]. Results of qRT-PCR showed that miR-21 concentrations were significantly higher in HPC than in APC in both CTRL and HI groups, as well as *TGFB1* in the HI group. Taken together, the current results demonstrated that HPC possesses better potential to modulate the PTEN-AKT pathway by miR-21 and reverse high glucose and high insulin-induced insulin resistance, as was suggested

by Ling et al., [115]. Tha main limitations of the presented study are the small sample size and limited information about the animals.

## 4. Materials and Methods

### *4.1. Sample Acquisition and Cell Isolation*

Coronary corium tissue samples were collected postmortem at a local slaughterhouse, the reason for the euthanization of the animal was unrelated to this study. Samples were obtained from one young (approximately 1-3 years old), of unknown sex and breed. As for the adipose tissue, it was dissected from an 8-year-old warm-blood mare. The tissue samples were immediately transported to the laboratory and the cells were isolated as previously described [117,118]. Briefly, adipose tissue and coronary corium tissue samples were washed 3 times with PBS and then dissected in a Petri dish with a #10 scalpel blade in 1×1mm squares. After dissection, the minced tissue was transferred to a sterile 50 mL tube with a solution of collagenase type I (1 mg/mL) in DMEM/F12 (Merck, Poznan, Poland). The tube containing minced coronary corium tissue was incubated at 37 °C for 2 hours with 2-dimensional agitation while the falcon containing minced adipose tissue was incubated under the same conditions for 40 minutes. After digestion, the coronary corium-containing solution was filtered with a 100 μm filter and then with a 70 μm filter, while the solution containing adipose tissue was not filtered. Subsequently, both samples were centrifuged for 10 min at 1,200×g, the supernatant was discarded and the cell pellet was then centrifuged again for 4 minutes at 300×g. After the isolation, HPC were phenotyped, as previously described. The HPC and ASC were cryopreserved in a freezing medium (89% FBS, 10% DMSO, 1% penicillin/streptomycin mix) and stored at -156 °C. Before the experiment started, the HPC were thawed and cultured in DMEM/F12 (Sigma Aldrich/Merck, Poznan, Po-land) 1% penicillin/streptomycin mix (P/S, Sigma Aldrich, Poznan, Poland) and 10% FBS (Foetal Bovine Serum, Sigma Aldrich/Merck, Poznan, Poland), while ASC was cultured in Dulbecco's modified Eagle medium (DMEM) containing 1000 mg/L glucose, supplemented with 1% penicillin/streptomycin mix (P/S, Sigma Aldrich, Poznan, Poland) and 5% of FBS. Both ASC and HPC cultures were in the 10th passage at the time of the experiment.

### *4.2. Hyperinsulinemia model*

For the hyperinsulinemia model, both ASC and HPC cells were starved for 2 hours without FBS, then 500 nmol of insulin was added and cells were incubated for 24 hours in a humidified incubator at 37 °C in 5% CO2. Subsequently, cells were harvested for further analysis.

### *4.3. Cell Imagining and Mitochondrial Network Visualization.*

The mitochondrial network of ASC and HPC was evaluated based on fluorescent staining. Briefly, cells were first incubated with the MitoRed dye solution (Sigma-Aldrich/Merck, Poznan, Poland) at 37 °C in a $CO_2$ incubator for 30 minutes and then fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich/Merck, Poznan, Poland) for 30 minutes at room temperature. After fixation, cells were washed 3 times with PBS and nuclei were stained with 4',6-Diamidine-2'-phenylindole dihydrochloride using ProLong™ Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, Warsaw, Poland). A confocal microscope (Leica TCSSPE, Leica Microsystems, KAWA.SKA Sp. z o. o., Zalesie Górne, Poland) was used to observe and imagine the cells at 630x magnification. All images were subsequently processed with Fiji software (ImageJ 1.52n, Wayne Rasband, National Institute of Health, Bethesda, MD, USA).

*4.4. Microcapillary Flow Cytometry Analysis*

ASC and HPC cells were analysed with the commercially available Muse™ Assay Kit and the Muse™ Cell Analyzer (Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. Briefly, after harvesting, cells were centrifuged at 300×g for 5 minutes, washed with PBS, and fixed overnight using a 70% ethanol solution at -20 °C. Subsequently, cells were centrifuged at 300×g for 5 minutes and rinsed again with PBS. The cells were incubated with the Muse™ Cell Cycle Reagent (Muse™ Cell Cycle As-say Kit; Merck, Warsaw, Poland) for 30 minutes and analyzed. Furthermore, the mitochondrial membrane potential was assessed with the Muse® MitoPotential Assay Kit (Luminex). Briefly, the solution containing the harvested cells was diluted with 1x Assay buffer and incubated with the MitoPotential working solution for 20 min at 37 °C. Then Muse 7-AAD was added to the samples and the resulting solution was incubated for 5 minutes at room temperature. Finally, the samples were analysed with the Muse™ Cell Analyzer. In terms of intracellular oxidative stress factor analysis, Muse® Oxidative Stress kit (Luminex) was used, according to the manufacturer's instruction. Briefly, samples were incubated at 37 °C for 30 minutes with the Muse® Oxidative Stress Working Solution. After incubation mixed thoroughly and analysed on the Muse™ Cell Analyser. Nitric oxide activity in ASC and HPC was evaluated using the Muse® Nitric Oxide Kit (Luminex), following the manufacturer's protocol. Briefly, the samples were incubated with Muse® Nitric Oxide working solution at 37 °C for 30 minutes, and then Muse® 7-AAD working solution was added and mixed with the samples. Subsequently, the samples were analysed with the Muse™ Cell Analyzer.

*4.5. RNA isolation and RT-qPCR*

To assess the expression of key markers for apoptosis, inflammation, autophagy, insulin signalling pathway, mitochondrial metabolism and miRNAs, total RNA was isolated from ASC and HPC cells, using the phenol-chloroform method as described before [119]. RNA concentrations and purity were calculated based on the absorbance at 260/280 nm with a nano spectrophotometer (Epoch, Biotek, Bad Friedrichshall, Germany). A 150 ng of total RNA was used for digestion with RNAse free DNAse I (Sig-ma-Aldrich/Merck, Poznan, Poland), subsequently, cDNA synthesis was performed using the PrimeScript RT Reagent Kit (Takara Bio Europe, Saint-Germaine, Laye, France), both digestion and synthesis reaction was completed on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The obtained cDNA was diluted with nuclease-free water in a 1:3 ratio and used for RT-qPCR analysis. The expression of mRNA and miRNA transcripts was detected using specific primers (Table 1) and the SensiFAST SYBR & Fluorescein Kit (Meridian Bioscience, London, UK). All RT-qPCR analyses were performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad), as previously described [120]. The relative expressions were calculated using the $2^{-\Delta\Delta CQ}$ method [121] with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene for the mRNA or U6 for miRNA. The control (CTRL) samples were used as a reference.

*4.6. The isolation of mitochondria and mtRNA*

Intact mitochondria were isolated from ASC and HPC cultures, using the Mitochondria Isolation Kit for Cultured Cells (ThermoFisher Scientific, Warsaw, Poland), according to the manufacturer's instructions. Then, mtRNA was isolated from the extracted mitochondria with the phenol-chloroform method, as described above [119].

*4.7. Statistical analysis*

At least three technical replicates were performed for each biological assay and the results are presented as a mean ± SD. The normality of the data was assessed with Kolmogorov-Smirnov or Shapiro-Wilk test and the variance was analyzed with the Fisher test. The statistical difference between the experimental groups was calculated using the unpaired Student's t-test or Mann-Whitney U test. The GraphPad Prism 8 software (La Jolla, CA, USA) was used to process the data and prepare the graphs. Differences between ASC and HPC were marked with an asterisk (*) while differences between the control group and the hyperinsulinemia group were marked with a dash (#). Differences with a probability of $P < 0.05$ were considered significant and the significance was marked with one asterisk/dash (*)/(#), the differences with a probability of $P < 0.01$ were marked with two asterisks/dash (**) and differences with a probability of $P < 0.001$ were marked with three asterisks (***).

## 5. Conclusions

In this study, our objective was to assess how hyperinsulinemia will affect two pools of stem cells with potential in the treatment of laminitis, the well-established ASC and the recently described HPC. Cell cycle dynamics, mitochondrial metabolism, oxidative stress, apoptosis, and miRNA expression were compared between the two populations, under standard culture conditions and hyperinsulinemia. Our findings indicate that HPC are characterised by higher activity in terms of mitochondrial dynamics, mitophagy, and mitochondrial biogenesis under standard culture conditions, yet the effect of hyperinsulinemia on these processes is similar in ASC and HPC. Furthermore, the obtained results indicate that HPC may possess a somewhat higher resistance towards oxidative stress than ASC. Additionally, the analysis of immunomodulatory markers expression revealed that HPC may help the cytokine storm for a more effective phenotype change to an immunosuppressing pool of cells with better migratory and healing abilities under hyperinsulinemia than ASC. Furthermore, we observed that HPC are more resistant to hyperinsulinemia and may have greater therapeutic potential in reducing EMS and/or Laminitis. Additionally, the current results demonstrated that HPC possesses better potential to modulate the PTEN-AKT pathway by miR-21 and reverse high glucose and high insulin-induced insulin resistance. Taken together, the obtained results indicate that HPC may be a very promising cell pool with therapeutic potential in laminitis treatment, and could be more effective than ASC. However, further research is necessary to fully elucidate HPC therapeutic potential.

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