UNIWERSYTET
WARMIŃSKO-MAZURSKI
W OLSZTYNIE

WYDZIAŁ BIOINŻYNIERII ZWIERZĄT

Dziedzina: Nauki rolnicze

Dyscyplina: Zootechnika i rybactwo

mgr inż. Aleksandra Wiktoria Cichowska-Likszo

Rozprawa doktorska

Białkowe markery jakości nasienia najądrzowego psa

Protein markers of canine epididymal semen

Praca wykonana w Katedrze Biochemii i Biotechnologii Zwierząt
pod kierunkiem

dr hab. inż. Marzeny Mogielnickiej-Brzozowskiej, prof. UWM

Olsztyn, 2026

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Wyrazy wdzięczności składam także Współautorom publikacji naukowych wchodzących w skład niniejszej rozprawy oraz wszystkim Osobom zaangażowanym w realizację badań.

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Dziękuję również mojej Rodzinie za obecność, dobre słowo oraz wsparcie, które dodawały mi siły w chwilach wymagających szczególnej determinacji.

SPIS TREŚCI

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PUBLIKACJA 2. 82

PUBLIKACJA 3. 108

Wykaz artykułów naukowych wchodzących w skład rozprawy doktorskiej

1. Mogielnicka-Brzozowska, M., **Cichowska, A.W.** (2024). Molecular Biomarkers of Canine Reproductive Functions. *Current Issues in Molecular Biology*, *46*, 6139–6168. https://doi.org/10.3390/cimb46060367.

   IF = 3,0[1], MNiSW[2] = 70 pkt.

2. **Cichowska, A.W.**, Wisniewski, J., Bromke, M.A., Olejnik, B., Mogielnicka-Brzozowska, M. (2023). Proteome Profiling of Canine Epididymal Fluid: In Search of Protein Markers of Epididymal Sperm Motility. *International Journal of Molecular Sciences*, *24*, 14790. https://doi.org/10.3390/ijms241914790.

   IF = 4,9[1], MNiSW[3] = 140 pkt.

3. **Cichowska-Likszo, A.W.**, Likszo, P., Fraser, L., Mlynarz, P., Mogielnicka-Brzozowska, M. (2026). ALB, GPX5 and PTGDS as potential biomarkers for the aging processes of the dog epididymis. *Reproduction, Fertility and Development*, *38*, RD25105. https://doi.org/10.1071/RD25105.

   IF = 2,1[1], MNiSW[2] = 140 pkt.

Sumaryczny IF = 10,0

Łączna liczba punktów MNiSW = 350

Badania zostały sfinansowane ze środków Uniwersytetu Warmińsko-Mazurskiego w Olsztynie (projekty badawcze nr 11.610.003-300, 11.610.003-110) oraz ze środków Ministra Edukacji i Nauki w ramach programu „Regionalna Inicjatywa Doskonałości”.

[1] Wskaźnik Impact Factor (IF) czasopisma w roku publikacji.
[2] Liczba punktów według Komunikatu Ministra Nauki i Szkolnictwa Wyższego w sprawie wykazu czasopism naukowych z dnia 5 stycznia 2024 roku.
[3] Liczba punktów według Komunikatu Ministra Nauki i Szkolnictwa Wyższego w sprawie wykazu czasopism naukowych z dnia 17 lipca 2023 roku.

Charakterystyka bibliometryczna dorobku kandydatki

BIBLIOTEKA UNIWERSYTECKA
UWM W OLSZTYNIE

Olsztyn, 19 maja 2026 r.

mgr inż. Aleksandra Cichowska-Likszo

Katedra Biochemii i Biotechnologii Zwierząt
Wydział Bioinżynierii Zwierząt
Uniwersytet Warmińsko-Mazurski w Olsztynie

Potwierdzenie analizy bibliometrycznej publikacji autorstwa mgr inż. Aleksandry. Cichowskiej-Likszo (na podstawie danych z bazy ***Bibliografii Publikacji Pracowników*** *Uniwersytetu Warmińsko-Mazurskiego w Olsztynie*).

Analiza cytowań przeprowadzona w dniu 19 maja 2026 r. przedstawia się następująco:

- w bazie *Web of Science Core Collection Basic Search* (WoS^{CC}_{BS}):
  - liczba cytowań – **9**
  - indeks Hirscha – ***h* = 2**
- w bazie *Scopus Author Search* ($Scopus_{AS}$):
  - liczba cytowań – **17**
  - indeks Hirscha – ***h* = 2**

Sumaryczny *Impact Factor* (IF) wg bazy *Journal Citation Reports* (JCR) – **IF_{Σ} = 17,9**

Liczba punktów wg Ministerstwa Edukacji i Nauki (MNiSW) – **875** pkt.

Tabelaryczne zestawienie punktów MNiSW i wskaźnika *Impact Factor* (IF):

Typ dokumentu	łączna liczba prac	liczba prac z IF	liczba prac z punktacją MEiN	łączna wartość IF	łączna wartość punktacji MEiN
ogółem	**24**	**6**	**14**	**17,9**	**875**
abstrakt, komunikat, streszczenie, plakat	4	0	0	0,0	0
artykuł w czasopiśmie polskim	2	2	2	2,7	240
artykuł w czasopiśmie zagranicznym	4	4	4	15,2	490
streszczenie w materiałach konferencyjnych (komunikat, poster)	4	0	0	0,0	0

redakcja materiałów konferencyjnych	1	0	0	0,0	0
książka	1	0	0	0,0	0
redakcja monografii naukowej	3	0	3	0,0	60
rozdział (fragment) w monografii naukowej	5	0	5	0,0	85

Zestawienia tabelaryczne - bibliografia, punkty MNiSW i Impact Factor

Lp.	Opis bibliograficzny	Punkty	IF
2026			
Artykuły z czasopism			
1	Cichowska-Likszo A., Likszo P., Fraser L., Młynarz P., Mogielnicka-Brzozowska M.: ALB, GPX5 and PTGDS as potential biomarkers for the aging processes of the dog epididymis, Reproduction Fertility and Development, vol. 38, nr 2, 2026, Numer artykułu: nr art. RD25105., s. 1-13, DOI:10.1071/RD25105	140	2,1
2	Mogielnicka-Brzozowska M., Cichowska-Likszo A., Likszo P., Fraser L., Popielarczyk W., Pieklik J., Kamińska M., Luvoni G.: Potential Proteins Associated with Canine Epididymal Sperm Motility, Cells, Multidisciplinary Digital Publishing Institute (MDPI), vol. 15, nr 1, 2026, Numer artykułu: nr art. 85, s. 1-23, DOI:10.3390/cells15010085	140	5,2
2025			
Książka			
1	Luberda-Bieńkowska Z., Cichowska-Likszo A., Kordan W.: Katedra Biochemii i Biotechnologii Zwierząt : 70 lat działalności, 2025, Wydawnictwo Naukowe FNCE, ISBN 978-83-68609-34-9, 185 s.	—	—
Streszczenie w materiałach konferencyjnych (komunikat, poster)			
1	Mogielnicka-Brzozowska M., Cichowska-Likszo A., Dopieralska W.: Proteom jąder i najądrzy psa w kontekście białek wiążących cynk, W: XIX Międzynarodowy Kongres Rozrodu Małych Zwierząt: Płodność, Ciąża, Noworodek. Wrocław 11-12 października 2025, 2025, Uniwersytet Przyrodniczy we Wrocławiu, ISBN 978-83-969819-2-9, s. 175	—	—
Abstrakt, komunikat, streszczenie, plakat			
1	Mogielnicka-Brzozowska M., Cichowska-Likszo A., Popielarczyk W., Fraser L., Strzeżek R.: Analysis of canine epididymal sperm proteins as motility biomarkers, Reproduction in Domestic Animals, Blackwell, vol. 60, nr Supplement 2, 2025, Numer artykułu: nr abs. 6441, s. 29, DOI:10.1111/rda.70071	—	—
2024			
Streszczenie w materiałach konferencyjnych (komunikat, poster)			

1	Mogielnicka-Brzozowska M., Cichowska A., Młynarz P.: ALB, GPX5 and PTGDS as potential markers of canine reproductive tract ageing, W: 25th International EVSSAR Congress - European Veterinary Society for Small Animal Reproduction 27th - 29th June 2024, Barcelona / Milani Chiara, Montserrat Rivera del Alamo Maria, Schäfer-Somi Sabine (red.), 2024, European Veterinary Society for Small Animal Reproduction, s. 88-88	—	—
Artykuły z czasopism			
1	Mogielnicka-Brzozowska M., Cichowska A.: Molecular Biomarkers of Canine Reproductive Functions, Current Issues in Molecular Biology, MDPI, vol. 46, nr 6, 2024, s. 6139-6168, DOI:10.3390/cimb46060367	70	3
2023			
Streszczenie w materiałach konferencyjnych (komunikat, poster)			
1	Mogielnicka-Brzozowska M., Żmudzińska A., Wiśniewski J., Młynarz P., Olejnik B., Cichowska A.: Canine epididymal spermatozoa ageing is reflected by variations in functional quality and proteomic characteristics, W: 48th World Small Animal Veterinary Association Congress and 28th FECAVA Eurocongress, Lisbon, 27-29 September 2023 : WSAVA 2023 : Posters E-Book, 2023, [s.n.], s. 202-202	—	—
2	Cichowska A., Wiśniewski J., Bromke M., Olejnik B., Mogielnicka-Brzozowska M.: In search for epididymal fluid protein markers of canine (Canis lupus familiaris) epididymal sperm motility, W: Innovative Food of High Quality for Human Health and Sustainability - an integrated program of innovation and research development in agricultural and veterinary science, 2023, Uniwersytet Warmińsko-Mazurski w Olsztynie, ISBN 978-83-8100-384-1, s. 11-11	—	—
Rozdział z monografii			
1	Pieklik J., Cichowska A., Mogielnicka-Brzozowska M.: Białka plemników nadjądrzowych psa związane z ruchliwością - ontologia genów, W: XX Konferencja Studenckich i Doktoranckich Kół Naukowych : Nauka i sztuka młodych wobec zagrożeń współczesnego świata / Wyrębek Henryk *[i in.]* (red.), 2023, Uniwersytet Przyrodniczo-Humanistyczny w Siedlcach, s. 139-143	20	—
2	Popielarczyk W., Cichowska A., Mogielnicka-Brzozowska M.: Potencjał wybranych białek plemników najądrzowych psa (Canis lupus familiaris) jako markerów ruchliwości, W: XX Konferencja Studenckich i Doktoranckich Kół Naukowych : Nauka i sztuka młodych wobec zagrożeń współczesnego świata / Wyrębek Henryk *[i in.]* (red.), 2023, Uniwersytet Przyrodniczo-Humanistyczny w Siedlcach, s. 144-147	20	—
3	Cichowska A., Mogielnicka-Brzozowska M.: Profilowanie oraz analiza bioinformatyczna proteomu płynu ogona najądrzy psa (Canis lupus familiaris), W: XX Konferencja Studenckich i Doktoranckich Kół Naukowych : Nauka i sztuka młodych wobec zagrożeń współczesnego świata / Wyrębek Henryk *[i in.]* (red.), 2023, Uniwersytet Przyrodniczo-Humanistyczny w Siedlcach, s. 134-138	20	—
Artykuły z czasopism			

1	Cichowska A., Wiśniewski J., Bromke M., Olejnik B., Mogielnicka-Brzozowska M.: Proteome Profiling of Canine Epididymal Fluid: In Search of Protein Markers of Epididymal Sperm Motility, International Journal of Molecular Sciences, MDPI, vol. 24, nr 19, 2023, Numer artykułu: nr art. 14790, s. 1-19, DOI:10.3390/ijms241914790	140	4,9
2	Mogielnicka-Brzozowska M., Woźniak M., Cichowska A., Fraser L., Kraziński B., Strzeżek R., Zielińska D.: Applicability of 2,4-dinitrophenylhydrazine (DNPH) method of protein oxidative damage measurement in the seminal plasma of canine (Canis lupus familiaris) and stallion (Equus caballus), Polish Journal of Veterinary Sciences, Polish Academy of Sciences Committee of Veterinary Sciences, vol. 26, nr 2, 2023, s. 177-184, DOI:10.24425/pjvs.2023.145020	100	0,8
2022			
Artykuły z czasopism			
1	Mogielnicka-Brzozowska M., Piątkowska E., Fraser L., Cichowska A., Załęcki M., Kraziński B., Słowińska M., Kordan W.: Zinc-binding proteins in stallion seminal plasma as potential sperm function regulators, Annals of Animal Science, Instytut Zootechniki, vol. 22, nr 3, 2022, s. 961-976, DOI:10.2478/aoas-2022-0015	140	1,9
Abstrakt, komunikat, streszczenie, plakat			
1	Mogielnicka-Brzozowska M., Żmudzińska A., Bromke M., Strzeżek R., Zielińska M., Olejnik B., Cichowska A.: Proteomic analysis of intracellular and membrane-associated fractions of canine (Canis lupus familiaris) epididymal spermatozoa, Reproduction in Domestic Animals, Blackwell, vol. 57, nr S4, 2022, Numer artykułu: P49, s. 85, DOI:10.1111/rda.14244	—	—
2	Mogielnicka-Brzozowska M., Piątkowska E., Cichowska A., Kraziński B., Fraser L., Kordan W.: Zinc-non-binding and zinc-binding proteins of stallion seminal plasma characterized by label-free quantification and gene ontology analysis, Reproduction in Domestic Animals, vol. 57, nr S1, 2022, Numer artykułu: P149, s. 96-97, DOI:10.1111/rda.14051	—	—
2021			
Redakcja monografii naukowej			
1	Cierechowicz A., Cichowska A., Chelińska M. (red.): 50 MKSKN 2021 Międzynarodowe Seminarium Kół Naukowych w Olsztynie Seminarium Obrazowania Studenckiej Myśli Naukowej T. 2 : Aktualne problemy badawcze : Nauki społeczne i humanistyczne, 2021, Wydawnictwo Naukowe FNCE, ISBN 978-83-66800-72-4, 535 s.	20	—
2	Cierechowicz A., Cichowska A., Chelińska M. (red.): 50 MSKN 2021 "Międzynarodowe Seminarium Kół Naukowych : koła naukowe szkołą twórczego działania" T. 1 : Aktualne problemy badawcze : Nauki biomedyczne i techniczne, 2021, Wydawnictwo Naukowe FNCE, ISBN 978-83-66800-71-7, 724 s.	20	—
Redakcja materiałów konferencyjnych			
1	Cierechowicz A., Cichowska A., Chelińska M. (red.): 50. Międzynarodowe Seminarium Kół Naukowych w Olsztynie : "Seminarium Obrazowania Studenckiej Myśli Naukowej". T. 3, Aktualne problemy badawcze : nauki biomedyczne, techniczne,	—	—

	społeczne i humanistyczne : księga abstraktów, 2021, Wydawnictwo Naukowe FNCE, ISBN 978-83-66800-71-7, 200 s.		
Abstrakt, komunikat, streszczenie, plakat			
1	Mogielnicka-Brzozowska M., Piątkowska E., Cichowska A., Kraziński B., Fraser L., Kordan W.: Zinc-non-binding and zinc-binding proteins of stallion seminal plasma characterized by label-free quantification and gene ontology analysis, W: ESDAR European Society for Domestic Animal Reproduction, 2021, s. 1-1	—	—
2020			
Redakcja monografii naukowej			
1	Mogielnicka-Brzozowska M., Cichowska A. (red.): 49 MSKN 2020 "Międzynarodowe Seminarium Kół Naukowych w Olsztynie : koła naukowe szkołą twórczego działania" T. 2 : Problemy społeczne i ekonomiczne przed erą koronawirusa, 2020, Wydawnictwo Naukowe FNCE, ISBN 978-83-66264-55-7, 374 s.	20	—
Rozdział z monografii			
1	Cichowska A.: Wpływ procedur laboratoryjnego postępowania z nasieniem na jakość nasienia oraz proteom plazmy nasienia i plemników, W: 49 MSKN 2020 "Międzynarodowe Seminarium Kół Naukowych : koła naukowe szkołą twórczego działania" T. 3, Nowoczesne rozwiązania proekologiczne w naukach zootechnicznych, weterynaryjnych, rolniczych i technicznych / Mogielnicka-Brzozowska Marzena (red.), 2020, Wydawnictwo Naukowe FNCE, ISBN 978-83-66264-56-4, s. 32-44	20	—
2017			
Rozdział z monografii			
1	Cichowska A., Mogielnicka-Brzozowska M.: Zastosowanie elektroforezy SDS-PAGE do identyfikacji białek płynów tkanek jąder i najądrzy kota domowego (Felis catus)-badania wstępne, W: Koła naukowe - szkołą twórczego działania : nauki weterynaryjne i rolnicze [praca recenzowana], 2017, Wydawnictwo ABADA, s. 87-92	5	—

Zestawienia tabelaryczne - analiza cytowań w bazie WoS^{CC} i Scopus

Lp.	Opis bibliograficzny	WoS^{CC}	Scopus$_{AS}$
2026			
Artykuły z czasopism			
1	Cichowska-Likszo A., Likszo P., Fraser L., Młynarz P., Mogielnicka-Brzozowska M.: ALB, GPX5 and PTGDS as potential biomarkers for the aging processes of the dog epididymis, Reproduction Fertility and Development, vol. 38, nr 2, 2026, Numer artykułu: nr art. RD25105., s. 1-13, DOI:10.1071/RD25105	0	0
2	Mogielnicka-Brzozowska M., Cichowska-Likszo A., Likszo P., Fraser L., Popielarczyk W., Pieklik J., Kamińska M., Luvoni G.: Potential Proteins Associated with Canine Epididymal Sperm Motility, Cells, Multidisciplinary Digital Publishing Institute (MDPI), vol. 15, nr 1, 2026, Numer artykułu: nr art. 85, s. 1-23, DOI:10.3390/cells15010085	0	0

2025			
Książka			
1	Luberda-Bieńkowska Z., Cichowska-Likszo A., Kordan W.: Katedra Biochemii i Biotechnologii Zwierząt : 70 lat działalności, 2025, Wydawnictwo Naukowe FNCE, ISBN 978-83-68609-34-9, 185 s.	—	—
Streszczenie w materiałach konferencyjnych (komunikat, poster)			
1	Mogielnicka-Brzozowska M., Cichowska-Likszo A., Dopieralska W.: Proteom jąder i najądrzy psa w kontekście białek wiążących cynk, W: XIX Międzynarodowy Kongres Rozrodu Małych Zwierząt: Płodność, Ciąża, Noworodek. Wrocław 11-12 października 2025, 2025, Uniwersytet Przyrodniczy we Wrocławiu, ISBN 978-83-969819-2-9, s. 175	—	—
Abstrakt, komunikat, streszczenie, plakat			
1	Mogielnicka-Brzozowska M., Cichowska-Likszo A., Popielarczyk W., Fraser L., Strzeżek R.: Analysis of canine epididymal sperm proteins as motility biomarkers, Reproduction in Domestic Animals, Blackwell, vol. 60, nr Supplement 2, 2025, Numer artykułu: nr abs. 6441, s. 29, DOI:10.1111/rda.70071	0	—
2024			
Streszczenie w materiałach konferencyjnych (komunikat, poster)			
1	Mogielnicka-Brzozowska M., Cichowska A., Młynarz P.: ALB, GPX5 and PTGDS as potential markers of canine reproductive tract ageing, W: 25th International EVSSAR Congress - European Veterinary Society for Small Animal Reproduction 27th - 29th June 2024, Barcelona / Milani Chiara, Montserrat Rivera del Alamo Maria, Schäfer-Somi Sabine (red.), 2024, European Veterinary Society for Small Animal Reproduction, s. 88-88	—	—
Artykuły z czasopism			
1	Mogielnicka-Brzozowska M., Cichowska A.: Molecular Biomarkers of Canine Reproductive Functions, Current Issues in Molecular Biology, MDPI, vol. 46, nr 6, 2024, s. 6139-6168, DOI:10.3390/cimb46060367	6	9
2023			
Streszczenie w materiałach konferencyjnych (komunikat, poster)			
1	Mogielnicka-Brzozowska M., Żmudzińska A., Wiśniewski J., Młynarz P., Olejnik B., Cichowska A.: Canine epididymal spermatozoa ageing is reflected by variations in functional quality and proteomic characteristics, W: 48th World Small Animal Veterinary Association Congress and 28th FECAVA Eurocongress, Lisbon, 27-29 September 2023 : WSAVA 2023 : Posters E-Book, 2023, [s.n.], s. 202-202	—	—
2	Cichowska A., Wiśniewski J., Bromke M., Olejnik B., Mogielnicka-Brzozowska M.: In search for epididymal fluid protein markers of canine (Canis lupus familiaris) epididymal sperm motility, W: Innovative Food of High Quality for Human Health and Sustainability - an integrated program of innovation and research development in agricultural and veterinary science, 2023, Uniwersytet	—	—

	Warmińsko-Mazurski w Olsztynie, ISBN 978-83-8100-384-1, s. 11-11		
Rozdział z monografii			
1	Pieklik J., Cichowska A., Mogielnicka-Brzozowska M.: Białka plemników nadjądrzowych psa związane z ruchliwością - ontologia genów, W: XX Konferencja Studenckich i Doktoranckich Kół Naukowych : Nauka i sztuka młodych wobec zagrożeń współczesnego świata / Wyrębek Henryk *[i in.]* (red.), 2023, Uniwersytet Przyrodniczo-Humanistyczny w Siedlcach, s. 139-143	—	—
2	Popielarczyk W., Cichowska A., Mogielnicka-Brzozowska M.: Potencjał wybranych białek plemników najądrzowych psa (Canis lupus familiaris) jako markerów ruchliwości, W: XX Konferencja Studenckich i Doktoranckich Kół Naukowych : Nauka i sztuka młodych wobec zagrożeń współczesnego świata / Wyrębek Henryk *[i in.]* (red.), 2023, Uniwersytet Przyrodniczo-Humanistyczny w Siedlcach, s. 144-147	—	—
3	Cichowska A., Mogielnicka-Brzozowska M.: Profilowanie oraz analiza bioinformatyczna proteomu płynu ogona najądrzy psa (Canis lupus familiaris), W: XX Konferencja Studenckich i Doktoranckich Kół Naukowych : Nauka i sztuka młodych wobec zagrożeń współczesnego świata / Wyrębek Henryk *[i in.]* (red.), 2023, Uniwersytet Przyrodniczo-Humanistyczny w Siedlcach, s. 134-138	—	—
Artykuły z czasopism			
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Wykaz skrótów

ACRBP – białko wiążące akrozynę (ang. *acrosin binding protein*

ACTB – aktyna cytoplazmatyczna 1 (ang. *actin, cytoplasmic 1*)

AFP – alfa-fetoproteina (ang. *alpha-fetoprotein*)

AKAPs – białka kotwiczące kinazę A (ang. *A-kinase anchoring proteins*)

ALB – albumina (ang. *albumin*)

ALBs – albuminy (ang. *albumins*)

ALH – amplituda bocznego wychylenia główki (ang. *the amplitude of lateral head displacement*)

ALPL – fosfataza alkaliczna (ang. *alkaline phosphatase*)

BCF – częstotliwość przecięcia toru ruchu witki (ang. *beat cross frequency*)

BOLL – homolog białka Boule wiążącego RNA (ang. *Boule homolog, RNA-binding protein*)

CARD6 – członek 6 rodziny białek zawierających domenę rekrutacji kaspaz (ang. *caspase recruitment domain family member 6*)

CEMIP – hialuronoglukozoaminidaza (ang. *hyaluronoglucosaminidase*)

CES5A – karboksyloesteraza 5A (ang. *carboxylesterase 5A*)

CLU – klasteryna (ang. *clusterin*)

CPSE – esteraza specyficzna dla prostaty psa (ang. *canine prostate-specific esterase*)

CRISP2 – sekrecyjne białko bogate w cysteinę 2 (ang. *cysteine-rich secretory protein 2*)

ELSPBP1 – najądrzowe białko wiążące plemniki 1 (ang. *epididymal sperm-binding protein 1*)

ExG1 – grupa eksperymentalna 1 (ang. *experimental group 1*)

ExG2 – grupa eksperymentalna 2 (ang. *experimental group 2*)

ExG3 – grupa eksperymentalna 3 (ang. *experimental group 3*)

ExGs – grupy eksperymentalne (ang. *experimental groups*)

GALNT6 – N-acetylogalaktozaminylotransferaza polipeptydowa (ang. *polypeptide N-acetylgalactosaminyltransferase*)

GO – ontologia genów (ang. *gene ontology*)

GPX1 – peroksydaza glutationowa 1 (ang. *glutathione peroxidase 1*)

GPX2 – peroksydaza glutationowa 2 (ang. *glutathione peroxidase 2*)

GPX3 – peroksydaza glutationowa 3 (ang. *glutathione peroxidase 3*)

GPX5 – wydzielnicza peroksydaza glutationowa najądrza (ang. *epididymal secretory glutathione peroxidase*)

GPX6 – peroksydaza glutationowa 6 (ang. *glutathione peroxidase 6*)

GPX8 – peroksydaza glutationowa 8 (ang. *glutathione peroxidase 8*)

GSM – grupa badawcza o dobrej ruchliwości plemników (ang. *good sperm motility*)

GSR – reduktaza glutationowa (ang. *glutathione reductase*)

GSS – syntetaza glutationowa (ang. *glutathione synthetase*)

HPGDS – hematopoetyczna syntaza D prostaglandyny (ang. *hematopoietic prostaglandin D synthase*)

KEGG – ang. *Kyoto Encyclopedia of Genes and Genomes*

LCN10 – lipokalina 10 (ang. *lipocalin 10*)

LCN9 – lipokalina 9 (ang. *lipocalin 9*)

LCNL1 – białko zawierające domenę lipokalinową/cytozolową wiążącą kwasy tłuszczowe (ang. *lipocln_cytosolic_FA-bd_dom domain-containing protein*)

LIN – współczynnik liniowości ruchu (ang. *linearity coefficient*)

LOC606925 – białko zawierające domenę KRAB (ang. *KRAB domain-containing protein*), KRAB – ang. *Krüppel-associated box*

LOC607874 – białko zawierające domenę cystatyny (ang. *cystatin domain-containing protein*)

LTF – laktotransferyna (ang. *lactotransferrin*)

MEDIUM – subpopulacja plemników charakteryzująca się ruchem średnim

MMPs – metaloproteinazy (ang. *metalloproteinases*)

nanoUPLC-Q-TOF/MS – nano-ultrawysokosprawna chromatografia cieczowa sprzężona z kwadrupolową spektrometrią mas z pomiarem czasu przelotu (ang. *nano ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry*)

NPC2 – wewnątrzkomórkowy transporter cholesterolu typu C2 (ang. *Niemann-Pick intracellular cholesterol transporter C2*)

OLFM4 – olfaktomedyna 4 (ang. *olfactomedin 4*)

OPN – osteopontyna (ang. *osteopontin*)

PLS3 – plastyna 3 (ang. *plastin 3*)

PMOT – ruchliwość postępowa (ang. *progressive motility*)

PRDX6 – peroksyredoksyna 6 (ang. *peroxiredoxin 6*)

PSM – grupa badawcza o obniżonej ruchliwości plemników (ang. *poor sperm motility*)

PTGDS – izomeraza D prostaglandyny H2 (ang. *prostaglandin-H2 D-isomerase*)

PTGES – syntaza prostaglandyny E (ang. *prostaglandin E synthase*)

PTGES2 – syntaza prostaglandyny E2 (ang. *prostaglandin E synthase 2*)

PTGIS – syntaza prostaglandyny I2 (ang. *prostaglandin I2 synthase*)

PTGS1 – syntaza prostaglandyny G/H 1 (ang. *prostaglandin G/H synthase 1*)

PTGS2 – syntaza prostaglandynowo-endonadtlenkowa 2 (ang. *prostaglandin-endoperoxide synthase 2*)

RAPID – subpopulacja plemników charakteryzujących się ruchem szybkim

SDS-PAGE – elektroforeza w żelu poliakrylamidowym w obecności dodecylosiarczanu sodu (ang. *sodium dodecyl sulphate polyacrylamide gel electrophoresis*)

SEM – błąd standardowy średniej (ang. *standard error of mean*)

SLOW – subpopulacja plemników charakteryzujących się ruchem wolnym

STATIC – subpopulacja plemników nieruchomych

STR – współczynnik prostoliniowości ruchu (ang. *straightness*)

TMOT – ruchliwość całkowita (ang. *total motility*)

VAP – średnia prędkość ruchu po uśrednionym torze (ang. *average path velocity*)

VCL – prędkość po torze zakrzywionym (ang. *curvilinear velocity*)

VSL – prędkość ruchu po linii prostej (ang. *straight-line velocity*)

WAPdcp* – białko zawierające domenę WAP (ang. *WAP domain-containing protein*), WAP – ang. *whey acidic protein*

**skrót wprowadzony na potrzeby niniejszej pracy*

Streszczenie

Cele pracy: a) przegląd biomarkerów molekularnych wskazanych dotychczas jako przydatne w ocenie jakości nasienia psa, b) analiza proteomu płynu ogona najądrza psa w zależności od ruchliwości plemników i wieku psa oraz c) selekcja potencjalnych markerów białkowych występujących w płynie najądrzowym psa, związanych z ruchliwością plemników najądrzowych i wiekiem zwierzęcia.

W pierwszej pracy usystematyzowano wiedzę dotyczącą biomarkerów molekularnych, wskazanych do tej pory jako użyteczne w ocenie funkcji reprodukcyjnych psów. Wykazano, że zarówno białka, jak i substancje niebiałkowe, takie jak lipidy, węglowodany czy jony, mogą stanowić cenne wskaźniki jakości nasienia psa.

W drugiej pracy po raz pierwszy przeprowadzono kompleksowe profilowanie proteomu płynu ogona najądrza psa z wykorzystaniem nanoUPLC-Q-TOF/MS. Wykazano zależne od ruchliwości plemników zmiany w składzie proteomu płynu najądrzowego oraz wyższą zawartość ALB, CRISP2, LCNL1 i PTGDS w płynie najądrzowym towarzyszącym plemnikom o dobrej ruchliwości. Wyniki dla ALB i PTGDS potwierdzono metodą Western blot, wskazując ich potencjalną rolę jako markerów ruchliwości plemników najądrzowych psa.

Trzecia praca koncentrowała się na wpływie wieku na skład białkowy płynu ogona najądrza psa. Wykazano, że proces starzenia prowadzi do zmian w zawartości ALB, GPX5 i PTGDS. Uzyskane wyniki sugerują, że białka te mogą pełnić funkcję biomarkerów procesów starzenia się najądrza psa.

Przeprowadzone badania pozwoliły scharakteryzować mikrośrodowisko białkowe płynu ogona najądrza psa oraz dostarczyły nowej wiedzy o jego związku z ruchliwością plemników i procesami starzenia się najądrza.

Słowa kluczowe: biomarkery; płyn najądrza; plemniki najądrzowe; proteom; pies

Abstract

The aims of the study: a) to review molecular biomarkers identified to date as useful indicators of canine semen quality, b) to analyze the proteome of canine cauda epididymal fluid in relation to sperm motility and the age of the dog, and c) to select potential protein markers present in canine epididymal fluid associated with epididymal sperm motility and animal age.

The first study systematized current knowledge on molecular biomarkers considered useful for the assessment of canine reproductive functions. It was demonstrated that both proteins and non-protein substances, such as lipids, carbohydrates, and ions, may serve as valuable indicators of canine semen quality.

In the second study, for the first time, comprehensive proteomic profiling of canine cauda epididymal fluid was performed using nanoUPLC-Q-TOF/MS. Motility-dependent changes in the composition of the epididymal fluid proteome were identified, together with higher content of ALB, CRISP2, LCNL1, and PTGDS in epididymal fluid associated with spermatozoa exhibiting good motility. The findings for ALB ang PTGDS were confirmed by Western blot analysis, indicating their potential role as markers of canine epididymal sperm motility.

The third study focused on the effect of age on the protein composition of canine cauda epididymal fluid. Ageing was shown to induce changes in the abundance of ALB, GPX5, and PTGDS. The obtained results suggest that these proteins may serve as biomarkers of ageing processes in the canine epididymis.

The conducted studies enabled the characterization of the protein microenvironment of canine cauda epididymal fluid and provided new knowledge about its relationship with sperm motility and epididymal ageing processes.

Keywords: biomarkers; epididymal fluid; epididymal sperm; proteome; dog

1. Wstęp

Ocena jakości nasienia stanowi jeden z podstawowych elementów diagnostyki płodności samców oraz istotny komponent biotechnik rozrodu stosowanych zarówno u zwierząt gospodarskich, jak i towarzyszących. W przypadku psa zagadnienie to nabiera szczególnego znaczenia ze względu na rosnącą wartość hodowlaną i społeczną tego gatunku. Pies stanowi cenny model badawczy dla innych przedstawicieli rodziny *Canidae*, w tym gatunków zagrożonych wyginięciem. Jednocześnie jest interesującym modelem translacyjnym w badaniach nad płodnością ssaków, umożliwiającym analizę mechanizmów molekularnych procesów reprodukcyjnych obserwowanych u innych gatunków, w tym człowieka (Kirchhoff, 2002; Ramos Angrimani i in., 2017).

Dotychczasowe badania koncentrowały się przede wszystkim na analizie jakości nasienia ejakulowanego. Nasienie najądrzowe stanowi cenny materiał biologiczny wykorzystywany w sytuacjach, gdy pozyskanie ejakulatu jest niemożliwe, między innymi w wyniku nagłej śmierci samca, w przypadkach azoospermii lub podczas działań związanych z zabezpieczeniem materiału genetycznego cennych osobników hodowlanych i gatunków zagrożonych wyginięciem.

W ostatnich latach obserwuje się dynamiczny rozwój badań nad molekularnymi podstawami funkcjonowania męskiego układu rozrodczego, w tym nad rolą najądrza w procesach dojrzewania, ochrony i przechowywania plemników. Najądrze nie pełni wyłącznie funkcji magazynowania gamet męskich, lecz stanowi wysoce wyspecjalizowane środowisko biologiczne warunkujące osiągnięcie przez plemniki pełnej dojrzałości funkcjonalnej niezbędnej do zapłodnienia (Cornwall, 2009; Björkgren i Sipilä, 2019). Proces dojrzewania plemników zachodzący w najądrzu obejmuje intensywne przemiany morfologiczne i biochemiczne, w tym modyfikacje profilu białkowego błon komórkowych plemników, regulację metabolizmu energetycznego oraz stablizację struktur komórkowych (Dacheux i Dacheux, 2014). Podczas transportu przez najądrze plemniki pozostają w stałym kontakcie z płynem najądrzowym zawierającym liczne białka, enzymy, lipidy oraz czynniki antyoksydacyjne uczestniczące w regulacji procesu dojrzewania i funkcji plemników (Dacheux i in., 2003; Dacheux i Dacheux, 2014; Gervasi i Visconti, 2017). Wszystkie te składniki są ze sobą ściśle powiązane, a ich efektywne współdziałanie zapewnia sygnały komórkowe niezbędne do uzyskania odpowiednich cech plemników (Dacheux i Dacheux, 2014; Bertol, 2016).

Spośród parametrów jakości nasienia, ruchliwość plemników uznawana jest za wysoce predykcyjny wskaźnik potencjału reprodukcyjnego samców (Dcunha i in., 2020). Plemniki ssaków opuszczające jądra są pozbawione zdolności do ruchu, a właściwość ta nabywana jest stopniowo podczas transportu przez najądrze (Dacheux i Dacheux, 2014; de Souza i in., 2017; Björkgren i Sipilä, 2019; James i in., 2020). Ze względu na istotne znaczenie ruchliwości zarówno w rozrodzie naturalnym, jak i technikach wspomaganego rozrodu, zrozumienie mechanizmów regulujących ten proces pozostaje jednym z kluczowych zagadnień współczesnej biologii rozrodu. Problem ten nabiera dodatkowego znaczenia w kontekście rosnącej częstotliwości zaburzeń płodności obserwowanych zarówno u ludzi, jak i u zwierząt.

Istotnym problemem naukowym i praktycznym pozostaje także wpływ procesów starzenia na funkcjonowanie najądrza i jakość plemników. Starzenie męskiego układu rozrodczego wiąże się z nasileniem stresu oksydacyjnego, zmianami w metabolizmie komórkowym, zaburzeniami homeostazy błonowej oraz kumulacją uszkodzeń DNA i białek (Aikten i Baker, 2006; Sampson i in., 2007). Procesy te mogą prowadzić do pogorszenia ruchliwości plemników oraz obniżenia płodności samców. Pomimo udokumentowanego wpływu procesu starzenia na jakość funkcjonalną i proteom plemników najądrzowych psa (Bhanmeechao i in., 2018; Zmudzinska i in., 2022b), nie wyjaśniono do tej pory wpływu zmian zachodzących wraz z wiekiem w składzie białkowym płynu najądrzowego oraz ich oddziaływań na plemniki, co jest niezwykle istotne.

Aby określić skład białkowy tak złożonego mikrośrodowiska najądrza, konieczne jest zastosowanie wysokoprzepustowych metod proteomicznych. Zaawansowana spektrometria mas połączona ze szczegółowymi analizami bioinformatycznymi może dostarczyć nowej wiedzy na temat potencjalnych mechanizmów działania poszczególnych białek i ich roli w starzeniu się najądrza. Takie podejście może umożliwić identyfikację poszczególnych białek jako biomarkerów starzenia się najądrza.

Dotychczasowa ocena jakości nasienia psa opierała się głównie na klasycznych parametrach, takich jak koncentracja, ruchliwość, żywotność czy morfologia plemników. Choć parametry te pozostają podstawą diagnostyki andrologicznej, ich wartość prognostyczna bywa ograniczona. W związku z tym współczesne badania koncentrują się na identyfikacji molekularnych biomarkerów jakości nasienia, które mogłyby dostarczyć bardziej precyzyjnych informacji dotyczących potencjału reprodukcyjnego samców.

Podejmowanie badań nad białkowymi markerami jakości nasienia najądrzowego psa jest uzasadnione zarówno z punktu widzenia nauk podstawowych, jak i praktyki klinicznej oraz hodowlanej. Identyfikacja markerów molekularnych może przyczynić się do opracowania bardziej precyzyjnych metod oceny potencjału reprodukcyjnego samców, wspomóc kwalifikację materiału biologicznego do kriokonserwacji i technik wspomaganego rozrodu oraz zwiększyć skuteczność zachowania cennych zasobów genetycznych (Niżański i in., 2016). Ponadto poznanie molekularnych mechanizmów regulujących dojrzewanie plemników i funkcjonowanie najądrza może przyczynić się do lepszego zrozumienia biologii rozrodu ssaków oraz do rozwoju badań z zakresu biologii rozrodu, proteomiki funkcjonalnej i medycyny translacyjnej.

2. Cel, zakres badań i hipotezy badawcze

Analiza dostępnej literatury wykazała wciąż ograniczoną wiedzę dotyczącą składu biochemicznego płynu najądrzowego psa i wpływu obecnych w nim składników na właściwości funkcjonalne plemników. Istotna jest też potrzeba poszukiwania markerów jakości nasienia najądrzowego, które mogłyby posłużyć do konstrukcji praktycznych testów o wartości predykcyjnej dla technik wspomaganego rozrodu. W związku z powyższym sformułowano następujące cele i hipotezy badawcze.

Cele pracy:

a) przegląd biomarkerów molekularnych wskazanych dotychczas jako przydatne w ocenie jakości nasienia psa **(publikacja 1)**,
b) analiza proteomu płynu najądrzowego psa w zależności od ruchliwości plemników oraz wieku psa **(publikacja 2 i 3)**,
c) selekcja potencjalnych markerów białkowych występujących w płynie najądrzowym psa, związanych z ruchliwością plemników najądrzowych i wiekiem zwierzęcia **(publikacja 2 i 3)**.

Hipotezy badawcze:

1) Skład białkowy płynu z ogona najądrza psa różni się w zależności od jakości ruchliwości plemników najądrzowych **(publikacja 2)**;
2) Niektóre białka obecne w płynie najądrzowym są powiązane z parametrami ruchliwości plemników i mogą być potencjalnymi markerami jakości nasienia najądrzowego psa **(publikacja 2)**;
3) Skład białkowy płynu z ogona najądrza psa zmienia się wraz z wiekiem zwierzęcia **(publikacja 3)**;
4) Zmiany w zawartości niektórych białek płynu najądrzowego mogą być związane z procesem starzenia się najądrza psa i wskazywać na ich potencjalne znaczenie jako markerów tego procesu **(publikacja 3)**;
5) Niektóre białka płynu najądrzowego wykazują zależność z parametrami ruchliwości plemników u psów w różnym wieku **(publikacja 3)**.

W celu weryfikacji powyższych hipotez zrealizowano następujące zadania badawcze:

1) **Zadanie badawcze nr 1 – Identyfikacja markerów białkowych ruchliwości plemników z płynu ogona najądrza psa**, obejmujące charakterystykę proteomiczną

płynu ogona najądrza psa w powiązaniu z ruchliwością plemników najądrzowych oraz analizę zależności pomiędzy zawartością wybranych białek a parametrami ruchu plemników najądrzowych w celu identyfikacji białek o potencjalnym znaczeniu markerowym **(publikacja 2)**;

2) **Zadanie badawcze nr 2 – Identyfikacja markerów białkowych starzenia się najądrza psa**, obejmujące charakterystykę proteomu płynu ogona najądrza psa w zależności od wieku zwierzęcia oraz wytypowanie białek związanych z procesami starzenia się najądrza jako potencjalnych biomarkerów tych procesów **(publikacja 3)**.

3. Materiał i metody

3.1. Odczynniki chemiczne

Odczynniki chemiczne użyte w badaniach zakupiono od firmy Sigma Chemical Company (St. Louis, MO, USA), o ile nie podano inaczej.

3.2. Zwierzęta doświadczalne i materiał badawczy

Materiał badawczy pozyskiwano od 23 psów mieszańców w wieku od 1 roku do 11 lat, o masie ciała od 9 do 30 kg. Psy przebywały w Schronisku dla Bezdomnych Zwierząt w Tomarynach (Polska) w tych samych warunkach środowiskowych i otrzymywały standardową dietę. Psy poddawano rutynowemu zabiegowi kastracji, przeprowadzanemu przez wykwalifikowanego lekarza weterynarii. Po orchiektomii jądra wraz z najądrzami transportowano w temperaturze 4°C do laboratorium Katedry Biochemii i Biotechnologii Zwierząt Uniwersytetu Warmińsko-Mazurskiego w Olsztynie (Polska). Pozyskane nasienie najądrzowe kolekcjonowano metodą aspiracji. Tkankę nacinano skalpelem, a płyn pobierano za pomocą pipety automatycznej, zgodnie z protokołem Ramos Angrimani i in. (2017) z modyfikacjami. Nasienie pobrane z prawego i lewego najądrza tego samego osobnika łączono.

Wszystkie procedury z udziałem zwierząt przeprowadzano zgodnie z Dyrektywą Parlamentu Europejskiego i Rady 2010/63/UE oraz obowiązującymi przepisami prawa polskiego dotyczącymi ochrony zwierząt wykorzystywanych do celów naukowych i edukacyjnych (Dz.U. 2017).

3.3. Ocena jakości plemników pozyskanych z ogona najądrza

Koncentrację plemników najądrzowych określano za pomocą komory Bürkera pod mikroskopem świetlnym (Olympus BX41TF, Tokio, Japonia). Do oceny parametrów ruchu plemników wykorzystywano system komputerowej analizy nasienia (system CASA, HTM-IVOS 12.3, Hamilton Thorne Biosciences, MA, USA). Procedura oraz ustawienia oprogramowania zostały opisane w pracach Mogielnickiej-Brzozowskiej i in. (2020) oraz Zmudzinskiej i in. (2022b).

Analizowano następujące parametry plemników: ruchliwość całkowitą (ang. *total motility*, TMOT [%]), ruchliwość postępową (ang. *progressive motility*, PMOT [%]), średnią prędkość ruchu po uśrednionym torze (ang. *average path velocity*, VAP [μm/s]), prędkość

ruchu po linii prostej (ang. *straight-line velocity*, VSL [μm/s]), prędkość ruchu po torze zakrzywionym (ang. *curvilinear velocity*, VCL [μm/s]), amplitudę bocznego wychylenia główki (ang. *the amplitude of lateral head displacement*, ALH [μm]), częstotliwość przecięcia toru ruchu witki (ang. *beat cross frequency*, BCF [Hz]), współczynnik prostoliniowości ruchu (ang. *straightness*, STR [%]) i współczynnik liniowości ruchu (ang. *linearity coefficient*, LIN [%]). W analizie wyróżniono subpopulacje plemników charakteryzujące się ruchem szybkim (RAPID [%]), średnim (MEDIUM [%]), wolnym (SLOW [%]) oraz plemniki nieruchome (STATIC [%]).

3.4. Izolacja płynu ogona najądrza

Płyn ogona najądrza oddzielano od plemników najądrzowych poprzez wirowanie (800 × *g*, 10 minut, 4°C) (Ramos Angrimani i in., 2017). Uzyskany supernatant (płyn najądrza) pobierano znad osadu zawierającego plemniki najądrzowe, przenoszono do nowych probówek, a następnie ponownie wirowano (15 000 × *g*, 10 minut, 4°C) (Dacheux i in., 2006). Próby przechowywano w temperaturze −80°C do czasu dalszych analiz.

3.5. Układ eksperymentalny

Materiał badawczy (płyn ogona najądrza psa) analizowano w dwóch układach eksperymentalnych:

I. w zależności od parametrów ruchliwości plemników najądrzowych, w dwóch grupach eksperymentalnych: o dobrej ruchliwości plemników (ang. *good sperm motility*, GSM; n = 13) oraz o obniżonej ruchliwości plemników (ang. *poor sperm motility*, PSM; n = 10). Grupa GSM obejmowała płyny ogona najądrza towarzyszące plemnikom wykazującym PMOT ≥55%, a grupa PSM obejmowała płyny ogona najądrza towarzyszące plemnikom wykazującym PMOT <55% **(publikacja 2)**;

II. w zależności od wieku osobników, z podziałem na trzy grupy eksperymentalne (ang. *experimental groups*, ExGs): psy młode w wieku 1–3 lat, n = 8 (ExG1); psy dorosłe w wieku 3,5–6 lat, n = 8 (ExG2); oraz psy stare w wieku 6,5–11 lat, n = 7 (ExG3) **(publikacja 3)**.

3.6. Przygotowanie płynu ogona najądrza do analizy proteomicznej

Szczegółowa procedura przygotowania płynu ogona najądrza do analiz proteomicznych została opisana w **publikacjach 2 i 3**. Całkowitą zawartość białka w płynach ogona najądrza oznaczano metodą Bradforda (1976). Białka wytrącano z roztworu,

poddawano redukcji i alkilacji, a następnie trawieniu trypsyną zgodnie z metodą opisaną przez Morelle i Michalskiego (2007), z niewielkimi modyfikacjami. Uzyskane peptydy poddawano analizie z użyciem nano-ultrawysokosprawnej chromatografii cieczowej sprzężonej z kwadrupolową spektrometrią mas z pomiarem czasu przelotu (ang. *nano ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry*, nanoUPLC-Q-TOF/MS).

3.7. Analiza nanoUPLC-Q-TOF/MS

Do rozdzielenia peptydów uzyskanych po trawieniu białek zastosowano system chromatografii cieczowej Waters Acquity klasy M (Waters Corporation, Milford, MA, USA), sprzężony ze spektrometrem mas Synapt G2-Si (Waters Corporation). Surowe dane uzyskane po analizie nanoUPLC-Q-TOF/MS opracowywano za pomocą oprogramowania Byonic (wersja 4.4, Protein Metrics, Cupertino, CA, USA). Wykryte peptydy porównywano z bazą danych proteomu psa SWISSPROT (CANLF) pobraną w lutym 2022 r.

3.8. Walidacja wyników techniką Western blot

Analizę Western blot przeprowadzano zgodnie z wcześniej opisaną metodą (Towbin i in., 1979). Białka rozdzielano metodą elektroforezy w 12% żelu poliakrylamidowym w obecności SDS (ang. *sodium dodecyl sulphate polyacrylamide gel electrophoresis*, SDS-PAGE), a następnie przenoszono na membrany. Po zakończonym transferze membrany blokowano, płukano i inkubowano przez noc z odpowiednimi przeciwciałami pierwszorzędowymi, a następnie drugorzędowymi.

3.9. Analiza bioinformatyczna i wizualizacja danych proteomicznych

W publikacjach 2 i 3 do analizy funkcjonalnej białek wykorzystano narzędzia PANTHER, ToppCluster, STRING oraz KOBAS 3.0 (Kaimal i in., 2010; Szklarczyk i in., 2019; Szklarczyk i in., 2023; Bu i in., 2021).

Wyniki przedstawiono w formie tabel, wykresów i map cieplnych z wykorzystaniem programów GraphPad PRISM (wersja 8.0.1 lub 10.4.2, GraphPad Software, San Diego, CA, USA) i OriginPro 2023 (OriginLab Corporation, Northampton, MA, USA).

3.10. Analizy statystyczne

Do przeprowadzenia analiz statystycznych wykorzystano oprogramowanie GraphPad PRISM (wersja 8.0.1 lub 10.4.2). W zależności od rodzaju danych i spełnienia założeń

normalności rozkładu stosowano test t-Studenta lub test U Manna–Whitneya **(publikacja 2)** oraz analizę wariancji ANOVA lub test Kruskala–Wallisa z testem post hoc Tukeya **(publikacja 3)**. Korelacje pomiędzy zawartością białek płynu ogona najądrza (intensywność MS) a parametrami ruchliwości plemników analizowano przy użyciu współczynnika rang Spearmana. Wyniki przedstawiono jako średnie ± SEM (ang. *standard error of mean* – błąd standardowy średniej). Za istotne statystycznie uznawano wartości $p < 0,05$.

Szczegółowy opis zastosowanej metodologii przedstawiono w **publikacjach 2 i 3**.

4. Wyniki i omówienie

Publikacja 1 pozwoliła na usystematyzowanie wiedzy na temat biomarkerów molekularnych funkcji reprodukcyjnych psa. Stwierdzono, że białka markerowe można podzielić na dwie grupy. Pierwszą stanowiły białka dobrze poznane: białka kotwiczące kinazę A (AKAPs), albuminy (ALBs), fosfataza alkaliczna (ALPL), klasteryna (CLU), esteraza specyficzna dla prostaty psa (CPSE), sekrecyjne białko bogate w cysteinę 2 (CRISP2), laktotransferyna (LTF), metaloproteinazy (MMPs), osteopontyna (OPN) oraz izomeraza D prostaglandyny H2 (PTGDS). Drugą grupę stanowiły białka słabiej poznane, do których należały m.in.: białko wiążące akrozynę (ACRBP), aktyna cytoplazmatyczna 1 (ACTB), hialuronoglukozoaminidaza (CEMIP), najądrzowe białko wiążące plemniki 1 (ELSPBP1), białko zawierające domenę cystatyny (LOC607874), wewnątrzkomórkowy transporter cholesterolu typu C2 (NPC2). Ze względu na obszerną wiedzę dotyczącą roli enzymów antyoksydacyjnych w ochronie plemników przed stresem oksydacyjnym i utrzymaniu jakości nasienia, potraktowano je jako oddzielną grupę białek markerowych. Do niebiałkowych markerów jakości nasienia psa zaliczono substancje lipidowe (kwasy tłuszczowe, fosfatydylocholina), węglowodany (glikozaminoglikany) oraz jony (cynk, wapń). Funkcje najważniejszych białek obejmowały poprawę kapacytacji plemników (AKAPs, CRISP2, OPN), reakcji akrosomalnej (CRISP2, OPN, CLU) oraz ruchliwości plemników (AKAPs, ALBs, LTF, CPSE). Niektóre białka mogą również stanowić potencjalne biomarkery nowotworów prostaty i jąder (ALPL, CLU, CPSE, OPN). Opisano też markery białkowe, których ekspresja była skorelowana z wiekiem psa (ACTB, LOC607874).

Jednocześnie wykazano, że pomimo rosnącej liczby badań dotyczących proteomu nasienia psa, nadal niewiele wiadomo na temat składu białkowego płynu najądrzowego oraz roli obecnych w nim białek w regulacji dojrzewania i właściwości funkcjonalnych plemników oraz procesów starzenia się najądrza. W celu uzupełnienia tej luki poznawczej przeprowadzono dwa zadania badawcze.

4.1. Zadanie badawcze nr 1 – Identyfikacja markerów białkowych ruchliwości plemników z płynu ogona najądrza psa

Na podstawie wartości PMOT plemników najądrzowych próby zakwalifikowano do dwóch grup badawczych GSM i PSM. Koncentracja plemników najądrzowych nie różniła się istotnie pomiędzy grupami ($p > 0,05$). Wartości TMOT, PMOT, VSL, BCF, STR oraz

LIN były istotnie wyższe w grupie GSM, natomiast VCL i ALH w grupie PSM ($p < 0,05$) **(Tabela 1, publikacja 2)**.

Przy użyciu analiz proteomicznych zidentyfikowano łącznie 915 białek, w tym 506 w grupie GSM i 409 w grupie PSM. Wykaz wszystkich białek zidentyfikowanych w poszczególnych grupach badawczych przedstawiono w **tabelach uzupełniających S1–S2 do publikacji 2**. Ze względu na uzyskany szeroki profil proteomiczny zastosowano kryterium selekcji oparte na częstości występowania białek. Do dalszych analiz włączono wyłącznie białka, które występowały u ponad 50% osobników w danej grupie badawczej. Wykazano obecność 6 białek unikatowych dla grupy GSM, 4 białek unikatowych dla grupy PSM oraz 11 białek wspólnych dla obu grup badawczych **(Rycina 1, publikacja 2)**. Do białek unikatowych dla grupy GSM należały: ACTB, ALPL, członek 6 rodziny białek zawierających domenę rekrutacji kaspaz (CARD6), CLU, N-acetylogalaktozaminylotransferaza polipeptydowa (GALNT6) oraz olfaktomedyna 4 (OLFM4). Natomiast do białek unikatowych dla grupy PSM należały: homolog białka Boule wiążącego RNA (BOLL), białko zawierające domenę KRAB (LOC606925), LOC607874 oraz białko zawierające domenę WAP (WAPdcp*). Wśród białek wspólnych dla obu grup badawczych zidentyfikowano: alfa-fetoproteinę (AFP), albuminę (ALB), karboksyloesterazę 5A (CES5A), CRISP2, ELSPBP1, wydzielniczą peroksydazę glutationową najądrza (GPX5), białko zawierające domenę lipokalinową/cytozolową wiążącą kwasy tłuszczowe (LCNL1), LTF, NPC2, plastynę 3 (PLS3) oraz PTGDS.

Analiza ontologii genów (*ang. gene ontology*, GO) wykazała podobne główne funkcje molekularne, procesy biologiczne i klasy białek w obu grupach badawczych **(Rycina 3, publikacja 2)**. Podobnie, w obu grupach badawczych dominowały białka związane z wiązaniem cząsteczek oraz aktywnością katalityczną. Największe różnice odnotowano w kategorii „proces biologiczny", gdzie w grupie PSM wyraźnie dominowały białka związane z lokalizacją cząsteczek. W obu grupach największy udział stanowiły białka transportowe/nośnikowe oraz enzymy uczestniczące w przemianach metabolitów **(Rycina 3, publikacja 2)**. Uzupełniająca analiza ToppCluster wykazała wspólne i specyficzne funkcje molekularne oraz składniki komórkowe dla obu grup badawczych **(Rycina 4, publikacja 2)**.

Analiza szlaków metabolicznych KEGG (ang. *Kyoto Encyclopedia of Genes and Genomes*) wykazała udział zidentyfikowanych białek w różnych procesach biologicznych i

metabolicznych **(Tabela 2, publikacja 2)**. W obu grupach badawczych obecne były białka ALB, GPX5 i PTGDS związane z metabolizmem kwasu arachidonowego oraz syntezą hormonów tarczycy. W grupie GSM dodatkowo wykazano wzbogacenie szlaku sygnałowego Hippo oraz ogólnych szlaków metabolicznych, związanych z obecnością białek ACTB, AFP, ALPL, GALNT6, GPX5 i PTGDS. Natomiast w grupie PSM wykazano większy udział białek związanych z metabolizmem cholesterolu (NPC2) i ochroną antyoksydacyjną (GPX5).

Na podstawie względnej zawartości białek (ang. *MS intensity* – intensywność sygnału MS), spośród 11 białek wspólnych dla obu grup badawczych wyodrębniono 4 polipeptydy charakteryzujące się istotnie wyższą zawartością w grupie GSM niż w grupie PSM ($p < 0,05$) **(Rycina 2, publikacja 2)**. Należały do nich: ALB ($p = 0,048$), CRISP2 ($p = 0,046$), LCNL1 ($p = 0,039$) oraz PTGDS ($p = 0,0221$). Analiza Western blot potwierdziła istotnie wyższą zawartość ALB ($p = 0,014$) i PTGDS ($p = 0,049$) w grupie GSM, natomiast nie wykazano istotnych różnic ($p > 0,05$) w zawartości CRISP2 pomiędzy grupami **(Rycina 7, publikacja 2)**. Niestety pomimo długich poszukiwań i korespondencji z wieloma producentami i dostawcami uzyskanie przeciwciała dla LCNL1 okazało się niemożliwe. Zależności pomiędzy ww. białkami wykazano w sieci interakcji białko–białko **(Rycina 5, publikacja 2)**. Szczególnie interesujące są zależności pomiędzy PTGDS, CRISP2 i LCNL1, które mogą wskazywać na współdziałanie tych białek w regulacji środowiska najądrza oraz ruchliwości plemników najądrzowych.

Analizowano również zależności między względną zawartością wybranych białek płynu ogona najądrza a poszczególnymi parametrami ruchu plemników najądrzowych. W przypadku białek wspólnych dla obu grup badawczych stwierdzono dodatnią korelację pomiędzy zawartością AFP a parametrem SLOW ($p = 0,044$) **(Rycina 6A, publikacja 2)**. Zawartość białek unikatowych dla grupy GSM korelowała zarówno dodatnio, jak i ujemnie z wybranymi parametrami ruchu plemników. Dodatnie korelacje stwierdzono pomiędzy zawartością CARD6 a VAP ($p = 0,049$), SLOW ($p = 0,047$) oraz pomiędzy zawartością OLFM4 a ALH ($p = 0,034$) **(Rycina 6B, publikacja 2)**. Ujemne korelacje występowały pomiędzy zawartością ALPL a STR ($p = 0,046$), a także pomiędzy zawartością OLFM4 a STR ($p = 0,049$) oraz LIN ($p = 0,021$) **(Rycina 6B, publikacja 2)**. W przypadku białek unikatowych dla grupy PSM obserwowano wyłącznie korelacje ujemne, obejmujące zależności pomiędzy zawartością BOLL a ALH ($p = 0,049$) oraz LOC607874 a VAP ($p = 0,046$) i VSL ($p = 0,049$) **(Rycina 6C, publikacja 2)**.

4.2. Zadanie badawcze nr 2 – Identyfikacja markerów białkowych starzenia się najądrza psa

Dokonano profilowania proteomicznego płynu ogona najądrza psów w odniesieniu do wieku osobników w trzech grupach eksperymentalnych: ExG1, ExG2, ExG3. Zidentyfikowano 121 białek unikatowych w grupie ExG1, 122 białka w ExG2 oraz 56 białek w ExG3. Wspólnych dla wszystkich trzech grup eksperymentalnych było 48 białek. Ponadto zidentyfikowano białka wspólne dla grup ExG1 i ExG2 – 16, dla ExG2 i ExG3 – 16 oraz dla ExG1 i ExG3 – 12 **(Rycina 1a, publikacja 3)**. Pełną listę białek zidentyfikowanych w poszczególnych grupach eksperymentalnych przedstawiono **w tabelach uzupełniających S1–S3 do publikacji 3**.

Analizowano białka, które występowały u ponad 50% osobników w danej grupie eksperymentalnej. Wykazano 5 białek unikatowych dla grupy ExG1, 1 białko unikatowe dla ExG2 oraz 1 białko unikatowe dla ExG3. Nie stwierdzono białek wspólnych między grupami ExG1 i ExG2. Stwierdzono natomiast 8 białek obecnych w grupach ExG2 i ExG3 oraz 4 białka w grupach ExG1 i ExG3. Dalszym analizom poddano 7 białek obecnych we wszystkich trzech grupach eksperymentalnych: ALB, CES5A, GPX5, LTF, NPC2, PLS3 oraz PTGDS **(Rycina 1b, publikacja 3)**.

Na podstawie względnej zawartości białek (*MS intensity*) przeprowadzono analizę półilościową białek wspólnych dla wszystkich grup eksperymentalnych. Istotne statystycznie różnice ($p < 0,05$) w zawartości pomiędzy grupami wykazano dla 3 białek: ALB, GPX5 i PTGDS. Zawartość ALB była najwyższa w ExG2, przy istotnej różnicy pomiędzy ExG1 a ExG2 ($p = 0,014$). Podobnie najwyższe zawartości GPX5 i PTGDS odnotowano w ExG2, z istotnymi różnicami między ExG1 i ExG2 (odpowiednio dla GPX5: $p = 0,006$; dla PTGDS: $p = 0,023$) **(Rycina 2, publikacja 3)**.

W celu potwierdzenia obecności i poziomu zawartości ALB, GPX5 i PTGDS w płynie ogona najądrza w poszczególnych grupach eksperymentalnych, przeprowadzono analizę Western blot. Potwierdzono istotne statystycznie różnice w zawartości ALB między trzema grupami eksperymentalnymi ($p < 0,001$) oraz najwyższą zawartość ALB w ExG2 i najniższą w ExG1 **(Rycina 3a, publikacja 3)**. Zawartość ALB była istotnie wyższa w grupie ExG2 niż w ExG1 ($p < 0,001$) oraz ExG3 ($p = 0,047$). Dodatkowo istotną różnicę w zawartości ALB stwierdzono pomiędzy grupami ExG1 i ExG3 ($p = 0,003$) **(Rycina 3a, publikacja 3)**.

Analiza wariancji ANOVA wykazała istotne statystycznie różnice w zawartości GPX5 pomiędzy grupami eksperymentalnymi ($p < 0,001$). Najwyższą zawartość GPX5 odnotowano w ExG3 **(Rycina 3b, publikacja 3)**. Istotne różnice w zawartości GPX5 wykazano pomiędzy grupami ExG2 i ExG3 ($p < 0,001$) oraz ExG1 i ExG3 ($p = 0,010$) **(Rycina 3b, publikacja 3)**.

Podobnie jak w przypadku ALB i GPX5, analiza wariancji ANOVA wykazała istotne statystycznie różnice w zawartości PTGDS pomiędzy grupami eksperymentalnymi ($p = 0,002$). Potwierdzono rozkład poziomów zawartości PTGDS w poszczególnych grupach eksperymentalnych, w tym najwyższą zawartość PTGDS obserwowaną w ExG2, a najniższą w ExG1 **(Rycina 3c, publikacja 3)**. Zawartość PTGDS była istotnie wyższa w grupie ExG2 niż w grupie ExG1 ($p = 0,002$). Dodatkowo istotną różnicę w zawartości PTGDS stwierdzono pomiędzy grupami ExG1 i ExG3 ($p = 0,024$) **(Rycina 3c, publikacja 3)**.

Analiza GO **(Rycina 4, publikacja 3)** wykazała podobne główne funkcje molekularne i procesy biologiczne dla wszystkich grup eksperymentalnych, jednak udział procentowy białek przypisanych do poszczególnych kategorii był odmienny. Może to odzwierciedlać związane z wiekiem zmiany funkcjonalne w mikrośrodowisku najądrza psa. We wszystkich trzech grupach (ExG1, ExG2, ExG3) dominowały białka związane z aktywnością katalityczną, wiązaniem cząsteczek i aktywnością antyoksydacyjną. Dodatkowo wyłącznie w grupach ExG2 i ExG3 wykazano obecność funkcji regulatora aktywności translacji oraz regulatora funkcji molekularnych. Na uwagę zasługuje również stopniowe zmniejszenie udziału białek przypisanych do aktywności antyoksydacyjnej od ExG1 do ExG3. W odniesieniu do kategorii „proces biologiczny" **(Rycina 4, publikacja 3)** w grupie ExG1 dominowały białka związane z lokalizacją cząsteczek oraz procesami metabolicznymi. W ExG2 dominowały białka związane z procesami komórkowymi i lokalizacją cząsteczek. Natomiast w ExG3 obserwowano dominujący udział białek w procesach związanych z lokalizacją cząsteczek i procesami komórkowymi. Ponadto wyłącznie w grupach ExG2 i ExG3 wykazano udział białek w procesach reprodukcyjnych (głównie związany z obecnością ELSPBP1).

Przy użyciu narzędzia STRING przeprowadzono również analizę sieci interakcji białko–białko dla GPX5 i PTGDS **(Rycina 5, publikacja 3)**. Wykazano, że GPX5 silnie oddziałuje głównie z innymi członkami rodziny peroksydaz glutationowych (GPX1, GPX2, GPX3, GPX6, GPX8) oraz innymi białkami zaangażowanymi w regulację stresu

oksydacyjnego, w tym z reduktazą glutationową (GSR), syntetazą glutationową (GSS) i peroksyredoksyną 6 (PRDX6) **(Rycina 5a, publikacja 3)**. Analiza sieci interakcji PTGDS wykazała natomiast powiązania tego białka z innymi prostaglandynami (PTGS1, PTGS2, PTGES, PTGES2, HPGDS) oraz z przedstawicielami rodziny lipokalin (LCNL1, LCN9, LCN10) **(Rycina 5b, publikacja 3)**. Szczególnie interesujące wydają się powiązania PTGDS z LCNL1, CRISP2 oraz ACRBP **(Rycina 5b, publikacja 3)**.

Przeprowadzono analizę korelacji między względną zawartością ALB, GPX5 i PTGDS w płynie ogona najądrza a parametrami ruchu plemników najądrzowych psów w grupach ExG1, ExG2 i ExG3. W ExG1 wykazano dodatnią korelację między zawartością GPX5 a parametrem RAPID ($p = 0,044$) oraz ujemną korelację między zawartością GPX5 a parametrem STATIC ($p = 0,035$) **(Rycina 6a, publikacja 3)**. W ExG2 wykazano ujemną korelację pomiędzy zawartością ALB a SLOW ($p = 0,033$) oraz dodatnią korelację z parametrem STATIC ($p = 0,047$) **(Rycina 6b, publikacja 3)**. Ponadto stwierdzono ujemną korelację między zawartością GPX5 a TMOT ($p = 0,033$). W grupie psów najstarszych (ExG3) wykazano dodatnią korelację pomiędzy zawartością GPX5 a parametrem SLOW ($p = 0,035$) oraz ujemną korelację pomiędzy zawartością PTGDS a VCL ($p = 0,017$) **(Rycina 6c, publikacja 3)**.

5. Podsumowanie i wnioski

W badaniach po raz pierwszy przeprowadzono profilowanie proteomiczne płynu ogona najądrza psa z wykorzystaniem metody nanoUPLC-Q-TOF/MS, dzięki czemu scharakteryzowano mikrośrodowisko białkowe determinujące dojrzewanie i funkcjonalność plemników najądrzowych. Dodatkowo analizy te umożliwiły identyfikację wielu nowych białek tworzących środowisko płynu najądrza, które do tej pory nie zostały wykryte u psa. Należały do nich m.in.: BOLL, CARD6, GALNT6, LOC606925, PLS3. Pierwszy raz dokonano analizy funkcjonalnej białek płynu najądrzowego w powiązaniu z parametrami ruchliwości plemników oraz wiekiem psa.

Wykazano, że skład białkowy płynu z ogona najądrza psa różni się w zależności od jakości ruchu plemników najądrzowych. Zidentyfikowano obecność określonych białek związanych z płynem najądrzowym towarzyszącym plemnikom o dobrych parametrach ruchliwości (GSM), jak również grupę białek unikatowych dla płynu najądrzowego towarzyszącego plemnikom o obniżonej ruchliwości (PSM).

Białka unikatowe dla grupy GSM (ACTB, ALPL, CARD6, CLU, GALNT6, OLFM4) były funkcjonalnie związane głównie z procesami dojrzewania i różnicowania plemników, organizacją struktur komórkowych oraz mechanizmami regulacyjnymi i ochronnymi sprzyjającymi utrzymaniu prawidłowej ruchliwości i funkcji plemników najądrzowych psa (Pedersen i in., 2001; Cabello-Agüeros i in., 2003; Zhang i in., 2005; Dufner i in., 2006; Anholt, 2014; Yao i in., 2017; Zmudzinska i in., 2022b). Białka unikatowe dla grupy PSM (BOLL, LOC606925, LOC607874, WAPdcp*) były powiązane głównie z procesami spermatogenezy, regulacją proliferacji i apoptozy komórkowej oraz starzeniem się najądrza, co może wskazywać na ich związek z obniżoną jakością plemników najądrzowych (Urrutia, 2003; Lin i in., 2005; Lindblad-Toh i in., 2005; Kostova i in., 2007; Raghavendra i in., 2022; Zmudzinska i in., 2022b; Peng i in., 2023; Dissanayake i in., 2025).

Szczególnie interesującą grupę stanowiły białka wspólne dla grup GSM i PSM, których względna zawartość różniła się istotnie pomiędzy grupami. Białka ALB, CRISP2, LCNL1 oraz PTGDS były związane głównie z dojrzewaniem plemników, ochroną antyoksydacyjną oraz regulacją właściwości błon i metabolizmu plemników (Stillwell i in., 1990; Breitbart, 2002; Chen i in., 2007; Jamsai i in., 2008; Kao i in., 2008; Lu i in., 2008; Roche i in., 2008; Zhou i in., 2010; Macıas-Garcıa i in., 2015; Zhou i in., 2015; Björkgren i

Sipilä, 2019; Lim i in., 2019; Mogielnicka-Brzozowska i in., 2019; Zmudzinska i in., 2022a; Celedon i in., 2025; Mogielnicka-Brzozowska i in., 2026).

Analiza korelacji wykazała, że wybrane białka płynu ogona najądrza mogą pozostawać w funkcjonalnym związku z poszczególnymi parametrami ruchliwości i ruchu plemników najądrzowych.

Wyższa zawartość ALB i PTGDS w grupie GSM potwierdzona techniką Western blot wzmacnia potencjalne znaczenie tych białek jako markerów dobrej jakości nasienia najądrzowego psa.

Starzenie się męskiego układu rozrodczego jest wynikiem wieloczynnikowych zmian zachodzących na poziomie molekularnym, komórkowym i tkankowym, obejmujących m.in. kumulację uszkodzeń DNA, białek oraz struktur lipidowych (Sampson i in., 2007; Sharma i in., 2015; Guelfi i in., 2024). Analiza proteomu płynu najądrzowego psa w kontekście wieku osobników dostarczyła nowych informacji dotyczących podstaw molekularnych procesów dojrzewania i starzenia najądrza oraz ich potencjalnego wpływu na właściwości plemników najądrzowych.

Wykazano, że skład białkowy płynu z ogona najądrza psa zmienia się wraz z wiekiem zwierzęcia. Różna liczba i rodzaj białek charakteryzowały płyny najądrzowe pochodzące od psów w różnym wieku. Wyodrębniono 3 białka wspólne dla wszystkich grup wiekowych, których zawartość zmieniała się istotnie wraz z wiekiem, co może wskazywać na ich znaczenie w procesach starzenia się najądrza psa. Należały do nich ALB, GPX5 oraz PTGDS.

Zawartość ALB była najwyższa w grupie psów dorosłych (ExG2), co potwierdzono techniką Western blot. Białko to odgrywa kluczową rolę w ochronie antyoksydacyjnej plemników poprzez absorpcję nadtlenków lipidów oraz redukcję stężenia jonów Zn^{2+} w plemnikach, wpływając na ich ruchliwość i żywotność (Lu i in., 2008; Roche i in., 2008; Mogielnicka-Brzozowska i in., 2019). Można przypuszczać więc, że zwiększona zawartość ALB w płynie najądrzowym w grupie psów dorosłych jest niezbędna dla optymalnej ochrony plemników w okresie największej aktywności rozrodczej samców.

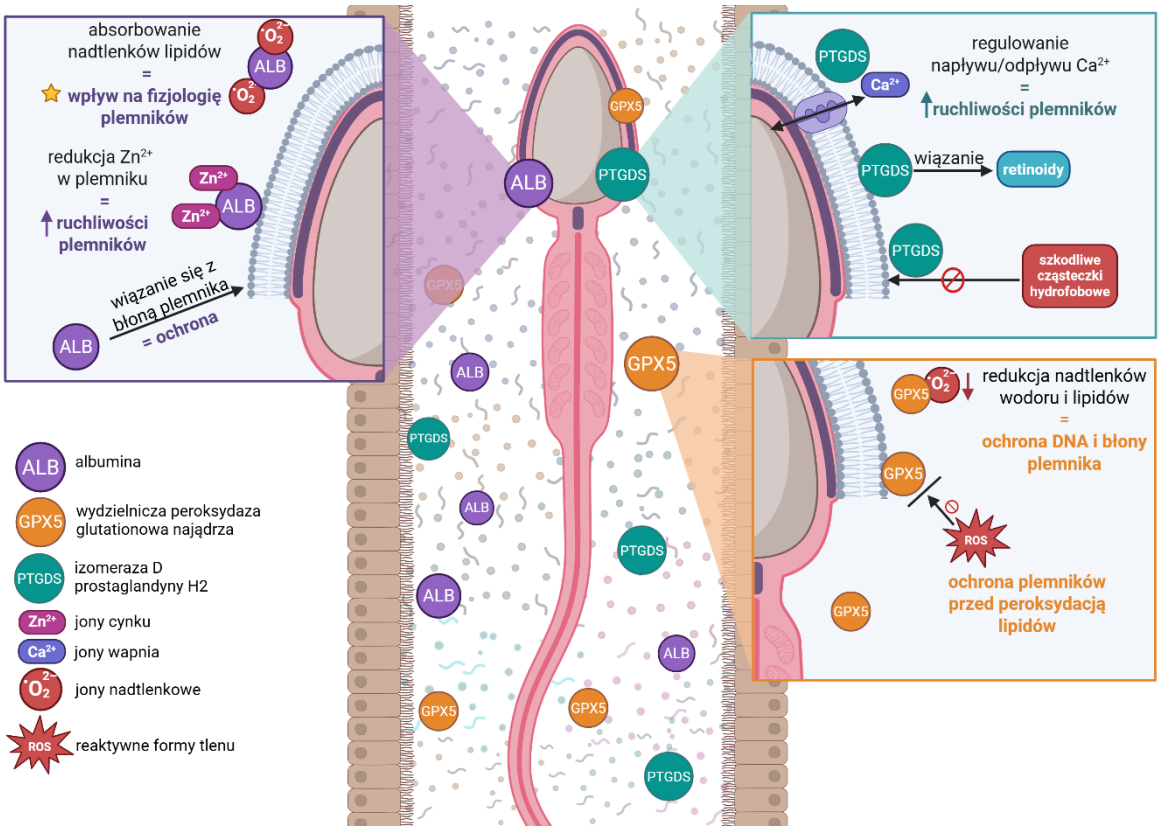
Podobnie najwyższe zawartości GPX5 odnotowano w grupie psów dorosłych (ExG2), jednak analiza Western blot nie potwierdziła tych wyników wskazując na najwyższą zawartość GPX5 w grupie psów najstarszych. Różnice te mogą wynikać z odmiennych

podstaw metodologicznych obu technik analitycznych (Kiernan i in., 2002; Yang i Herold, 2017; Stevens i Pukala, 2020). Najwyższa zawartość GPX5 w płynie najądrzowym najstarszych psów może odzwierciedlać nasiloną odpowiedź na stres oksydacyjny towarzyszący starzeniu się najądrza (Aitken, 2009; Pintus i Ros-Santaella, 2021).

Również w przypadku PTGDS najwyższą zawartość wykazano w grupie psów dorosłych (ExG2), co potwierdziła analiza Western blot. Uzyskane wyniki wskazują, że aktywność PTGDS jest największa u samców będących w wieku najintensywniejszej aktywności rozrodczej, co jest uzasadnione biorąc pod uwagę zdolność tego białka do ochrony plemników przed szkodliwymi cząsteczkami hydrofobowymi oraz regulacji transportu jonów Ca^{2+} przez błony plemników, co moduluje ruchliwość tych komórek (Zhou i in., 2010; Van Tilburg i in., 2021). W wyniku analizy sieci interakcji wykazano wielopoziomowe zależności pomiędzy PTGDS a LCNL1, CRISP2 i ACRBP. Szczególnie istotne wydaje się silne funkcjonalne powiązane PTGDS z różnymi kluczowymi białkami zaangażowanymi w metabolizm plemników, w tym z innymi członkami rodziny prostaglandyn oraz rodziny lipokalin.

Przeprowadzone analizy korelacji wskazują, że wraz z wiekiem mogą zmieniać się zależności pomiędzy zawartością wybranych białek płynu najądrzowego a poszczególnymi parametrami ruchliwości i ruchu plemników.

Profilowanie proteomiczne płynu ogona najądrza psa umożliwiło identyfikację ALB, GPX5 i PTGDS jako potencjalnych markerów molekularnych procesów starzenia najądrza. Białka te są związane z utrzymaniem homeostazy oksydacyjnej, odgrywają istotną rolę w ochronie plemników oraz regulacji ich metabolizmu. Zmiany w najądrzu psa wywołane starzeniem się organizmu mogą być widoczne w różnicach w zawartości wskazanych białek w płynie ogona najądrza. Na podstawie uzyskanych wyników można przypuszczać, że u młodych psów systemy ochronne oparte na ALB, GPX5 i PTGDS nie są jeszcze w pełni rozwinięte, natomiast najwyższa zawartość tych białek w grupie dorosłych osobników może odpowiadać okresowi największej aktywności rozrodczej. Z kolei zmiany zawartości tych białek obserwowane u najstarszych psów mogą odzwierciedlać mechanizmy kompensacyjne związane ze stresem oksydacyjnym, nasilającym się wraz z postępującymi procesami starzenia. Schematyczny model funkcji ALB, GPX5 i PTGDS jako markerów procesów starzenia najądrza psa przedstawiono na **Rycinie 1**.



Rycina 1. Schematyczny model funkcji ALB, GPX5 i PTGDS jako markerów procesów starzenia się najądrza psa.

Źródło: opracowanie własne przy użyciu BioRender.com.

Wnioski:

1. Przeprowadzone badania wykazały, że płyn najądrza psa stanowi bogate źródło białek uczestniczących w procesach dojrzewania, ochrony i regulacji funkcji plemników.
2. Zastosowanie nowoczesnych metod proteomicznych umożliwiło identyfikację licznych białek potencjalnie związanych z ruchliwością plemników najądrzowych.
3. Wykazano, że skład proteomiczny płynu ogona najądrza psa różni się w zależności od parametrów ruchliwości plemników najądrzowych, co wskazuje na istotny udział środowiska najądrza w kształtowaniu zdolności reprodukcyjnych samców.
4. Białka ALB, GPX5 oraz PTGDS z płynu ogona najądrza mogą stanowić potencjalne biomarkery jakości plemników oraz funkcji najądrza psa.
5. Uzyskane wyniki potwierdziły istotną rolę procesów oksydacyjnych i mechanizmów antyoksydacyjnych w utrzymaniu prawidłowej funkcji plemników.

6. Stwierdzono, że proces starzenia się najądrza wpływa na zmiany ekspresji wybranych białek związanych z ochroną i dojrzewaniem plemników, co może prowadzić do obniżenia płodności samców.

7. Zidentyfikowane biomarkery mogą znaleźć zastosowanie w diagnostyce zaburzeń płodności, ocenie jakości nasienia oraz monitorowaniu zmian związanych ze starzeniem układu rozrodczego psa.

8. Wyniki badań rozszerzają wiedzę dotyczącą molekularnych mechanizmów funkcjonowania najądrza psa oraz stwarzają podstawy do dalszych badań nad proteomiką i biomarkerami płodności u zwierząt.

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7. Spis rycin

Rycina 1. Schematyczny model funkcji ALB, GPX5 i PTGDS jako markerów procesów starzenia się najądrza psa.

8. Wykaz materiałów uzupełniających

Materiały uzupełniające do publikacji stanowiących podstawę rozprawy dostępne są pod poniższymi adresami:

1. Publikacja 2

https://www.mdpi.com/article/10.3390/ijms241914790/s1

2. Publikacja 3

https://connectsci.au/rd/article-supplement/267809/pdf/rd25105_ac/

9. Kopie publikacji wchodzących w skład rozprawy doktorskiej oraz oświadczenia kandydatki i współautorów

PUBLIKACJA 1.

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Review

Molecular Biomarkers of Canine Reproductive Functions

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Abstract: The aim of the current study is to review potential molecular biomarker substances selected so far as useful for assessing the quality of dog semen. Proteins, lipids, carbohydrates, and ions can serve as molecular biomarkers of reproductive functions (BRFs) for evaluating male reproductive health and identifying potential risk factors for infertility or reproductive disorders. Evaluation of BRF levels in semen samples or reproductive tissues may provide insights into the underlying causes of infertility, such as impaired sperm function, abnormal sperm–egg interaction, or dysfunction of the male reproductive tract. Molecular biomarker proteins may be divided into two groups: proteins that are well-studied, such as A-kinase anchoring proteins (AKAPs), albumins (ALBs), alkaline phosphatase (ALPL), clusterin (CLU), canine prostate-specific esterase (CPSE), cysteine-rich secretory protein 2 (CRISP2), lactotransferrin (LTF), metalloproteinases (MMPs), and osteopontin (OPN) and proteins that are not well-studied. Non-protein markers include lipid-based substances (fatty acids, phosphatidylcholine), carbohydrates (glycosaminoglycans), and ions (zinc, calcium). Assessing the levels of BRFs in semen samples may provide valuable information for breeding management and reproductive assessments in dogs. This review systematizes current knowledge that could serve as a starting point for developing practical tests with the use of biomarkers of canine reproductive functions and their predictive value for assisted reproductive technique outcomes and semen preservation.

Keywords: canine; semen; biomarkers; proteins; lipids; carbohydrates

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1. Introduction

Different reproductive biotechniques nowadays play a significant role in dog breeding. These biotechniques offer numerous benefits and advancements that enhance breeding programs and address various challenges faced by breeders. Fertility prediction and control in dogs using molecular biomarkers of reproductive functions (BRFs) is a key aspect of responsible breeding, health management, and population control. According to the Merriam-Webster Dictionary [1] definition, a biomarker is any biological component that can function as an indicator of a physiological pathway. Biomarkers are by definition objective, quantifiable rates of biological processes [2]. The development of simple molecular tests for advanced epididymal and ejaculated semen quality evaluation would be highly beneficial. Advanced molecular tests can more accurately assess the quality of semen samples (fresh and preserved), including sperm concentration, motility, morphology, and viability, leading to higher success rates in artificial insemination and natural breeding [3]. Additionally, knowing the exact quality of dog semen allows breeders to use it more effectively, ensuring that only high-quality doses are used for breeding, increasing the likelihood of conception. Additionally, modern semen evaluation can prevent costly unsuccessful breeding attempts and reduce the financial burden associated with poor-quality semen. Breeders can command higher prices for stud services if they can demonstrate that their sires produce high-quality ejaculates, backed by modern testing. Better semen quality evaluation improves the success rates of semen cryopreservation, ensuring that stored samples remain viable and effective for future use. High-quality semen is critical

for successful in vitro fertilization (IVF) and subsequent embryo transfer, leading to better outcomes in reproductive technologies. With detailed information on quality of male used for reproduction, breeders can create more precise and effective breeding plans tailored to the specific needs and conditions of their breeding program. Modern semen evaluation with the use of molecular biomarkers contributes to a better understanding of canine reproduction and fertility, leading to further advancements in veterinary reproductive medicine. Implementing new technologies requires expanding knowledge and further research on canine semen storage techniques and procedures for handling semen. This is important for using valuable male reproductors, rare breeds of dogs and endangered canine species.

Thanks to access to increasingly modern research equipment, knowledge related to canine reproduction is developing in parallel to the expansion of areas of knowledge regarding human reproduction. The increasing demands of dog breeders and the changing living environment of these animals accompanying humans force a change in the approach of owners, veterinarians, and scientists to issues related to the reproduction of these animals. Modern technologies facilitate both detailed examination of the health of the reproductive organs and measurement of the quality of semen obtained from the male. Today, breeders expect accurate information on the health of their most valuable males because the success of their breeding and its profitability depends on it. Research has been ongoing for years to identify biomarkers of semen quality that could be easily linked to key parameters for the fertilization process and the specificity of the insemination dose. The most common substances contained in semen for this purpose include proteins, lipids, carbohydrates, and ions. All of these components are tightly interlinked, and their effective cooperation provides cellular signals required for sufficient sperm functional features. Relationships are sought between the level of a given substance in the animal's reproductive organ fluids, sperm, blood, and selected semen quality parameters. Most often, the relationship between sperm motility, the condition of sperm plasma membranes, and DNA integrity is taken into account.

In canines, proteins from epididymal fluid or seminal plasma may coat sperm plasma membranes via phospholipid binding, exerting protective effects [4]. Additionally, these proteins, when bound, may also act as different ion carrier proteins or proteins with enzymatic functions [5,6]. Their role may be to alter ion concentrations in seminal fluid and spermatozoa, acting as decapacitation factors or protease inhibitors, as well as serine proteases affecting the activity of other proteins [6]. Moreover, these proteins might influence various aspects of sperm functions, for example, sperm membrane permeability and sperm motility, and they may modulate fertilization-associated events [7]. Lipids are involved in a wide range of reproductive events, from spermatogenesis and maturation to fertilization. As major compartments of sperm membranes, lipids maintain their integrity, control fluidity, and provide functional membrane microstructural domains and signaling molecules. Usually, different types of lipids do not function separately; rather, they act together in complex signaling pathways [8]. The maintenance of proper sperm function is also dependent on the balance between carbohydrates and ions, working intracellularly and extracellularly. Therefore, when searching for BRFs of dogs, it is necessary to take not only an individual approach but also a holistic, comprehensive approach to the analyzed molecules. Due to the growing interest in dog breeding over the years, as well as the dog being a model of the male reproductive system [9,10], it seems advisable to conduct a comprehensive examination of dog semen, including high-throughput OMICs (proteomics, lipidomics, metabolomics) and molecular biology tools, considering different variables such as age, breed, and pathological and environment conditions.

The aim of the current study is to review potential molecular biomarker substances selected so far as useful for assessing the quality of dog semen.

2. Protein Markers

Protein profiling of a reproductive tissue or a sperm cell, defined as proteomics, provides new possibilities and promising potential in dog reproduction research [11]. The

detection and identification of protein biochemical properties, which influence their functional features and result in reproductive success, is important. Identification of proteins influencing sperm motility or sperm membrane integrity may be used in biomedical strategies for semen assessment, infertility treatment, and semen conservation improvement [12]. Some proteins that are abundant in semen or reproductive tissue were recognized many years ago in canines. Their functions in reproduction are well known. However, certain proteins have been newly discovered in canine semen and tissue and are not yet well-known; however, they possess a potential that needs to be recognized more widely. In this article, the characteristics of both types of proteins are provided, and their potential as markers in dog breeding is explored.

2.1. Well-Studied/Highly Abundant Proteins

Some proteins have been well studied and are recognized as being correlated with canine semen quality parameters.

A-Kinase Anchoring Proteins (AKAPs) share the property of binding to the regulatory subunit of protein kinase A (PKA) [13]. ProAKAP4 (Pro A-Kinase Anchoring Protein 4) is expressed as a precursor of the AKAP4 (A-Kinase An-choring Protein 4) [14]. ProAKAP4 and AKAP4 are involved in a signaling cascade including adenosine monophosphate cyclic, protein kinase A, tyrosine kinase, and phosphatase [15]. These proteins are actively phosphorylated on their tyrosine and serin residues during capacitation, which allows us to consider them as biomarkers of the capacitation status [16,17]. ProAKAP4 is produced during the round spermatid stage and is incorporated into the fibrous sheath of the developing sperm flagellum [18]. ProAKAP4 is cleaved during the condensed spermatid stage, resulting in its conversion into active AKAP4 [13,18]. It has been described as a functional marker of sperm in mammals [19,20]. AKAP4 is associated with good-quality spermatogenesis and is involved in the regulation of sperm motility [14,21]. Le Couazer and Bencharif (2021) [14] showed, for the first time, the localization of proAKAP4 and AKAP4 in the fibrous sheath of the dog's spermatozoid and their expression in the spermatic part of the dog's ejaculate (not in the urethral or prostatic fractions) [22]. Additionally, in humans, the absence or weak expression of proAKAP4 and AKAP4 was associated with poor sperm motility [23]. There are kits (Dog 4MID® kits, 4BioDx, Lille, France) available on the market allowing for objective validation of semen quality by quantifying the proAKAP4 in dog semen. The translation of fundamental discoveries around proAKAP4 and AKAP4 semen quality markers into a practical tool (such as 4MID® kits) is of great importance in breeding and veterinary practice [20]. However, further detailed research is needed on the expression of proAKAP4 and AKAP4 in canine semen and its association with canine reproductive functions.

Albumins (ALBs) are proteins with a molecular mass of about 65 kDa [24]. In addition to being the main component of blood serum, they are also known to be an important component of the seminal plasma of the canine [25] epididymal spermatozoa [26] and epididymal fluid [27]. A semi-quantitative analysis of ALBs in canine epididymal fluid showed its higher abundance in the good sperm motility group [27]. In the reproductive system, they are mainly secreted by the testes, epididymides, and prostate [24]. The addition of heterogenous bovine serum albumin (BSA) to the ejaculated canine spermatozoa preserves sperm viability and function [28] and improves sperm capacitation and acrosome reaction [29]. Studies of homologous ALBs (found in the seminal plasma) have shown that they are implicated in fertilization-associated events, such as sperm capacitation [30] and zona pellucida penetration [31]. The seminal plasma ALB content is highly correlated with the motility parameters of animal spermatozoa [32]. The exact mechanism of action of ALBs is not known. It has been postulated that they may act via two mechanisms. One is via their antioxidant properties and the other is by binding bivalent cations to regulate their number in the reproductive fluids [5,33]. Even a simple mechanism of binding seminal plasma or epididymal fluid ALBs to the plasma membrane of ejaculated or epididymal spermatozoa plays a protective role in sperm motility [27,32,34]. Due to their antioxidant

properties, ALBs can absorb lipid peroxides from reproductive fluids, which contribute to their protective effect on the structures involved in sperm motility and sperm membrane integrity [25]. It has also been demonstrated that ALBs possess a strong ability to bind zinc ions, thus reducing their concentrations in the spermatozoa and enhancing the motility of the cells [32].

Alkaline phosphatase (ALPL) is a dephosphorylation enzyme present at very high concentrations in canine reproductive tracts [35]. In dogs, most of the ALPL is produced in the epididymis, but it is also found in seminal plasma [36,37]. It also plays a role in the transport of sugars and other organic molecules across biological membranes [38]. Its activity has been demonstrated in cytoplasmic droplets, which raises the hypothesis that ALPL catalyzes dephosphorylation and transport of phosphate groups between the epididymal sperm and epididymal fluid. ALPL might be a potential biomarker for prostate and testicular cancer [39]. This enzyme inhibitor can reduce the growth and invasion of cancer cells [39]. Measurement of the enzymatic activity of ALPL in the canine seminal plasma has been used for the diagnosis of incomplete ejaculation or azoospermia in dogs [38], and a reduced concentration of ALPL in the seminal plasma suggests bilateral obstruction of the vas deferens or epididymis [40].

Clusterin (CLU) is a common SP protein in many species [41–43]. CLU is an extracellular chaperone secreted in high amounts by epididymides and testes [44,45]. Its presence in the canine epididymal sperm and fluid has been established [26,27]. CLU might be adsorbed from the epididymal fluid and cover epididymal sperm plasma membranes, positively influencing sperm motility [27]. However, it may also be an indicator of low semen quality because secretion of this protein is enhanced in case of cellular damage or heat shock [42,46,47]. It is overexpressed in several human cancers, such as prostate cancer [48]. Morphologically defective sperm extensively bind CLU to its plasma membrane [49]. This protein has a role in inhibiting cell apoptosis, mediation of female tolerance to seminal antigens, and sperm maturation processes [50]. It also participates in membrane remodeling and DNA reparation [48]. CLU participates in sperm maturation by affecting lipid transport and membrane remodeling [48].

Canine prostate-specific esterase (CPSE) accounts for more than 90% of the proteins secreted by the prostate and about 30% of the canine SP proteins. The enzyme has a molecular weight of 29 kDa and it can be dissociated into two subunits with molecular masses of 12–14 kDa and 15 kDa [51]. The CPSE identified in canine seminal plasma is a multifunctional protein due to its zinc-binding and phosphorylcholine-binding properties [4,6]. The enzyme has been detected in the post-acrosomal region and sperm tail of ejaculated spermatozoa of dogs [51]. It can bind to phosphorylcholine of the sperm plasma membrane and coat ejaculated spermatozoa, which could be implicated in sperm fertilization-related events. It is noteworthy that CPSE is utilized as a marker of glandular secretion and serves as a promising diagnostic tool for non-neoplastic canine prostatic disorders [40]. Interestingly, CPSE is an enzyme homologous to human prostate-specific antigen (PSA), which is a direct indicator of the development of prostate cancer. Both CPSE and PSA are members of the kallikrein family, a group of serine proteases [52]. This family also includes the horse prostate kallikrein (HPK), which, like human PSA, has the ability to bind zinc and mercury ions [52]. Findings by our team confirmed that CPSE can bind zinc ions, a property previously demonstrated only by Isaacs and Coffey (1984) [51]. This underscores the role of zinc ions in the proteases' activity in dog semen.

Members of the cysteine-rich secretory protein (CRISP) family have been found in spermatids [53,54] as well as in the acrosome and tail of ejaculated sperm [55–58]. CRISP expression is high in the testis, and it is present at the junction between germ and Sertoli cells within the seminiferous epithelium [55–58]. CRISP2 specifically regulates calcium flow through ryanodine receptors [59,60], is implicated in cell–cell adhesion, and is capable of binding to steroids [61,62]. A decrease in CRISP2 content in sperm is associated with infertility in humans [53,54] and horses [63]. It was found that the content of this protein in the canine epididymal spermatozoa was low in very young and senile dogs [26].

Lactotransferrin (LTF) is an iron-binding protein that regulates the availability and catalytic activity of iron [64]. In canines, LTF was found in seminal plasma, and further studies showed that it originates in the epididymis [4,27], similar to other animal species e.g., mice, boars, and stallions [65]. It was also found to be a component of canine epididymal sperm [26,34]. LTF binds to phosphorylcholine-containing phospholipids of the sperm plasma membrane in dogs [4]. LTF has antibiotic properties in the reproductive system, conveyed by its ability to sequester iron and prevent the harmful effects of pathogens on spermatozoa [64,65]. Additionally, LTF can bind lipopolysaccharides, heparin, glycosaminoglycans, DNA, and ions like Mn^{3+}, Co^{3+}, Cu^{2+}, and Zn^{2+} [65]. The exact function of LTF in the canine reproductive system is not known, but since it is present in high amounts, it must be important for canine reproductive functions. The addition of LTF to a cryoprotective extender can significantly improve the function of frozen ram sperm [66].

Metalloproteinases (MMPs) are mostly expressed in the epididymis, where they contribute to the modification of the sperm membrane and the regulation of sperm maturation and storage [67]. MMPs aid in the processing of proteins on the sperm surface, which is crucial for sperm function and fertilization [68]. MMP-2, MMP-9, proMMP-2, and proMMP-9 were identified in canine seminal plasma [69]. Saengsoi et al., (2011) [69] suggested that higher activation of proMMP-2, proMMP-9, and MMP-9 may be caused by an abnormal spermatogenesis process, whereas MMP-2 may benefit sperm motility and viability. Studies have shown associations between MMP amounts in seminal plasma and parameters such as sperm concentration, motility, and morphology [70]. Zinc-binding abilities have also been demonstrated for MMPs [70]. The levels of MMPs and their tissue inhibitors are correlated with sperm motility and sperm DNA fragmentation in men [71,72]. Alterations in MMP expression or activity may indicate abnormalities in sperm function [71,72]. MMPs have been proposed as predictive biomarkers for the success of assisted reproductive techniques (ART), such as IVF and intracytoplasmic sperm injection (ICSI) [73]. Assessing MMP amounts in semen or reproductive tissues may help predict fertilization outcomes and the likelihood of successful embryo implantation.

Osteopontin (OPN) is expressed in the male reproductive tract, including the testes and epididymis [74]. It has been implicated in various aspects of sperm function, such as sperm maturation, motility, and capacitation, which are essential for successful fertilization. Lower OPN concentrations have been linked to poorer sperm motility and morphology [75]. OPN levels in seminal plasma vary depending on factors such as breed, age, and reproductive health status [76]. OPN has been identified in the seminal plasma of dogs, indicating its secretion by the male reproductive tract and its presence in the ejaculate [75–77]. Erikson et al., (2007) [78] suggested that OPN localized to the post-acrosomal region on sperm membranes may participate in bovine fertilization by interacting with egg integrins. OPN may also regulate sperm adhesion to the female reproductive tract, their migration towards the egg, and sperm–oocyte interaction [79,80]. OPN has been shown to play a major role in tumorigenesis, tumor invasion, and metastasis, with reported associations with breast, prostate, and ovarian cancer [81].

The prostaglandin-H2 D-isomerase, also known as prostaglandin-D2-synthase (PTGDS) or lipocalin-type prostaglandin-D-synthase, is an enzyme that converts the cyclooxygenase product of prostaglandin H2 (PGH2) to prostaglandin D2 PGD2 [82]. It is an enzyme that binds small non-substrate lipophilic molecules such as retinoids [83]. It also acts as a scavenger of harmful hydrophobic molecules [83]. Moreover, lipocalins bind to specific cell-surface receptors and may form macromolecular complexes [84]. PTGDS also affects plasma membrane permeability, resulting in changes in the input of ions from the outside, which may be correlated to the regulation of sperm motility and access to Ca^{2+} ions by the sperm cell [83,85,86]. PTGDS has been found in the epididymal fluid and seminal plasma of rams [86] and cats [87]. A positive correlation between PTGDS content in human sperm and cell progressive motility has been shown [88]. PTGDS is interconnected with several essential proteins involved in sperm metabolism, such as CRISP2 and lipocalin cytosolic FA-bd domain-containing protein (LCNL1). However, the exact function of PTGDS in sperm metabolism is unknown. A pro-

teomic study revealed that PTGDS is present in greater abundance in dog epididymal fluid surrounding sperm showing good motility [27].

Well-studied molecular biomarker proteins that coat canine sperm and change its surface properties, biochemistry, and metabolism are shown in Figure 1.

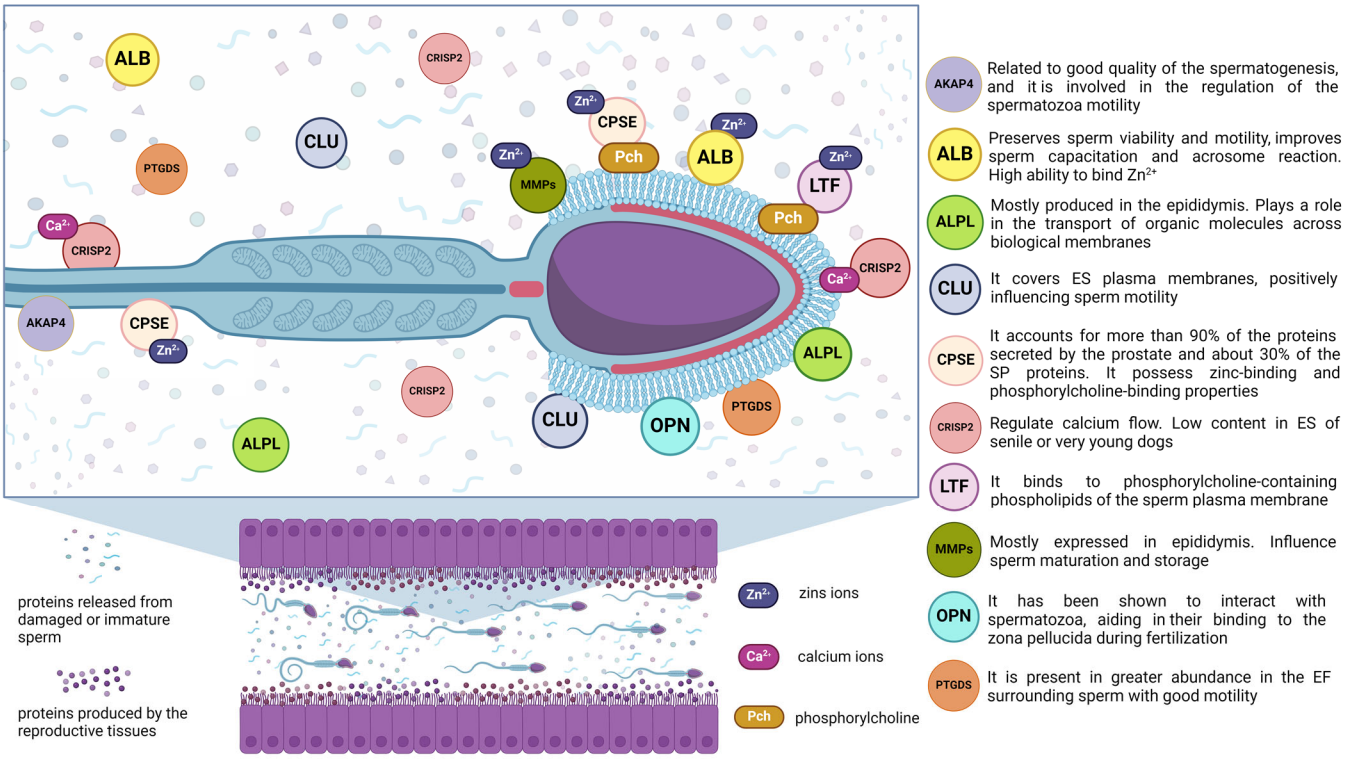


Figure 1. Well-studied/highly abundant molecular biomarker proteins that coat canine sperm and change its surface properties, biochemistry, and metabolism. AKAP4—A-kinase anchoring protein; ALB—albumin; ALPL—alkaline phosphatase; CLU—clusterin; CPSE—canine prostate-specific esterase; CRISP2—cysteine-rich secretory protein 2; LTF—lactotransferrin; MMPs—metalloproteinases; OPN—osteopontin; PTGDS—prostaglandin-H2 D-isomerase; SP—seminal plasma; EF—epididymal fluid; ES—epididymal spermatozoa. Created with BioRender.com.

2.2. Poorly Studied/Newly Recognized/Low-Abundance Proteins

Canine reproductive tissues, fluids, and sperm contain newly recognized proteins that could serve as potential protein markers for semen quality.

Acrosin binding protein (ACBP) is a calcium-binding protein detected in the semen of dogs (Bernese Mountain) [29]. The ACBP is a binding protein for both the precursor and intermediate forms of serine protease acrosin, a protein that is specifically localized in the acrosomes of germ and sperm cells [89]. ACBP improves sperm capacitation, acrosome reaction, and semen quality [29]. ACBP can be used as a molecular marker for pachytene spermatocytes, and for round, elongating, and elongated spermatids. ACBP can be used to monitor either normal spermatogenesis in the testicular tissues, or germ cell development in vitro [90]. ACBP may be a good marker for predicting boar sperm freezing capacity [91].

Actin binding protein (ACTB) is a structural protein that builds the cytoskeleton and is present in the flagellar and acrosomal membranes of spermatozoa. It is also responsible for cell volume changes [92,93]. Based on its location, its role was proposed to be in sperm capacitation and motility [92–94]. ACTB was previously found in the canine epididymal spermatozoa, and its expression was correlated with the dog's age [26]. Additionally, it was found in canine epididymal fluid, and it is highly abundant in semen with good sperm motility [27]. The exact mechanism of ACTB's influence on sperm motility needs to be evaluated in the future.

Abnormal spindle-like microcephaly-associated protein homolog (ASPM) is expressed in a variety of embryonic and adult tissues and is upregulated in cancer [95]. It has been found in canine epididymal spermatozoa [26]. ASPM misfunction affects chromosome segregation, which leads to reduced ability of fetal stem cells to produce neurons [95]. ASPM plays a role in sperm flagellar function [96].

Caspase recruitment domain containing protein 6 (CARD6) is found in the canine epididymal fluid [27]. Its function is to regulate cell proliferation, immune response, and cell apoptosis [97]. CARD6 may be involved in microtubular transport mechanisms, and proteins that interact with it may be targeted to the microtubule-organizing center, resulting in either their inactivation or translocation [98].

Hyaluronoglucosaminidase (CEMIP), also named KIAA1199, is a unique protein found in the epididymal spermatozoa of young dogs [26]. It is expressed in the human testis, and its biological role in cancer biology has been studied in humans [99]. It has not been described in other species, including dogs. This protein causes significant changes in cell morphology and actin cytoskeletal dynamics. It exerts its effects through regulation of the canonical Wnt and P38/MAPK signaling pathways [100] and the expression of CEMIP may be regulated depending on cell mortality rather than cell age [99].

Epididymal sperm-binding protein 1 (ELSPBP1) was first described in humans and dogs as a sperm-binding protein of epididymal origin that binds to the spermatozoa during their transit through the epididymis [101,102]. Since then, orthologs have been identified in horses [103], pigs [104,105], and bovines [102]. More recently, ELSPBP1 was shown to negatively correlate with bull fertility [106] and was associated with the dead sperm population [107]. It has been found in epididymal spermatozoa, and its presence is age-dependent [26].

The function of a family with sequence similarity 135 member A (FAM 135A) is the regulation of cellular proliferation, cell differentiation, development, and growth control [108]. Expression of the protein was observed in the testis, epididymis, prostate, and seminal vesicles in humans [109]. This protein has also been found to be a unique protein in the epididymal spermatozoa of older dogs [26].

N-acetylgalactosaminyltransferase-like proteins (GALNTL) were found in male reproductive organs in rodents and cattle [110–113]. In canines, it has been shown that polypeptide N-acetylgalactosaminyltransferase 6 (GALNT6) is important for epididymal sperm motility [27] and participates in mucin-type-O-glycan biosynthesis [26]. We can point out more reports about GALNTL5. In mice, it is localized in the head–tail coupling apparatus of cauda epididymal spermatozoa [112], and its expression is positively correlated with sperm motility [114].

Lipocalin cytosolic FA-bd domain-containing protein (LCNL1) is a member of the lipocalin family. These proteins transport or store small molecules, such as vitamins, hormones, and secondary metabolites [115]. LCN family proteins are important for sperm maturation, and they are expressed in different regions of the epididymis [116,117]. LCNL1, present in canine epididymal fluid, is important for sperm motility, although the exact mechanism is unknown [27].

Cystatin domain-containing protein (LOC607874) may serve as potential biomarker of canine aging [26]. A record for *Canis lupus familiaris* LOC607874 mRNA is found in the National Center for Biotechnology Information (NCBI). The cystatin-related epididymal-specific (*CRES*) gene is almost entirely restricted to the epididymis and much less is expressed in the testis, mainly in spermatids [118]. A *CRES* gene, which shares similarities with well-known protein inhibitors called cystatins, was found in the mouse epididymis [119]. This protein is unique to the epididymal spermatozoa of older dogs [26] and its presence in the epididymal fluid has been associated with poor sperm motility [27].

Niemann-Pick type C2 protein (NPC2), intracellular cholesterol transporter 2, also called epididymal secretory protein E1, is found in the canine epididymal spermatozoa [26]. In canines, epididymal secretory protein E1 (CE1/NPC2) is encoded by genes similar to those in humans [120]. The CE1 protein is a highly abundant, conserved, secretory

protein [121] and its mRNA is found in large amounts in the epididymal duct epithelium, while the protein is found in the duct lumen [122]. Recently, *CE1* has been identified as a gene that is important in the etiology of Niemann-Pick type C disease [123]. This protein is involved in cholesterol efflux from lysosomes [123]. In human ejaculated spermatozoa, a human sperm antigen (HE2), human homolog of CE1, was found in the acrosome and equatorial region of the cells [124]. NPC2 epididymal sperm-membrane-coating ability has been suggested [34] because similar results were shown by Araujo et al. [29,125], who found NPC2 to be an ejaculated sperm component.

Olfactomedin 4 (OLFM4) is an olfactomedin domain-containing glycoprotein [126]. OLFM-1, -2, -3, -4 are known to regulate cellular growth, differentiation, and pathological processes [127]. The absence of *OLFM4* gene expression is associated with the progression of human prostate cancer [128]. OLFM4 is found in human spermatozoa [129]. The OLFM-4 precursor is found in human epididymosomes [130]. The role of OLFM4 in sperm physiology is unknown. This protein was found to be expressed in the epididymal fluid of dogs and was correlated with good sperm motility [27].

Pleckstrin homolog, MyTH4, and FERM domain-containing H1 (PLEKHH1) is a protein constituent of the cytoskeleton with a role in intracellular signaling [131]. It binds phosphatidylinositol lipids within biological membranes and plays a role in recruiting proteins to membranes. PLEKHH1 expression is found in human testis, epididymis, seminal vesicles, and prostate [132]. This protein has also been found in the canine epididymal spermatozoa [26]; however, its function has not yet been established.

The tubulin (TUBB) family proteins are associated with structural cell organization and flagella movement [29,133–135]. These proteins have also been identified in the sperm membrane of Morada Nova rams [136] and mice [137]. In humans, tubulins are associated with low sperm motility [138,139]. A study by Araujo et al., (2022) [29] indicated a high abundance of tubulin alpha-3E chain in Maremmano-Abruzzese Sheepdogs and tubulin alpha-3 chain in Bernese Mountain Dogs [29]. Tubulins in seminal plasma are linked to damaged sperm in the ejaculate, possibly due to sample handling [140].

Poorly studied/newly recognized/low-abundance molecular biomarker proteins in canines, which represent less studied proteins that change the biochemistry and metabolism of sperm, are shown in Figure 2.

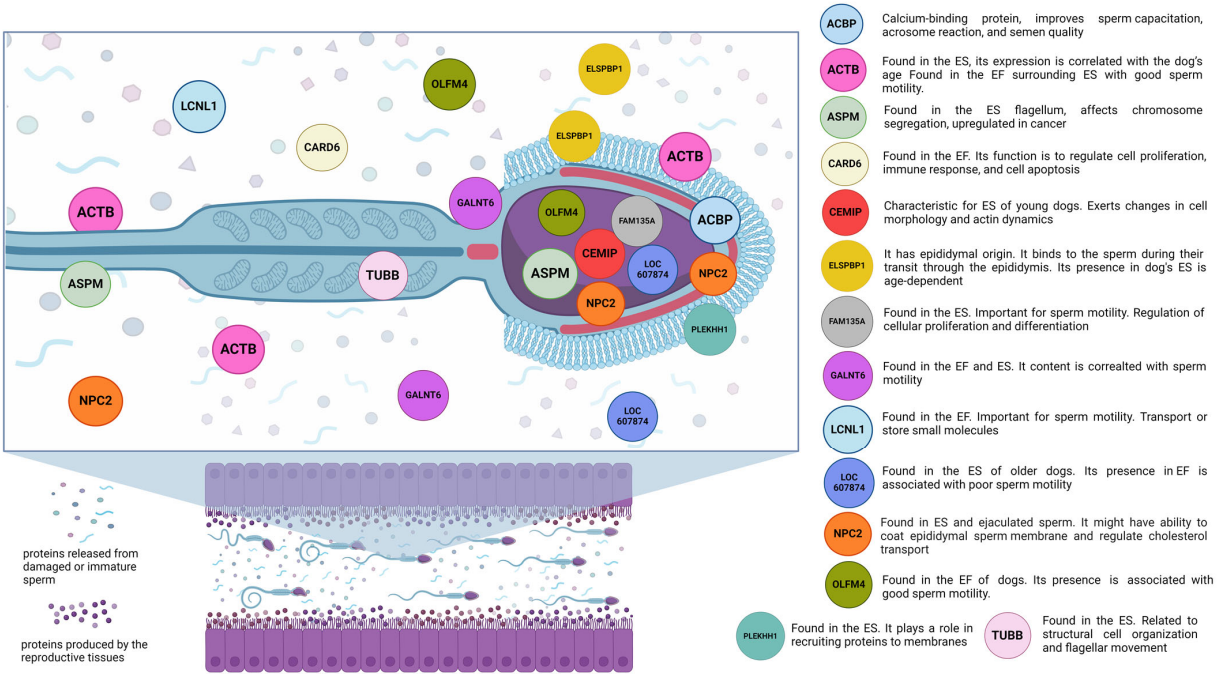


Figure 2. Poorly studied/newly recognized/low-abundance molecular biomarker proteins in canines that change sperm biochemistry and metabolism. ACBP—Acrosin binding protein; ACTB—Actin binding protein;

ASPM—Abnormal spindle-like microcephaly-associated protein homolog; CARD6—Caspase recruitment domain containing protein 6; CEMIP—Hyaluronoglucosaminidase; ELSPBP1—Epididymal sperm-binding protein 1; FAM135—A family with sequence similarity 135 member A; GALNT6—Polypeptide N-acetylgalactosaminyltransferase 6; LCNL1—Lipocalin cytosolic FA-bd domain-containing protein; LOC607874—Cystatin domain-containing protein; NPC2—Niemann-Pick type C2 protein; OLFM4—Olfactomedin 4; PLEKHH1—Pleckstrin homolog, MyTH4, and FERM domain-containing H1; TUBB—Tubulin; EF—epididymal fluid; ES—epididymal spermatozoa. Created with BioRender.com.

2.3. Antioxidant Enzymes

Glutathione peroxidase (GPX) is a well-known, highly abundant protein in the canine epididymis [141]. GPX5 is highly expressed in the epididymis and is then secreted into the lumen. Its role is to protect sperm from lipid peroxidation [141]. GPX is found in, among others, bull, boar, and dog seminal plasma [142–144] and in boar epididymal spermatozoa [144]. This protein also participates in important metabolic pathways in feline seminal plasma [87]. The above-mentioned protein works in a SOD/CAT system. Antioxidant enzyme activities of mammalian spermatozoa comprise those provided by superoxide dismutase (SOD) and GPX. SOD has been shown to play a key role in the intracellular scavenging of superoxide anions [145]. The superoxide anion generated by the inner mitochondrial membrane is dis-mutated to hydrogen peroxide by the Mn-SOD present in the mitochondrial matrix and by the Cu/Zn-SOD present in the cytosol. In turn, hydrogen peroxide is eliminated by either GPX or catalase to avoid cell damage. Because it has been shown that sperm from most mammalian species lack catalase activity, sperm mostly rely on GPX activity to eliminate intracellularly generated hydrogen peroxide [146]. It has been reported that both SOD and GPX play a central role in protecting mammalian sperm against oxygen-radical-induced damage leading to motility loss [146]. SOD could be a valid marker for resistance to cryodamage because the time observed for complete loss of motility due to lipoperoxidative damage to sperm under experimental conditions is strongly correlated with the SOD activity of the sperm sample [147]. A study of dogs confirmed the presence of endogenous antioxidants in the seminal plasma of pre-spermatic, spermatic, and post-spermatic fractions, with SOD representing the major enzymatic antioxidant in all dog ejaculate fractions, whereas GPX activity was present in the sperm-rich and post-spermatic fractions, while CAT activity was deficient [148]. Another study [149], however, demonstrated CAT activity in dog ejaculates and showed that the addition of SOD and CAT in the dilution extender of canine semen improved sperm quality. The addition of a GPX and SOD combination to dog semen can protect sperm viability and DNA integrity [150]. The addition of SOD, CAT, and GPX in the extender allows the preservation of semen quality in cold storage for both fertile and hypofertile dogs [151].

Antioxidant enzymes functioning as molecular biomarkers that participate in sperm biochemistry and metabolism in canines are shown in Figure 3.

Table 1 contains a summary of the most promising protein molecular biomarkers of canine reproductive functions.

Table 1. Summary of the most promising protein molecular biomarkers of canine reproductive functions described in the review. T—testis; EP — epididymis; EF—epididymal fluid; ES—epididymal spermatozoa; S—ejaculated spermatozoa; SP—seminal plasma; P—prostate; BS—blood serum, CD—cytoplasmic droplets.

Biomarker	Origin of Biomarker	Influence on Reproductive Functions	Implementation in Clinical/Breeding Practice	References
Well-studied proteins/highly abundant proteins				
AKAP4/ProAKAP4 (A-kinase anchoring protein)	T, S	- regulation of spermatogenesis - regulation of sperm capacitation - regulation of sperm motility	- biomarkers of capacitation status - associated with good-quality of spermatogenesis - involved in the regulation of spermatozoa motility - translation of semen quality markers into a practical tool (such as 4MID® kits, 4BioDx, Lille, France)	[14,16,17,21]

Table 1. *Cont.*

Biomarker	Origin of Biomarker	Influence on Reproductive Functions	Implementation in Clinical/Breeding Practice	References
ALBs (Albumins)	BS, T, EF, ES, SP, P	- preserves sperm viability - improves sperm capacitation and acrosome reaction - involved in zona pellucida penetration - regulates sperm motility - antioxidant properties	- The addition of heterogenous bovine serum albumin to the ejaculated canine spermatozoa preserves sperm viability and function -improves sperm capacitation and acrosome reaction	[28,29]
ALPL (Alkaline phosphatase)	CD, BS, EP, SP	- dephosphorylation enzyme - transport organic molecules across biological membranes	- potential biomarker for prostate and testicular cancer - ALPL inhibitors can reduce the growth and invasion of cancer cells—measurement of ALPL enzymatic activity in the canine SP has been used in the diagnosis of incomplete ejaculation or azoospermia in dogs - a lower concentration of ALPL in the SP suggests bilateral obstruction of the vas deferens or epididymis	[37,39,40]
CLU (Clusterin)	T, ES, EF, SP, P	- positively influences sperm motility - inhibiting cell apoptosis - mediation of female tolerance to seminal antigens - sperm maturation processes	- an indicator of low semen quality because secretion of this protein is enhanced in case of cellular damage or heat shock	[42,46,47]
CPSE (Canine prostate-specific esterase)	S, SP, P	- multifunctional protein due to its zinc-binding and phosphorylcholine-binding properties - coat ejaculated spermatozoa, which could be implicated in sperm fertilization-related events	- marker of glandular secretion and serves as a promising diagnostic tool for non-neoplastic canine prostatic disorders	[4,6,40]
CRISP2 (Cysteine-rich secretory protein)	T, ES, S	- specifically regulates calcium flow through ryanodine receptors - is implicated in cell–cell adhesion and is capable of steroid binding	- decrease in CRISP2 content in sperm is associated with infertility in humans and horses - its content is low in the ES of very young and senile dogs	[26,53,54,59–63]
LTF (Lactotransferrin)	EP, SP, ES	- regulates the availability and catalytic activity of iron - has antibiotic properties in the reproductive system - able to bind lipopolysaccharides, heparin, glycosaminoglycans, and DNA, and ions like Mn^{3+}, Co^{3+}, Cu^{2+}, and Zn^{2+}	- its addition to a cryoprotective extender can significantly improve the function of frozen ram sperm	[64–66]
MMPs (Metalloproteinases)	E, SP, S	- contribute to the modification of the sperm membrane and the regulation of sperm maturation and storage - higher activation of proMMP-2, proMMP-9, and MMP-9 may be caused by an abnormal spermatogenesis process, whereas MMP-2 may benefit sperm motility and viability	- associations between MMP amounts in SP and parameters such as sperm concentration, motility, and morphology - the levels of MMPs and their tissue inhibitors are correlated with sperm motility and sperm DNA fragmentation in men - alterations in MMP expression or activity may indicate abnormalities in sperm function - MMPs have been proposed as predictive biomarkers for the success of assisted reproductive techniques (ART) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI)	[67,70–73]
OPN (Osteopontin)	T, EP	- implicated in various aspects of sperm function such as sperm maturation, motility, and capacitation, which are essential for successful fertilization - OPN localized to the post-acrosomal region on sperm membranes may participate in bovine fertilization by interacting with egg integrins	- lower OPN concentrations are linked to poorer sperm motility and morphology - it plays a major role in tumorigenesis, tumor invasion, and metastasis in prostate cancer	[75,76,78]
PTGDS (Prostaglandin-H2 D-isomerase)	EF, SP	- an enzyme that binds small non-substrate lipophilic molecules such as retinoids	- has a positive effect on human sperm's progressive motility - PTGDS is present in greater abundance in the dog EF surrounding sperm showing good motility	[27,83,88]
		Poorly studied/newly recognized/low-abundance proteins		
ACBP (Acrosin binding protein)	S	- improves sperm capacitation, acrosome reaction, and semen quality	- molecular marker for monitoring spermatogenesis in testicular tissues or germ cell development in vitro - marker for predicting boar sperm freezing capacity	[29,90,91]
ACTB (Actin binding protein)	ES, EF	- sperm capacitation and motility	- potential marker of canine ES aging - found in canine EF (highly abundant in EF of dogs with good sperm motility)	[26,27,92–94]
ELSPBP1 (Epididymal sperm-binding protein 1)	EP, ES	- binds to spermatozoa during their transit through the epididymis	- negatively correlated with bull fertility - is associated with the dead sperm population - is found in the epididymal spermatozoa, and its presence is age-dependent	[26,106,107]
LOC607874 (Cystatin domain-containing protein)	T, EP, ES, EF	- sperm motility	- potential biomarker of aging - its presence in EF has been associated with poor sperm motility	[26,27,118]
NPC2 (Niemann-Pick type C2 protein)	EP, S	- intracellular cholesterol transporter - gene is important in the etiology of Niemann-Pick type C disease - involved in cholesterol efflux from lysosomes	- higher content of NPC2 in poor-freezability semen leads to higher efflux of cholesterol - potential freezability marker of semen	[26,123,152]
OLFM4 (Olfactomedin 4)	S, EF, Epididymosomes	- regulates cellular growth, differentiation, and pathological processes	- the absence of its gene expression is associated with the progression of human prostate cancer - found in the EF of dogs, and is correlated with good sperm motility	[27,28,128]
TUBB (Tubulin)	SP, S	- associated with structural cell organization and flagella movement	- presence in SP may be correlated to sample handling	[29]
		Antioxidant enzymes		
GPX5 (Glutathione peroxidase 5)	EP, SP, ES	- protects sperm from lipid peroxidation - works in the SOD/CAT system	- marker for resistance to cryodamage - the combination of GPX5 and SOD as an additive in the dilution extender of canine semen protects sperm viability and DNA integrity - addition of SOD, CAT, and GPX to the extender allows the preservation of semen quality in cold storage for both fertile and hypofertile dogs	[141,147,150,151]
SOD (Superoxide dismutase)	EP, SP, ES	- protects sperm from lipid peroxidation - works in the SOD/CAT system	as above	as above
CAT (Catalase)	SP	- enzymatic decomposition of hydrogen peroxide into water and oxygen - works in the SOD/CAT system	- addition of SOD and CAT to the dilution extender of canine semen improves sperm quality - addition of CAT to the extender allows the preservation of semen quality in cold storage for both fertile and hypofertile dogs	[119,149]

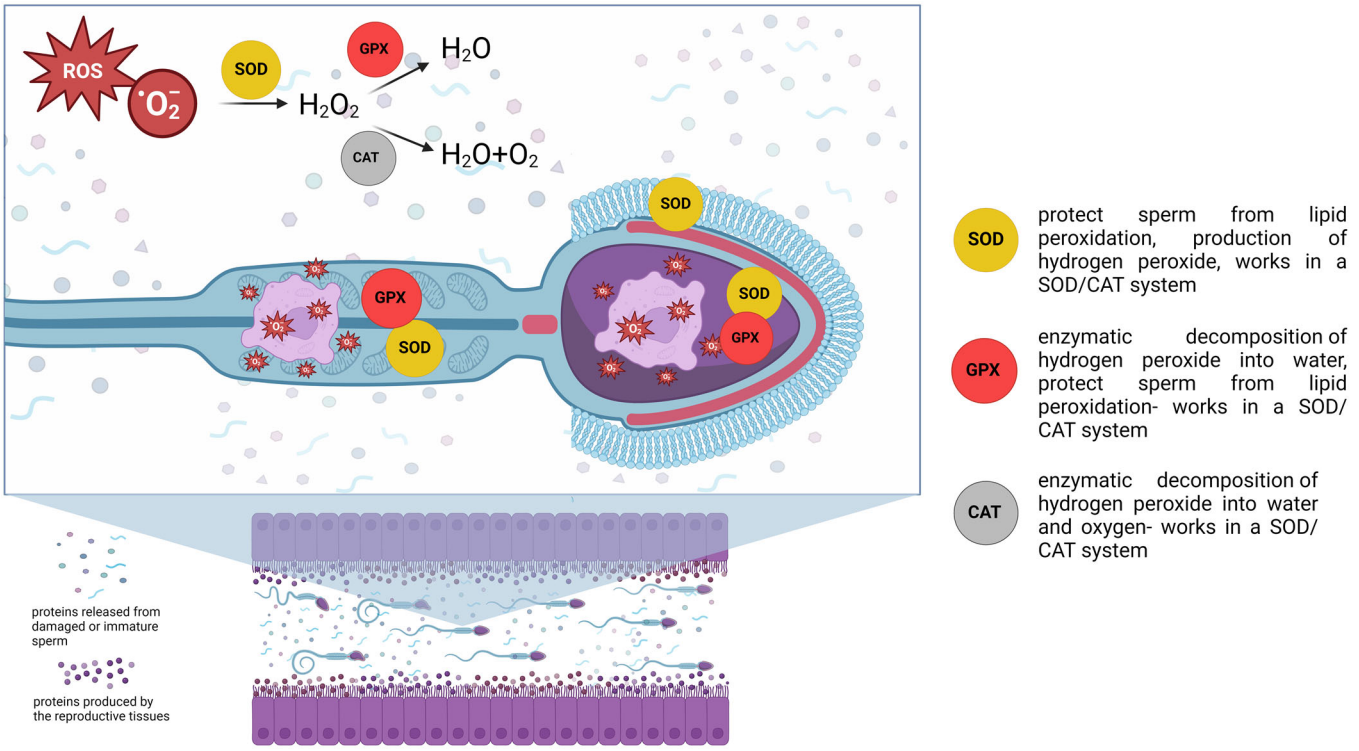


Figure 3. Antioxidant enzymes that function as molecular biomarkers and participate in sperm biochemistry and metabolism in canines. SOD—superoxide dismutase; GPX—glutathione peroxidase, CAT—catalase, ROS—reactive oxygen species.

3. Non-Protein Markers

3.1. Lipids

Lipids are essential building compartments of cells, including most of all their plasma membranes, the endoplasmic reticulum, the nuclear membranes, endosomes, and lysosomes [153]. Lipids form highly specialized sperm plasma membranes. Phospholipids, fatty acids, glycolipids, and neutral lipids (such as cholesterol and diacylglycerols) are among the lipids found in plasma membranes [8,154]. Changes in lipid composition within membranes generally correspond to their physiological needs [8]. The sperm plasma membrane's lipid profile varies during sperm transfer along the epididymal tract [155]. This facilitates the preservation of cellular stability and integrity, which are crucial for preventing sperm damage, as well as the progressive acquisition of functional competence by sperm, such as motility and the ability to fertilize [156]. Lipid reorganization of the sperm plasma membrane during sperm maturation occurs due to the region-specific environment surrounding sperm. Therefore, remodeling of the sperm plasma membrane's lipid profile is influenced by substances present in the epididymal fluid [157–159]. In relation to the above findings, the arrangement of fatty acids in the sperm membrane appears to be a significant factor that could potentially elucidate the quality of sperm, including their cryotolerance [160–162]. Only a few studies have been conducted on canine semen's lipid composition and its impact on semen quality.

3.1.1. Fatty Acids

Angrimani et al., (2017) [159] identified differences between fatty acid profiles of epididymal spermatozoa and epididymal fluid derived from different regions of canine epididymis (caput, corpus, cauda). The authors study also showed that the fatty acids content correlate with sperm motility parameters and sperm plasma membrane integrity. Díaz et al., (2014) [163] identified canine seminal plasma fatty acids. Lucio et al., (2017) [164]

conducted an analysis of the lipid profile of dog-ejaculated sperm, which the authors used to identify lipid markers of sperm motility.

Results obtained by Angrimani et al., (2017) [159] indicate a progressive increase in the dog sperm motility and plasmatic membrane integrity from caput to cauda epididymis. The same pattern was also observed for the concentrations of fatty acids (saturated, monounsaturated, and polyunsaturated).

Saturated Fatty Acids

According to Angrimani et al., (2017) [159], epididymal sperm had a higher concentration of saturated fatty acids (SFAs) compared to the epididymal fluid. Among fatty acids in canine seminal plasma, SFAs were observed to be the most abundant [163]. Caprylic and stearic fatty acids are the most abundant SFAs found in cauda epididymal sperm, as identified in a study by Angrimani et al., (2017) [159], while palmitic and stearic fatty acids are the most abundant in canine seminal plasma, according to Díaz et al., (2014) [163]. Caprylic fatty acid plays a dual role in the sperm membrane by exhibiting both bactericidal properties and protective functions, as per a study by [165]. Additionally, caprylic fatty acid was found to be effective in maintaining the viability of rooster sperm during in vitro storage when used as an extender [166]. It has been shown that stearic fatty acid regulates sperm function and provides the energy necessary for sperm motility and metabolism [167]. However, stearic fatty acid was also identified in human spermatozoa and seminal plasma and was negatively correlated with sperm motility [168]. High levels of stearic fatty acid were observed in lipid profiles of asthenozoospermic spermatozoa [169–171]. In humans, a positive correlation was observed between palmitic fatty acid and both sperm concentration and total sperm count, suggesting its potential significance in sperm production [172].

Monounsaturated Fatty Acids

For monounsaturated fatty acids (MUFAs), pentadecenoic fatty acid concentration was higher in the dog cauda epididymal sperm and fluid compared to epididymal caput and corpus, and the concentration increased from caput to cauda epididymal regions [159]. Pentadecenoic fatty acid has also been identified in canine seminal plasma, albeit in small amounts [163]; however, knowledge about its influence on epididymal sperm maturation and further semen quality is lacking. Oleic fatty acid is a characteristic component of the seminal plasma in both canines [163] and humans [168,173]. This fatty acid, along with stearic fatty acid, is associated with sperm metabolism and has a positive effect on both the motility and viability of boar spermatozoa [174].

Polyunsaturated Fatty Acids

Among polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) is present in the dog cauda epididymal spermatozoa [159]. Cauda epididymal fluid also contains higher DHA levels than the remaining regions of the epididymis, although it was lower than in the dog cauda epididymal sperm [159]. The contents of DHA in mammalian sperm differ among species and range from low percentages in rats and rabbits (1%) to almost half of the total content of PUFAs in human sperm [160,175–177]. Waterhouse et al., (2006) [178] suggested that, when freeze-thawed, boar sperm with higher levels of DHA in their membranes were more cryotolerant, which was attributed to the higher membrane fluidity. Docosahexaenoic acid adds unique fluidity to the sperm plasma membrane [179], thus affecting its integrity, which is essential for sperm motility and acrosome reaction [179,180]. Moreover, DHA positively influences testicular testosterone secretion and enhances sperm antioxidant capacity [181]. A positive correlation between DHA in the sperm plasma membrane and sperm motility in boars was demonstrated [182], and a low content of this fatty acid was observed in human asthenospermic semen [160]. Based on the above findings, Angrimani et al., (2017) [159] proposed that increasing DHA concentration in the epididymal sperm and fluid is important for the final steps of epididymal maturation in dogs due to its direct involvement in fertilization.

It should be noted that the highest concentration of individual fatty acids occurred both in the sperm and fluids derived from the cauda epididymis—the place where sperm achieve the highest maturity level. Changes in the structure of the sperm plasma membrane's lipids occur during sperm transfer through the epididymis. These changes are associated with the binding of fatty acids as well as other components, such as proteins, from the epididymal fluid [44,155]. This suggests that during epididymal maturation, the sperm takes up essential lipids, which may be vital for acquiring functional features required for its mission in the female reproductive tract. The highest concentration of fatty acids in the dog cauda epididymal sperm may contribute to the enhanced stability of the cell membrane [159]. Sperm cells are highly susceptible to oxidative stress due to the concentration of PUFAs found within the plasma membranes [183]. Unsaturated fatty acids add elasticity and fluidity to the plasma membrane, which are crucial for the proper development of sperm functional features such as motility [184]. However, their presence stimulates free radical activity and, thus, lipid peroxidation, which are the main causes of membrane disintegration [185]. A higher concentration of SFAs may reduce sperm damage during storage at low temperatures [176].

3.1.2. Phospholipids

Among the group of phospholipids, very early reports have shown that the dog spermatozoa contain large amounts of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, ethanolamine plasmalogen, and choline plasmalogen [186]. Phosphatidylcholine and phosphatidylethanolamine were identified in the motile spermatozoa and less frequently in the spermatozoa of asthenozoospermic individuals [164]. The reduction in phosphatidylcholine and phosphatidylethanolamine content in the sperm of asthenozoospermic individuals stems from the extensive degradation and impairment of PUFAs caused by oxidation induced by free radicals [170,187]. Canine sperm motility is indicated by the lipid markers phosphatidylcholine and phosphatidylethanolamine, according to the above-mentioned reports. Interestingly, phosphatidylethanolamine in the sperm membranes has the capacity to bind to lipocalin 2 in the female reproductive tract. In turn, lipocalin 2 can stimulate the rearrangement of lipid rafts and the efflux of cholesterol [188].

Plasmalogens

Among the group of lipids, plasmalogens are of great interest based on their protective functions against oxidative damage in sperm. Shan et al., (2021) [8], in their thorough review, explained the mechanism of plasmalogen antioxidant properties. Moreover, in canines, plasmenyl phosphatidylcholine (40:5) and plasmanyl phosphatidylcholine (40:4) were reported as lipid sperm motility markers [164] due to their increased content in motile sperm and cells with the lowest percentage of acrosomal membrane damage. These findings might provide evidence that the presence of plasmalogens in the motile sperm may be part of a protective mechanism protecting the sperm plasma membrane against lipid peroxidation and preventing damage induced by reactive oxygen species (ROS) [164].

Sphingomyelins

Sphingomyelins are part of biological membranes [189], including the sperm plasma membrane [190,191], and function as bioactive lipids [189]. Sphingomyelin has an affinity for cholesterol [192]. Interestingly, this combination may generate low-density lipid rafts in the plasma membrane, thereby modifying the lateral arrangement of proteins [193]. An early study by Darin-Bennet et al., (1974) [186] showed that dog spermatozoa contain large amounts of sphingomyelin; however, the latest reports on this matter do not indicate the significance of sphingomyelin in dog's reproductive functions.

Phosphorylcholine

The coating on the sperm plasma membrane is formed by phosphorylcholine moieties of phospholipids [194]. Protein complexes with the involvement of phosphorylcholine

moieties also form the coating of the dog sperm plasma membrane during ejaculation and protect the spermatozoa against the adverse conditions in the female reproductive tract, as well as against cold shock during semen freezing [4,195]. These findings suggested that the attachment of phospholipids containing phosphorylcholine is an important mechanism of protein coating on the dog sperm plasma membrane [4,195]. Mogielnicka-Brzozowska et al., (2017) [4] have shown that the phosphorylcholine-binding proteins (PchBPs) in the canine seminal plasma are high molecular weight aggregates. Mass spectrometric analysis of canine seminal plasma PchBPs identified five different types of proteins: LTF, ALB, PTGDS, CPSE, and lipocalin-like 1 protein isoform.

3.1.3. Cholesterol

Cholesterol is an important structural component maintaining the stability and fluidity of the cell membrane [196]. Cellular efflux of cholesterol is required to maintain homeostasis and is an essential step during the capacitation of spermatozoa [197]. Cholesterol is the precursor of steroid hormones, such as testosterone, produced by the Leydig cells, and plays an important role in normal spermatogenesis [198]. Cholesterol concentration in spermatozoa was shown to be associated with cryotolerance, motility, and morphology of sperm cells. Therefore, cholesterol in sperm cells provides protection against sperm damage induced by freeze/thaw processes [199]. Canine sperm membranes are not highly sensitive to cold damage due to their relatively high cholesterol:phospholipid ratio and the PUFA content of the membrane [200]. Schäfer-Somi and Palme (2016) [25] showed that in dogs, higher concentrations of cholesterol are found in the seminal plasma of ejaculates with good freezability than in the seminal plasma of ejaculates with bad freezability. The removal of seminal plasma from good-quality ejaculates decreased post-thaw motility, increased the percentage of morphologically abnormal sperm, and increased post-thaw DNA damage [201]. The movement of cholesterol from the sperm plasma membrane involves specific molecules with a high affinity for cholesterol, for example, NPC2, which is closely associated with the lipid structure of the cell membrane [202,203]. It was found that the NPC2 protein contains a functional cholesterol-binding site through which it exerts its high affinity for cholesterol and binds to the sperm membrane [204]. In humans, NPC2 is associated with cholesterol efflux from the sperm membrane during epididymal sperm maturation [205]. Its role in the removal of cholesterol from the lipid rafts in bull spermatozoa was suggested [206]. In mice, NPC2 is associated with the maintenance of cholesterol content in the sperm membrane [203]. These findings are consistent with results showing that higher content of NPC2 in poor-freezability semen leads to higher efflux of cholesterol [152]. This phenomenon affects the fluidity of the sperm plasma membrane [207] and increases the influx of calcium ions, which leads to impaired membrane integrity [207,208]. Based on NPC2's cholesterol-binding ability, a possible protective mechanism of NPC2 in reducing boar sperm membrane lipid loss during cryopreservation was proposed [152]. NPC2 was specified as a potential freezability marker of semen [152]. These investigations show that cholesterol content in semen might be a potential marker of canine semen resistance to cold shock [25]. Moreover, the positive correlation between seminal plasma cholesterol and semen quality in humans suggests that the cholesterol profile of seminal plasma can be used as an indicator of semen quality [209].

Lipids exhibit significant complexity and dynamism, undergoing continuous alterations in response to physiological and environmental factors [210]. Lipidomics is a field of research on the complete lipid profile (lipidome) within a cell, tissue, or organism. It also provides a quantitative analysis of the identified lipids [211]. Lipidomics can be used to study signal processing, lipid metabolism, molecular mechanisms, and biomarker discovery [210]. Despite the growing popularity and development of lipidomic tools, a lack of knowledge remains. The lipid composition of canine semen has also not been fully explored. This study would shed new light on understanding the impact of various lipids on reproductive processes in dogs. Combined with other molecular biology methods, lipidomics can be used to highlight the lipid markers of male fertility [183]. The identi-

fied biomarkers can be subjected to further research and used to determine, in detail, the molecular basis of male fertility in the future.

3.2. Carbohydrates

The effect of carbohydrates on the reproductive functions of dogs has not been described in detail. The available literature is dominated primarily by reports on the addition of sugars (glucose, fructose) to semen extenders [212] or basal medium [213]. Sugars have long been incorporated into semen diluents as exogenous energy substrates, osmotic constituents, and cryoprotective agents [214]. The carbohydrate composition of dog semen (sperm and seminal fluids) and the impact of individual sugar components on semen quality need to be investigated to fill the existing lack of current knowledge. Research based on modern, accurate measurement methods may allow the identification of new sugar markers of dogs' reproductive functions.

Heparin

Heparin belongs to the class of glycosaminoglycans [215]. Early studies showed that glycosaminoglycans are involved in events preceding the acrosome reaction [216,217] and stimulate acrosome reaction in sperm in vitro [218–221]. Interestingly, it has been speculated that sperm motility in the bitch reproductive tract is maintained by the glycosaminoglycans present in the uterine fluid, and the glycosaminoglycans in the oviductal and uterine fluids of estrous bitches are associated with in vivo capacitation of the dog sperm [222]. The role of heparin in reproductive processes can be considered in three ways: (a) heparin as an addition to the sperm capacitation medium; (b) heparin as a ligand bound to proteins present in the fluids of the reproductive system; and (c) heparin-binding proteins (HBPs) as a semen additive.

Heparin as an additive in the in vitro medium is effective in stimulating metabolic enzymes, prolonging sperm motility, and successfully inducing hyperactivation even in the sperm of asthenozoospermic dogs [223]. Additionally, Risopatrón et al., (2005) [224] showed the positive effect of the addition of heparin to the capacitating medium on the acrosome reaction of the canine sperm. The presence of heparin in an in vitro medium induces acceleration in the fertilizing ability of the bull epididymal sperm [225].

The ability of sperm to bind heparin is attributed to the presence of seminal plasma proteins. These proteins attach to the sperm surface (especially to lipids containing the phosphoryl-choline group) after ejaculation, which increases the number of heparin-binding sites on the cell [226–228]. The sperm is then allowed to respond to heparin [227]. Heparin binding to bovine seminal plasma proteins induces the efflux of cholesterol and phospholipids from sperm, leading to capacitation [229,230]. The binding of heparin to receptors localized in the proximal region of the sperm head results in alterations in the structure of the plasma membrane covering that specific area [216,231]. In consequence, this mechanism influences the modulation of the acrosomal reaction by zona pellucida glycoproteins [228]. Due to this, heparin is known as a potent enhancer of capacitation [218]. De Souza et al., (2006) [232] described HBPs of the canine seminal plasma. They showed that approximately 50% of the canine seminal plasma proteins are bound to heparin. Of the dog seminal plasma HBPs, CPSE is predominant [232], similar to the group of ZnBPs [6]. Moreover, there is a high homology between the CPSE and the PSA from the seminal plasma, and PSA has been identified as a heparin-binding protein. PSA is the protein marker for human prostate cancer [233–235]. Moreover, high concentrations of OPN were identified in the dog seminal plasma and recognized as HBP [77]. The functions of CPSE and OPN in canine reproduction are described above. The capacity of some proteins to bind several different ligands should be taken into account. It has been shown that boar seminal plasma ZnBPs have the ability to also bind heparin, which suggests that these proteins participate in events associated with the regulation of capacitation and acrosome reactions in boar sperm [7]. Seminal plasma HBPs play an essential role in the fertilization process, and any variations in the structures, content, and functions of these proteins may be associated with infertility [236].

Treatment of bull semen with seminal plasma HBPs improved sperm motility, viability, acrosome integrity, in vitro capacitation, and acrosome reaction by reducing the oxidative stress induced by cold shock and, consequently, damage to the sperm membrane [237].

This clearly indicates a positive effect of heparin and HBPs on dog semen quality and its participation in the fertilization process, suggesting that heparin and its binding proteins are distinguished as having marker potential.

3.3. Ions

The maintenance of proper sperm function is dependent on the balance of intracellular and extracellular ions and proper ion channel functioning. Sperm cells react rapidly to environmental fluctuations and the regulation of ion balance across their membrane has been shown to be of critical importance for sperm functional features such as motility and fertilization capacity [238]. Ion channels contribute significantly to modulating membrane potential through the maintenance of intracellular pH and osmotic balance [239]. Sperm motility acquisition, capacitation, acrosome reaction, hyperactivation, and transport in the female reproductive tract are ion channel-dependent processes, and the intracellular milieu is regulated by the activity of these ion channels [240]. The control of sperm membrane potential is essential for all these events, and it is regulated by complex signaling pathways that involve the activation of many ion transporters and ion channels present in the sperm membrane [241,242]. Thus, ion channel dysfunctions, interference with ion homeostasis, and sperm membrane potential alterations have important consequences for fertilization [238].

3.3.1. Zinc Ions

Zinc is an essential biological component that plays significant roles in several processes, including signaling, enzymatic activities, and regulation of sexual maturation, ensuring the stability of membrane lipid bilayers and the quaternary structure of the chromatin, and mitochondrial oxidative stress [243–246]. Zinc ions regulate a number of events that are crucial for fertilization, such as normal testicular development, spermatogenesis, and sperm function [247]. Any alterations in zinc can cause abnormalities in the functioning of the organism and have been associated with several diseases in dogs (see review [248]).

It has been shown that most of the zinc in canine seminal plasma originates from the prostate [249]. Large amounts of free zinc in seminal plasma inhibit sperm motility, while reduced free zinc improves their motility [250,251]. Zinc released by the prostate into seminal plasma during ejaculation can be utilized in two different manners. It can be incorporated into sperm cells and thus perform a protective role towards sperm chromatin and membrane stabilization and sperm motility [252–254]. Free zinc ions can also bind to different protein ligands that are implicated in fertilization processes in the female reproductive tract [195,255–257], reducing the zinc fraction available for sperm cells [258]. Free zinc availability in semen is regulated by high molecular proteins, which bind this element and reduce its amount in seminal plasma [259]. The authors' recent studies have shown that high molecular weight protein complexes of canine seminal plasma possess the ability to bind zinc ions, and this has a positive effect on the motility of spermatozoa stored at low temperatures [7].

Zinc–protein complexes were isolated from seminal plasma in canines and characterized for the first time by Mogielnicka-Brzozowska et al., (2012) [258]. This study showed that canine zinc-binding proteins (ZnBPs) occurred in their native state as high molecular aggregates. Mogielnicka-Brzozowska et al., (2015) [6] also showed that under denaturing and reducing conditions, these macromolecules disaggregate. Spectrometric analysis revealed that seminal plasma zinc ions play both structural and regulatory roles in the activity of CPSE, which is crucial for maintaining the normal function of prostate and sperm cells. Prostasomes have also been studied in the context of zinc. Prostasomes are small lipid membrane-confined extracellular vesicles in the mammalian reproductive tract fluids [260] with the ability to bind zinc ions [261–263]. Canine prostasome proteins have a very high

affinity for zinc ions, which suggests the important role of the vesicles in the metabolism of zinc in the canine semen [263]. It was shown that the addition of prostasomes to dog ejaculates had a positive effect on sperm quality (sperm plasma membrane and acrosome integrity) during storage at low temperatures [263].

There are some works systematizing knowledge on the role of zinc in male reproduction [248,264–267]. However, zinc ions found in dog semen should be further investigated for their potential as markers of semen quality.

3.3.2. Calcium Ions

Ca^{2+} levels are low in freshly ejaculated cells; however, during capacitation, Ca^{2+} levels increase due to the influx of extracellular calcium ions over the sperm plasma membrane after the opening of voltage-gated calcium channels [268]. Calcium ionophore is a substance that enables the transport of Ca^{2+} ions across the spermatozoa membrane by forming stable complexes with divalent cations [200]. Szász et al., (2000) [269] suggested that Ca^{2+}-ionophore-induced membrane changes in dog sperm cells are valuable parameters for predicting the suitability of dog ejaculates for cryopreservation. Calcium ions regulate several reproductive processes and events that are crucial for proper fertilization: spermatogenesis, testosterone secretion, sperm capacitation, hyperactivation, motility, and acrosome reaction (in review [270]). Despite reports indicating the important role of calcium ions in the regulation of reproductive events in humans, knowledge of the function of calcium in the canine reproductive tract is very limited. With the current state of knowledge, it is difficult to define calcium as a BRF in dogs. However, according to studies conducted in humans, calcium ions should not be underestimated, and further research is needed to explain the molecular basis of their action as a potential BRF in dogs.

Lipids, carbohydrates, and ions and their binding proteins that are involved in canine sperm functions are shown in Figure 4.

Table 2 contains a summary of the most promising lipid, carbohydrate, and ion molecular biomarkers of canine reproductive functions.

Table 2. Summary of the most promising non-protein molecular biomarkers of canine reproductive functions described in the review. EF—epididymal fluid; ES—epididymal spermatozoa; S—ejaculated spermatozoa; SP—seminal plasma; P—prostate.

Biomarker	Origin of Biomarker	Influence on Reproductive Functions	Implementation in Clinical/Breeding Practice	References
Lipids				
Saturated fatty acids (SFAs)				
Caprylic fatty acid	ES	- bactericidal properties - sperm membrane protection	- fatty acids supplementation for gamete manipulation techniques such as in vitro sperm maturation for immature canine spermatozoa - contraception, such as the use of inhibitors of the enzymes that are responsible for fatty acid biosynthesis, promoting lipid disruption of sperm - formulation of diluents and extenders, and improving cryopreservation protocols	[159,165,176]
Stearic fatty acid	ES, SP, S	- provides the energy for sperm motility and metabolism	as above	[159,167–171,176]
Palmitic fatty acid	SP	- positive effect on sperm production	as above	[159,172,176]
Monounsaturated fatty acids (MUFAs)				
Oleic fatty acid	SP	- regulation of sperm metabolism - positive effect on sperm motility and viability	- formulation of better diluents and extenders, and improving cryopreservation protocols	[159,174,176]
Polyunsaturated fatty acids (PUFAs)				
Docosahexaenoic acid (DHA)	ES, EF	- important for epididymal maturation - provides fluidity to the sperm plasma membrane affecting its integrity - positive effect on sperm cryotolerance	- fatty acids supplementation for gamete manipulation techniques such as in vitro sperm maturation for immature canine spermatozoa - contraception, such as the use of inhibitors of the enzymes that are responsible for fatty acid biosynthesis, promoting lipid disruption of sperm - formulation of better diluents and extenders, and improving cryopreservation protocols	[159,176,179,182]
Phospholipids				
Phosphatidylcholine Phosphatidylethanolamine	S	- sperm motility	- identification of asthenozoospermia	[164,170]

Table 2. *Cont.*

Biomarker	Origin of Biomarker	Influence on Reproductive Functions	Implementation in Clinical/Breeding Practice	References
		Plasmalogens		
Plasmenyl phosphatidylcholine (40:5) Plasmanyl phosphatidylcholine (40:4)	S	- sperm motility - antioxidant properties	- formulation of diluents and extenders, and improving cryopreservation protocols	[8,164]
		Neutral lipids		
Cholesterol	SP	- maintaining the stability and fluidity of the cell membrane - protection against sperm damage induced by freeze/thaw processes	- potential marker of canine semen crytolerance - higher concentrations of cholesterol in the SP of ejaculates with good freezability - indicator of semen quality	[25,199,201,209]
		Carbohydrates		
Heparin	oviductal and uterine fluids	- preceding and stimulation of sperm acrosome reaction in vitro - binding to SP proteins and performing different functions	- as an additive in the in vitro/capacitating medium: stimulates metabolic enzymes, prolongs sperm motility, and induces hyperactivation (even in the sperm of asthenozoospermic dogs), and has a positive effect on the acrosome reaction	[218–225]
		Ions		
Zinc	SP, P	- sperm motility regulation - binding to SP proteins and performing different functions	- high levels of free zinc in SP inhibit sperm motility, while reduced free zinc levels improve sperm motility	[250,251,258]

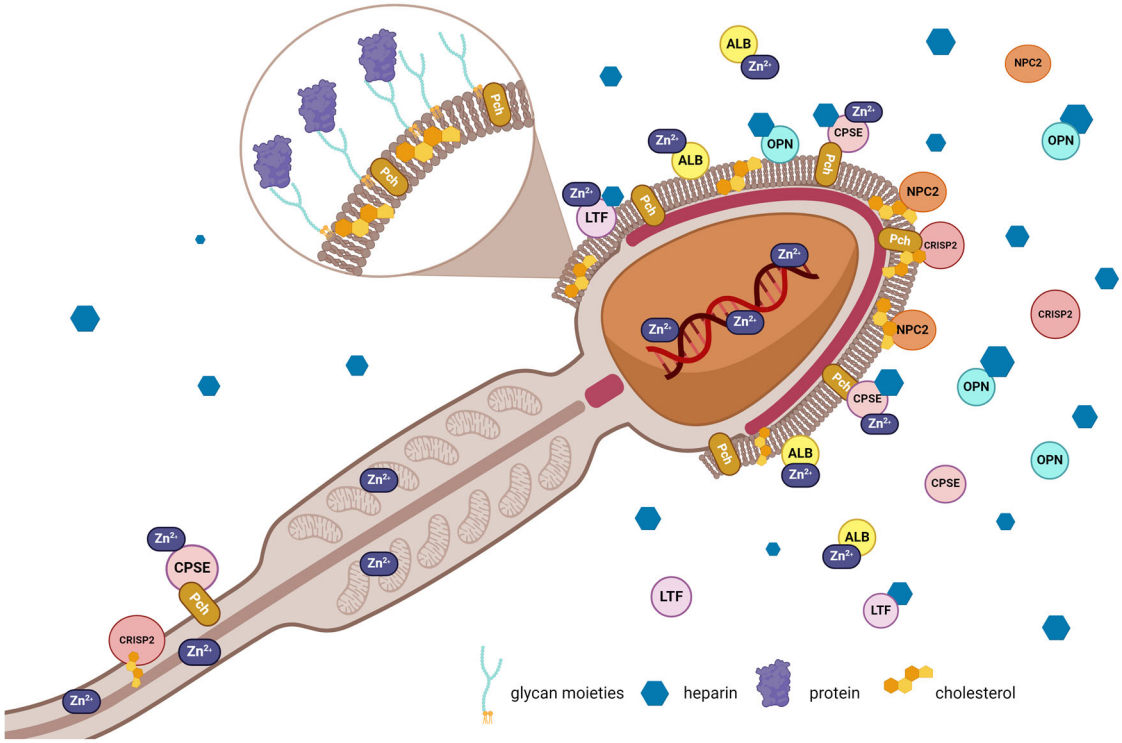


Figure 4. Lipids, carbohydrates, and ions and their binding proteins that are involved in canine sperm functions. ALB—albumin; CPSE—canine prostate-specific esterase; CRISP2—cysteine-rich secretory protein 2; LTF—lactotransferrin; NPC2—Niemann-Pick type C2 protein; OPN—osteopontin; Pch—phosphorylcholine; Zn^{2+}—zinc ions. Created with BioRender.com.

4. Differences in Biomarkers between Breeds

Purebred domestic dogs were created for backcrossing and inbreeding to determine desirable phenotypic or behavioral characteristics. These alterations may influence semen quality and impair biotechnologies of reproduction results [29]. Many canine breeds dealing with genetically specific diseases directly correlated with inbreeding in these breeds [271]. Some breeds may be more affected by reproductive impairments such as decreased semen quality or compromised fertility rates [272,273]. Araujo et al., (2022) [29] showed the influence of dog breed on the proteome of the spermatozoa and seminal plasma (for Golden Retriever, Great Dane, Bernese Mountain dog, and Maremmano-Abruzzese sheepdog). ALB was useful for the separation of the breeds based on spermatozoa proteins. Maremmano-Abruzzese Sheepdog had an increased abundance of ALB in the spermatozoa, and the Bernese Mountain dog had a low abundance of this protein. Tubulin alpha-3E

chain, ACRBP, and tubulin alpha-3 chain were considered relevant in dogs' seminal plasma. High abundances of the tubulin alpha-3E chain and the tubulin alpha-3 chain were found in the Maremmano-Abruzzese sheepdog and the Bernese Mountain dog, respectively. ACBP protein was found in higher abundance in the Bernese Mountain dog. According to the practical expertise of Araujo et al., (2022) [29], the Bernese Mountain dogs have low semen quality, which may be hypothesized as a pattern characteristic of this breed. The variations found between distinct canine breeds suggest that some cellular and molecular phenotypes associated with semen quality, intra-species cellular modulation, and motility could be breed-specific, similar to molecular markers of reproductive functions. Hallberg et al., (2024) [274] investigated the correlation between biomarkers for testicular cell function in the Bernese Mountain dog. Based on the markers discussed in our study, ALPL and CPSE levels vary between dogs based on semen quality and fertility. We found studies on the influence of breed and individual variation on the quality of frozen canine semen (Beagle, Schnauzer, Boxer, and Doberman) [275]. Based on the in vitro evaluation of the semen of different dog breeds, the quality of fresh semen does not show significant variation across the breeds studied; however, the integrity and viability of the sperm membrane and longevity of the thawed semen varies significantly both between individuals and between breeds. The canine spermatozoa cryotolerance varies based on the individual, and this variation may present some genetically inherited components that manifest as a characteristic of the seminal freezing of the breeds. These findings suggest that further studies on semen proteins that may be potential BRFs and functional investigations of breed-specific proteins in purebred dogs need to be performed to improve reproduction biotechnologies.

5. Characteristic Features of Molecular Biomarkers of Reproductive Processes

Most BRFs in dogs have certain unique characteristics. They are produced in the testicles, epididymis, or prostate, or are derived from blood and secreted into the reproductive organ fluids. These biomarkers interact with the sperm plasma membrane with the participation of proteins, lipids, carbohydrates, or ions. This interaction changes the functional characteristics of sperm. BRFs can be an integral part of sperm's structure or may only be temporarily attached and detached from it, influencing sperm physiology. These biomarkers may also have functions associated with antioxidant properties. The levels of BRFs in semen or fluids surrounding sperm can vary depending on the physiological characteristics of the cells or reproductive organs. The content of molecular biomarkers directly or indirectly affects the condition of reproductive tissues or semen quality parameters, which in turn affects reproductive success. The levels of biomarkers can be measured accurately and reproducibly.

6. Conclusions

This review systematizes current knowledge that could serve as a starting point for developing practical tests for the use of biomarkers of canine reproductive functions and their predictive value for assisted reproductive technique outcomes and semen preservation. The literature review allowed for the systematization of the features of a typical molecular marker of reproductive processes in dogs. From the entire pool of molecular markers of reproductive functions in dogs, it is possible to distinguish a pool of markers that can be used with great probability in the future to improve reproductive processes in dogs, including semen conservation. Their functions include improving sperm capacitation, the acrosomal reaction, and sperm motility. They may be potential biomarkers of prostate and testicular cancer. There are markers whose expressions were correlated with the dog's age. The addition of these biomarkers to the canine spermatozoa before or after preservation techniques preserves sperm viability and function, preventing cold shock consequences. Some described substances may be predictive biomarkers for the success of ART, such as IVF and ICSI. However, each type of marker must be treated individually in relation to these matters and this requires further in-depth research.

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Załącznik nr 4 do Załącznika nr 1
do Uchwały Nr 362
Senatu Uniwersytetu Warmińsko-Mazurskiego w Olsztynie
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wprowadzającej procedurę postępowania
w sprawie nadania stopnia doktora

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Przewodniczący Rady Naukowej Dyscypliny
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Oświadczam, że w pracy pod tytułem **Mogielnicka-Brzozowska M., Cichowska A.W. (2024). Molecular Biomarkers of Canine Reproductive Functions.** ***Current Issues in Molecular Biology*****, *46*, 6139–6168** mój wkład merytoryczny w jej przygotowanie polegał na: opracowaniu koncepcji i zakresu pracy, wyszukiwaniu i selekcji literatury naukowej, krytycznej analizie i syntezie literatury, interpretacji danych literaturowych, wizualizacji (przygotowanie tabel i rycin), przygotowaniu manuskryptu i odpowiedzi na recenzje oraz korekcie manuskryptu po recenzjach.

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do Uchwały Nr 362
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Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydatki mgr inż. Aleksandry Wiktorii Cichowskiej-Likszo polegający na opracowaniu koncepcji i zakresu pracy, wyszukiwaniu i selekcji literatury naukowej, krytycznej analizie i syntezie literatury, interpretacji danych literaturowych, wizualizacji (przygotowanie tabel i rycin), przygotowaniu manuskryptu i odpowiedzi na recenzje oraz korekcie manuskryptu po recenzjach.

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Proteome Profiling of Canine Epididymal Fluid: In Search of Protein Markers of Epididymal Sperm Motility

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Abstract: Sperm maturation in the epididymis is based on interactions with proteins from epididymal fluid (EF). The aim of the study was to profile canine EF proteome and investigate correlations between EF protein content and epididymal spermatozoa (ES) motion parameters. Twenty-three male dogs were divided into two groups: good sperm motility (GSM) and poor sperm motility (PSM). The total motility and progressive motility differed significantly ($p = 0.031$; $p < 0.001$, respectively) between the GSM group and the PSM group. The semen samples were centrifuged to separate the EF apart from the ES. The canine EF proteins were analyzed using nano-liquid chromatography, which was coupled with quadrupole time-of-flight mass spectrometry (NanoUPLC-Q-TOF/MS) and bioinformatic tools for the first time. A total of 915 proteins were identified (GSM—506; PSM—409, respectively). UniProt identification resulted in six unique proteins (UPs) in the GSM group of dogs and four UPs in the PSM group. A semi-quantitative analysis showed a higher abundance ($p < 0.05$) of four differentially expressed proteins in the GSM group (ALB, CRISP2, LCNL1, PTGDS). Motility-dependent variations were detected in the EF proteome and were related to important metabolic pathways, which might suggest that several proteins could be potential ES motility biomarkers.

Keywords: canine; epididymal fluid; epididymal spermatozoa; motility; proteome

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1. Introduction

The essential role of the epididymis in male fertility has been described extensively [1–3]. As sperm passes through the epididymis, it undergoes a maturation process that is characterized by morphological and physiological modifications necessary to obtain full capacity for fertilization. These modifications comprise changes in the biochemical composition of plasmalemma, including the addition, removal, or transformation of proteins and lipids, as well as final chromatin condensation and the acquisition of functional features, such as progressive motility [4,5]. This functional epididymal sperm (ES) maturation occurs in response to modifications in the epididymal fluid (EF) microenvironment [6]. The ES surface during transit along the epididymis is directly exposed to an epididymal milieu rich in numerous proteins, which may interact with the sperm in a specific manner [4,7]. This may have an impact on the functional features of the male reproductive cells [8]. Epididymal fluid proteins are thought to have a significant role in the subsequent steps leading to acquiring the ES functional capability for fertilization [9].

The mammalian spermatozoa leaving the testicles are immobile. In order to reach the oocyte, the sperm must be able to move progressively forward. Therefore, motility is a significant functional sperm parameter in both natural and assisted reproduction. Of all known semen quality parameters, sperm motility is considered to be a highly predictive

marker of male fertility [10]. The sperm acquire this property gradually while they pass through the epididymal tract. Considering that approximately 17.5% of couples worldwide face fertility problems [11], as well as the development of animal husbandry and the breeders' attitude towards reproductive success, it seems advisable to understand the mechanisms of sperm motility regulation by molecules present in EF.

A complete profiling of proteins present in a tissue or a cell is defined as proteomics [12]. Currently, this field of science is commonly used and has promising potential in animal reproduction research. The detection and identification of proteins found in EF may reveal unknown interactions between the components of these fluids and the sperm, which may affect the functional features of male gametes. Identification of the sperm motility-modulating proteins may be used in biomedical applications in strategies of infertility treatment, semen assessment, and conservation centers, both in animals and humans [13]. The development of a procedure for the identification of protein markers, which proves the quality of semen and its usefulness for reproduction on a dog model, would allow for the expansion of knowledge, not only in the aspect of dog breeding and preservation of endangered canine species but also in the field of human reproduction.

Taking into consideration that the epididymis provides a unique environment in which sperm acquire the fertilizing ability [7], it was decided to: (i) Provide profiling of the canine EF proteome; (ii) Investigate correlations between cauda EF proteins and selected ES motion parameters in the search for protein markers of ES motility.

2. Results

2.1. Epididymal Spermatozoa Quality Assessment

The concentration of ES in the studied groups of dogs ranged as follows: GSM—from 21.4 to 56.4 $\times 10^8$ spermatozoa/mL (34.74 $\pm$ 2.5 $\times 10^8$ spermatozoa/mL, mean $\pm$ SEM) and PSM—from 17.1 to 40.1 $\times 10^8$ spermatozoa/mL (33.17 $\pm$ 2.3 $\times 10^8$ spermatozoa/mL, mean $\pm$ SEM). There were no statistically significant differences ($p < 0.05$) between GSM and PSM groups in ES concentration.

The TMOT and PMOT differed significantly between the GSM group and PSM group ($p = 0.031$; $p < 0.001$, respectively). The obtained results showed significant differences in the VSL, VCL, ALH, and BCF values ($p = 0.030$; $p = 0.011$; $p = 0.003$; $p < 0.001$, respectively) between the studied groups. Moreover, significant differences in the percentage of STR ($p < 0.001$) and LIN ($p < 0.001$) between the studied groups of dogs were observed (Table 1). There were no statistically significant differences ($p < 0.05$) between GSM and PSM groups in VAP values or in rapid, medium, slow, or static motion parameters (Table 1).

Table 1. Average motion parameters of the ES of dogs (*Canis lupus familiaris*) divided into two groups according to values of ES progressive motility: GSM and PSM.

Sperm Parameters	Good Sperm Motility (n = 13)	Poor Sperm Motility (n = 10)	*p*-Value
Total motility (TMOT, %)	92.46 $\pm$ 0.4	87.9 $\pm$ 2.3	0.031
Progressive motility (PMOT, %)	62.08 $\pm$ 1.5	37.5 $\pm$ 5.2	0.001
Average path velocity (VAP, µm/s)	135.9 $\pm$ 3.5	127.8 $\pm$ 7.5	0.784
Straight line velocity (VSL, µm/s)	116.1 $\pm$ 3.3	99.06 $\pm$ 6.3	0.030
Curvilinear velocity (VCL, µm/s)	194.5 $\pm$ 5.2	217.3 $\pm$ 8.8	0.011
Amplitude of lateral head displacement (ALH, µm)	6.48 $\pm$ 0.2	7.68 $\pm$ 0.3	0.003
Beat cross frequency (BCF, Hz)	18.51 $\pm$ 1.0	14.0 $\pm$ 0.5	0.001
Straightness (STR, %)	84.46 $\pm$ 0.7	76.3 $\pm$ 0.8	0.001
Linearity (LIN, %)	61.92 $\pm$ 1.6	49.1 $\pm$ 1.4	0.001
Rapid (%)	82.23 $\pm$ 1.6	81.38 $\pm$ 1.94	0.744
Medium (%)	10.31 $\pm$ 1.8	8.37 $\pm$ 0.88	0.405
Slow (%)	4.31 $\pm$ 0.4	5.63 $\pm$ 0.82	0.112
Static (%)	3.15 $\pm$ 0.4	4.62 $\pm$ 0.73	0.080

Values are represented as the mean $\pm$ SEM. Differences between the GSM and PSM groups for TMOT, PMOT, STR, LIN, and rapid, medium, slow, static parameters were compared using Student's *t*-test. Differences between the GSM and PSM groups for VAP, VSL, VCL, ALH, and BCF parameters were compared using the Mann–Whitney U test. Significant differences with $p < 0.05$.

2.2. Epididymal Fluid Protein Mass Spectrometry Analysis

2.2.1. Qualitative Analysis

A total of 915 proteins were identified in both groups of dogs (GSM—506; PSM—409) by nanoUPLC-Q-TOF/MS (Supplementary Tables S1 and S2).

UniProt database-supported identification resulted in six unique proteins (UPs) that were identified in the GSM group of dogs and four UPs that were identified in the PSM group. Eleven proteins were common for GSM and PSM groups. UPs and common proteins were considered to be statistically significant (when present in 50% + 1 animals). The polypeptides, which were identified in the GSM group as UPs, were: Actin, cytoplasmic 1 (ACTB), Alkaline phosphatase (ALPL), Caspase recruitment domain family member 6 (CARD6), Clusterin (CLU), Polypeptide N-acetylgalactosaminyltransferase (GALNT6), and Olfactomedin 4 (OLFM4) (Figure 1). Four polypeptides were identified in the PSM group as UPs: Boule homolog, RNA-binding protein (BOLL), KRAB domain-containing protein (LOC606925), Cystatin domain-containing protein (LOC607874), and WAP domain-containing protein (N/A/WAPdcp) (Figure 1).

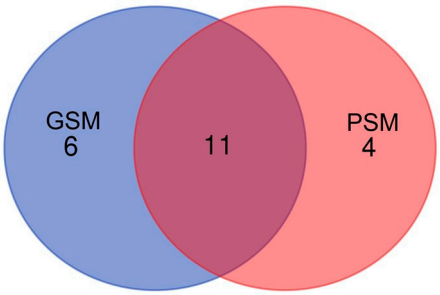


Figure 1. A Venn diagram showing the number of UPs and common proteins that were identified in the cauda EF of dogs (*Canis lupus familiaris*) divided into two groups according to values of ES progressive motility: GSM and PSM.

Protein identification resulted in 11 proteins common for both groups, GSM and PSM: Alpha-fetoprotein (AFP), Albumin (ALB), Carboxylesterase 5A (CES5A), Cysteine-rich secretory protein 2 (CRISP2), Epididymal sperm-binding protein 1 (ELSPBP1), Epididymal secretory glutathione peroxidase (GPX5), Lipocln_cytosolic_FA-bd_dom domain-containing protein (LCNL1), Lactotransferrin (LTF), NPC intracellular cholesterol transporter 2 (NPC2), Plastin 3 (PLS3), and Prostaglandin-H2 D-isomerase (PTGDS) (Figure 1).

2.2.2. Semi-Quantitative Analysis

A semi-quantitative analysis of protein abundance, based on MS intensity measurement, was also included in the study. When comparing the MS intensity of proteins common for both studied groups (GSM and PSM), statistically significant differences ($p < 0.05$) were found for four proteins. For all of the four proteins, MS intensities were higher in the GSM group (ALB: $7314 \pm 1518 \times 10^4$; CRISP2: $2235 \pm 882.9 \times 10^4$; LCNL1 $3270 \pm 1268 \times 10^4$; PTGDS: $4646 \pm 904.7 \times 10^4$; mean ± SEM) when compared with the PSM group (ALB: $3030 \pm 1594 \times 10^4$; CRISP2: $628 \pm 202.6 \times 10^4$; LCNL1: $260.8 \pm 60.65 \times 10^4$; PTGDS: $2323 \pm 280.9 \times 10^4$; mean ± SEM) (Figure 2).

No significant differences ($p < 0.05$) between GSM and PSM groups were found when comparing the MS intensity of AFP, CES5A, ELSPBP1, GPX5, LTF, NPC2, and PLS3 (Figure 2).

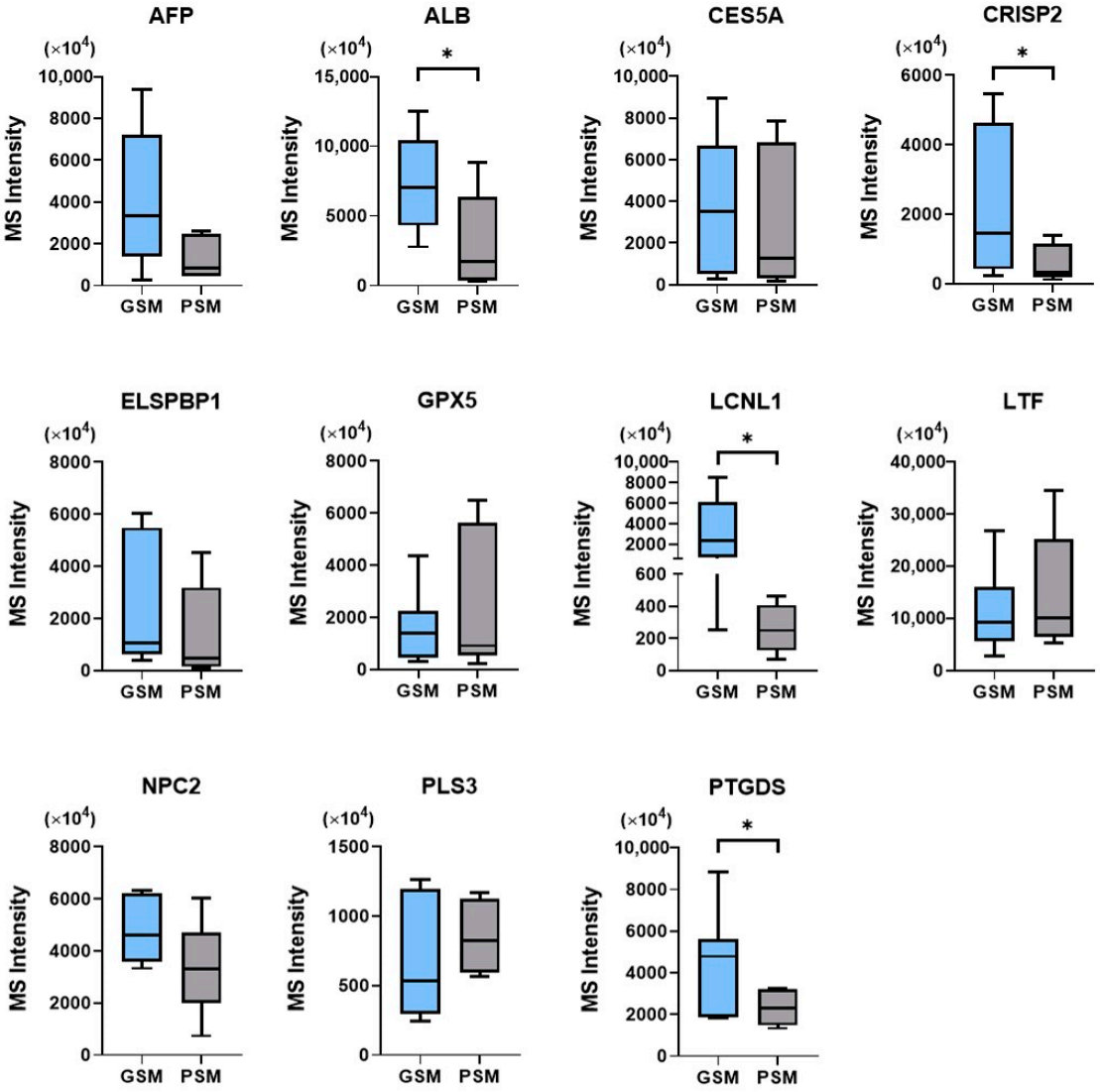


Figure 2. Box plots representing the abundance (MS intensity) distribution of common proteins in cauda EF of dogs (*Canis lupus familiaris*) divided into two groups according to values of ES progressive motility: GSM and PSM. (*) indicates significant differences between GSM and PSM groups ($p < 0.05$). AFP—Alpha-fetoprotein, ALB—Albumin, CES5A—Carboxylesterase 5A, CRISP2—Cysteine-rich secretory protein 2, ELSPBP1—Epididymal sperm-binding protein 1, GPX5—Epididymal secretory glutathione peroxidase, LCNL1—Lipocln_cytosolic_FA-bd_dom domain-containing protein, LTF—Lactotransferrin, NPC2—NPC intracellular cholesterol transporter 2, PLS3—Plastin 3, PTGDS—Prostaglandin-H2 D-isomerase.

2.3. Bioinformatic Analysis

2.3.1. Gene Ontology and ToppCluster Analysis

A GO analysis of proteins found in GSM and PSM groups showed similar main molecular functions, biological processes, and protein classes.

Based on GO enrichment, the most dominant molecular functions of the proteins in the GSM group were binding (63%) and catalytic activity (37%) (Figure 3A: MF). In addition, a GO analysis of the proteins in the PSM group showed that dominant molecular function terms were binding (75%) and catalytic activity (25%) (Figure 3B: MF). For the proteins found in the GSM group, the dominant biological processes were localization (30%), cellular process (20%), and a response to stimulus (10%) (Figure 3A: BP). For the proteins found in the PSM group, the most dominant biological process was localization (56%). In other identified biological processes (biological process involved in interspecies

interaction between organisms, cellular process, immune system process, response to stimulus), PSM group proteins had an equal share (11%) (Figure 3B: BP).

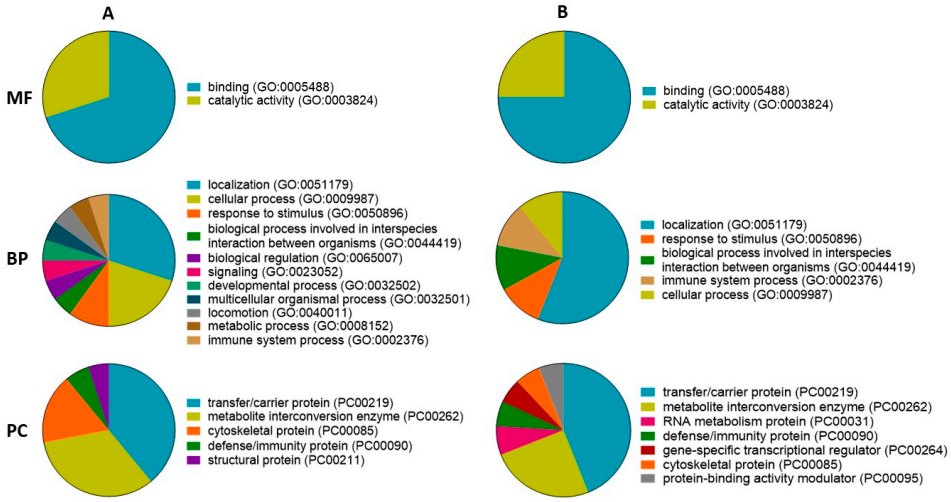


Figure 3. GO enrichment of proteins presented in cauda EF of dogs (*Canis lupus familiaris*) divided into two groups according to values of ES progressive motility: GSM (**A**) and PSM (**B**). The following GO terms were distinguished: molecular function (MF), biological process (BP), and protein class (PC).

The most dominant protein classes of proteins in the GSM group were transfer/carrier protein (39%), metabolite interconversion enzyme (33%), and cytoskeletal protein (16%) (Figure 3A: PC). Moreover, for proteins found in the PSM group, transfer/carrier protein (44%) and metabolite interconversion enzyme (25%) were the most dominant protein classes (Figure 3B: PC).

A detailed analysis of the biological categories that differ between experimental groups of dogs (GSM, PSM) and GO terms (molecular function and cellular component) was performed using the ToppCluster tool. This analysis resulted in a network representation of shared and GSM- and PSM-specific GO terms generated by comparing multiple protein lists (Figure 4).

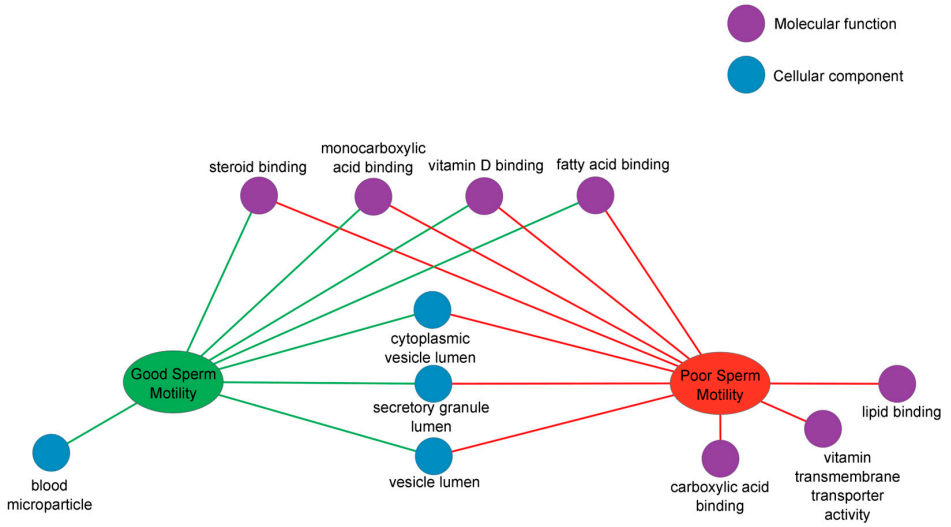


Figure 4. The ToppCluster analysis of proteins associated with the molecular function (purple circle) and cellular component (blue circle). The functional map illustrates shared and group-specific functional annotation terms generated in multicluster protein functional enrichment analysis for proteins identified in the cauda EF of dogs (*Canis lupus familiaris*) divided into two groups according to values of ES progressive motility: GSM (green ellipse) and PSM (red ellipse).

The ToppCluster analysis detailed the molecular functions previously identified during GO analysis using PANTHER. For proteins found in the PSM group, ToppCluster indicates carboxylic acid binding, lipid binding, and vitamin transmembrane transporter activity (Figure 4). Four molecular functions were common for proteins in the GSM and PSM groups: steroid binding, monocarboxylic acid binding, vitamin D binding, and fatty acid binding (Figure 4). The analysis indicated that proteins found in both GSM and PSM groups were components of cytoplasmic vesicle lumen, secretory granule lumen, and vesicle lumen (Figure 4).

2.3.2. The Kyoto Encyclopedia of Genes and Genomes Pathway Analysis

The KEGG pathway analysis showed statistically significant pathways for both GSM and PSM groups. Mainly arachidonic acid metabolism (PTGDS, GPX5) and thyroid hormone synthesis (GPX5, ALB) were significant for both GSM and PSM groups (Table 2). The Hippo signaling pathway (ACTB, AFP) and metabolic pathways (PTGDS, GALNT6, GPX5, ALPL) were characteristic for the GSM group (Table 2), while cholesterol metabolism (NPC2) and glutathione metabolism (GPX5) were specific for the PSM group (Table 2).

Table 2. The KEGG pathway analysis of the cauda EF proteins of dogs (*Canis lupus familiaris*) divided into two groups according to values of ES progressive motility: GSM and PSM ($p < 0.05$).

GSM				
ID	**Pathway Name**	**Protein Counts**	**$-Log^{10}$ (Corrected *p*-Value)**	**Protein Names**
cfa00590	Arachidonic acid metabolism	2	4.69×10^{-4}	PTGDS | GPX5
cfa04918	Thyroid hormone synthesis	2	6.47×10^{-4}	GPX5 | ALB
cfa04390	Hippo signaling pathway	2	2.79×10^{-3}	ACTB | AFP
cfa01100	Metabolic pathways	4	4.48×10^{-3}	PTGDS | GALNT6 | GPX5 | ALPL
PSM				
ID	**Pathway Name**	**Protein Counts**	**$-Log^{10}$ (Corrected *p*-Value)**	**Protein Names**
cfa00590	Arachidonic acid metabolism	2	2.16×10^{-4}	PTGDS | GPX5
cfa04918	Thyroid hormone synthesis	2	2.99×10^{-4}	GPX5 | ALB
cfa04979	Cholesterol metabolism	1	1.71×10^{-2}	NPC2
cfa00480	Glutathione metabolism	1	1.85×10^{-2}	GPX5

ACTB—Actin, cytoplasmic 1, AFP—Alpha-fetoprotein, ALB—Albumin, ALPL—Alkaline phosphatase, GALNT6—Polypeptide N-acetylgalactosaminyltransferase, GPX5—Epididymal secretory glutathione peroxidase, NPC2—NPC intracellular cholesterol transporter 2, PTGDS—Prostaglandin-H2 D-isomerase. Significant differences with $p < 0.05$.

2.3.3. Protein–Protein Interaction Network Analysis

The PPI network analysis revealed that PTGDS could interact with different proteins involved in numerous biological processes (Figure 5). A total of 31 nodes and 135 edges were identified for PTGDS, which revealed PTGDS associations with other members of prostaglandins family, but also with CRISP2, LCNL1, and SERPING1 (Figure 5).

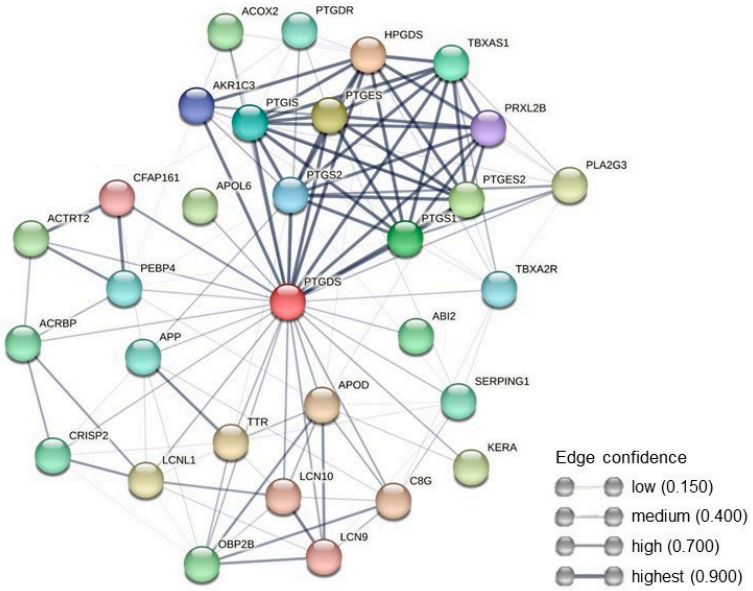


Figure 5. PPI network for Prostaglandin-H2 D-isomerase (PTGDS). The nodes represent proteins, and the edges represent the number of protein–protein interactions. The edges indicate both functional and physical protein associations. Line thickness indicates the strength of data support.

2.4. Correlations between Selected Cauda Epididymal Fluid Proteins and Epididymal Spermatozoa Motion Parameters

Spearman's rank correlation coefficient was used to present the relationships between ES motion parameters and the content (MS intensity) of individual cauda EF proteins. Figure 6A shows Spearman's rank correlation coefficients between ES motion parameters and the content of cauda EF polypeptides, which is common for both GSM and PSM groups of dogs. No correlations between cauda EF protein contents and ES motion parameters were found, except for a positive correlation between AFP content and the percentage of the slow motion parameter ($r = 0.57$; $p < 0.05$) (Figure 6A).

Spearman's rank correlation coefficients were analyzed to evaluate the relationship between the content of the EF proteins unique for the GSM group of dogs and individual ES motion parameters. Positive correlations were observed between CARD6 content and VAP ($r = 0.83$; $p < 0.05$), but also with a slow motion parameter ($r = 0.77$; $p < 0.05$) and between OLFM4 content and ALH ($r = 0.82$; $p < 0.05$) (Figure 6B). Negative correlations were found between ALPL content and STR ($r = -0.72$; $p < 0.05$), and also OLFM4 and STR ($r = -0.77$; $p < 0.05$) and LIN ($r = -0.85$; $p < 0.05$) (Figure 6B).

Spearman's rank correlation coefficients were also analyzed between the ES motion parameters and the content of the EF polypeptides unique for the PSM group of dogs. Only negative correlations were observed: between BOLL content and ALH ($r = -0.76$; $p < 0.05$), LOC607874 content and VAP ($r = -0.74$; $p < 0.05$), and VSL ($r = -0.71$; $p < 0.05$) (Figure 6C).

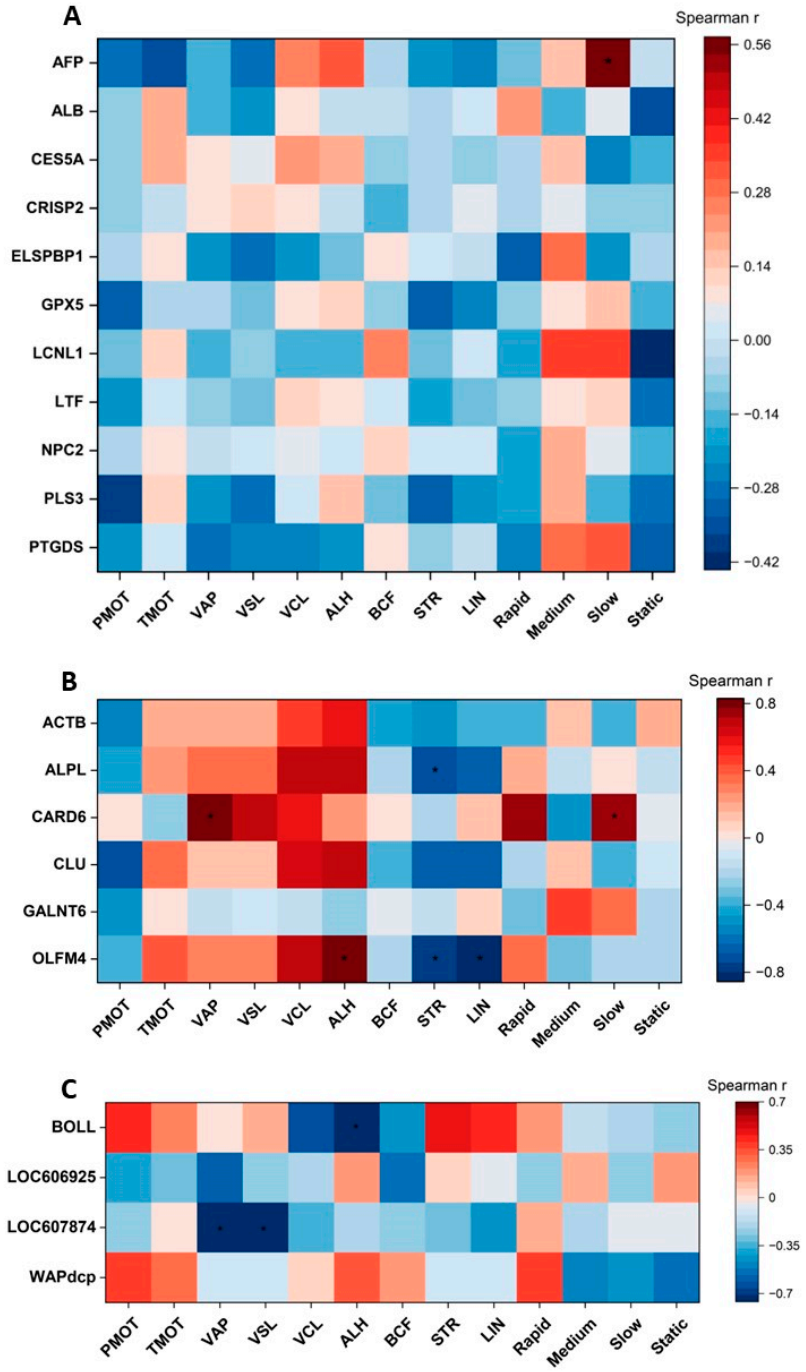


Figure 6. Heatmap showing Spearman's rank correlation coefficients between the values of the ES motion parameters and the abundance (MS intensity) of the cauda EF proteins (**A**) common for both GSM and PSM groups; (**B**) unique for GSM group; and (**C**) unique for PSM group of dogs (*Canis lupus familiaris*). (*) indicates significant correlations ($p < 0.05$). ACTB—Actin, cytoplasmic 1, AFP—Alpha-fetoprotein, ALPL—Alkaline phosphatase, ALB—Albumin, BOLL—Boule homolog, RNA binding protein, CARD6—Caspase recruitment domain family member 6, CES5A—Carboxylesterase 5A, CLU—Clusterin, CRISP2—Cysteine-rich secretory protein 2, ELSPBP1—Epididymal sperm-binding protein 1, GALNT6—Polypeptide N-acetylgalactosaminyltransferase, GPX5—Epididymal secretory glutathione peroxidase, LCNL1—Lipocln_cytosolic_FA-bd_dom domain-containing protein, LTF—Lactotransferrin, LOC606925—KRAB domain-containing protein, LOC607874—Cystatin domain-containing protein, NPC2—NPC intracellular cholesterol transporter 2, OLFM4—Olfactomedin 4, PLS3—Plastin 3, PTGDS—Prostaglandin-H2 D-isomerase, WAPdcp—WAP domain-containing protein. Motion parameter abbreviations are explained in Table 1.

2.5. Validation of Results by Western Blot

To validate and verify the abundance of proteins identified by mass spectrometry, a Western blot (WB) analysis was performed (Figure 7A,B). WB analysis showed a significantly higher abundance of ALB and PTGDS in the GSM group when compared to the PSM group (Figure 7B). WB analysis did not reveal any significant difference in CRISP2 abundance between GSM and PSM groups (Figure 7B).

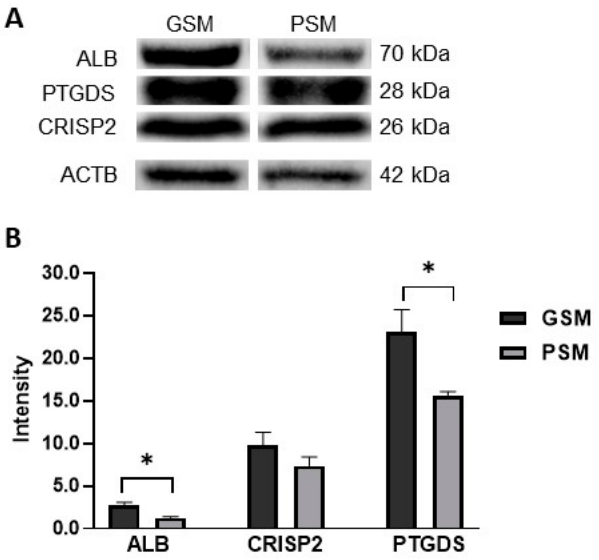


Figure 7. Western blot validation of change in abundances of cauda EF proteins of dogs (*Canis lupus familiaris*) divided into two groups according to values of ES progressive motility: GSM and PSM (**A**). Each column indicates the normalized value (mean ± SEM) of protein abundance from three replicates. Protein abundance represents band intensity, which is significantly different among the analyzed groups. (*) indicates significant differences between GSM and PSM groups at $p < 0.05$ (**B**). ALB—Albumin, ACTB—Actin, cytoplasmic 1, CRISP2—Cysteine-rich secretory protein 2, PTGDS—Prostaglandin-H2 D-isomerase.

3. Discussion

Proteins found in EF are known to exert an important influence on sperm function and metabolism [1,14–17]. Each epididymal segment is considered an individual "organ" and creates its unique microenvironment, with its own genes, regulatory proteins, and signal transduction pathways [18]. As part of the search for protein markers of epididymal semen quality, our goal was to check whether some proteins present in the dog's EF may be related to the quality of spermatozoa present in this fluid as measured by the motion parameters.

The study demonstrated that certain proteins were associated with an EF surrounding ES exhibiting good motility parameters, while another group of proteins was found to be indicative of the EF of ES with poor motility. Proteins unique to the EF of the GSM group may potentially exert a specific positive influence on sperm motion parameters, as shown in the current study. Some UPs found in the canine EF in which spermatozoa were showing poor motion parameters may exert an inhibitory effect on sperm motility. The exact mechanism of this action is specific for each protein and must be discussed separately. However, we cannot rule out that there are functional relationships between some proteins, which was shown in the PPI network.

In the study, proteome profiling of the canine EF was performed using NanoUPLC-Q-TOF/MS for the first time. There are no such reports in world literature, only EF mass spectrometry research in humans [19], boars [20], bulls [21–24], rams [25,26], and monkeys [27].

In the analyzed groups of semen differing in motility, it was found that the EF homologous with sperm of good motility possesses some UPs, such as ACTB, ALPL, CARD6, CLU, GALNT6, and OLFM4, which are important for the ES metabolism.

ACTB builds the cytoskeleton, flagellar, and acrosomal membrane of spermatozoa [28,29]. It is responsible for cell volume changes, and its role was proposed in sperm capacitation and motility [28–30]. ACTB was previously found in the canine ES, and its expression was correlated with the dog's age [31]. It seems to be a part of the ES structure, but a high abundance of it was found in the EF of the GSM group. The exact mechanism of the EF ACTB influence on ES motility needs to be evaluated in the future.

In dogs, most ALPL is produced in the epididymis [32]. Alkaline phosphatase appears in very high concentrations in canines [33] EF. It plays a role in the transport of sugars and other organic molecules across biological membranes [34]. Its activity has been demonstrated in cytoplasmic droplets, which raises the hypothesis that ALPL catalyzes dephosphorylation and transport of phosphate groups between the ES and EF. The source of the presence of this enzyme in EF could be the intense release of it from the cytoplasmic droplets during the maturation of the ES in the epididymis.

Immune response, cell proliferation, and apoptosis are controlled by proteins containing a caspase recruitment domain (CARD) [35]. CARD6 may be involved in microtubular transport mechanisms. Accordingly, proteins that interact with CARD6 may be targeted to the microtubule-organizing center, resulting in either their inactivation or translocation to a site where they can perform their functions [36]. This protein was found in the canine EF for the first time, and its function in the canine reproductive processes is unknown.

CLU is an extracellular chaperone secreted in high amounts by epididymides [37] and testes [38]. Its presence in the canine ES was established in an earlier study [31]. It participates in sperm maturation by membrane remodeling, takes part in DNA reparation, and it may inhibit cell apoptosis [39]. It could be hypothesized that CLU might be adsorbed from the EF and cover ES plasma membranes while somehow positively influencing ES motility.

Currently, GALNTL was found in male reproductive organs in rodents and cattle [40–43]. A KEGG pathway analysis indicated that GALNT6 participates in mucin-type-O-glycan biosynthesis in the canine ES [31]. GALNTL5 was localized in the head–tail coupling apparatus of cauda ES in mice [42], and the expression of GALNTL5 was positively correlated with sperm motility [44]. In this study, we showed that GALNT6 is also important for ES motility in canines.

OLFM4 is a glycoprotein for which the absence of gene expression has been shown in human prostate cancer [45,46]. Olfactomedins play essential roles in cell development and differentiation [47]. Olfactomedin-4 precursor was found in human epididymosomes [48]. The role of OLFM4 in ES physiology is unknown, along with its influence on the above-mentioned reproductive cell motility.

Summarizing, it may be hypothesized that the above-mentioned EF proteins exert their essential impact on the ES good motility by participating in sperm cell differentiation, sperm membrane remodeling, and regulating sperm motility.

In EF homologous with poor motility of ES, the following proteins were found to be unique: BOLL, LOC606925, LOC607874, and WAPdcp.

RNA-binding proteins (RBPs), including BOLL, are a group of proteins capable of regulating a plethora of cellular posttranscriptional processes, the perturbation of which leads to impaired spermatogenesis [49]. A reduced number of BOLL mRNA transcripts is associated with spermatogenic failure [50,51]. The presence of BOLL in the EF of the canine was shown for the first time in this study. It might be suggested that it was released into the EF from immature sperm or sperm with impaired spermatogenesis, resulting in reduced sperm motility.

LOC606925 is a protein containing a Krueppel-associated box (KRAB) domain. It was found in about one-third of eukaryotic Krueppel-type C2H2 zinc finger proteins (ZFPs) [52]. The members of the KRAB–ZFPs family are involved in processes including apoptosis,

cell proliferation, and tumorigenesis [52]. It has been reported that they are abundantly expressed in the testicles [53,54]. Although this protein might also be released from the ES into the EF milieu, its function and influence on sperm motility have not been determined.

LOC607874 is a cystatin-C-like protein, the content of which was associated with ageing of the canine epididymis [31]. The gene connected with this protein is specific for epididymis [55]. Considering that the presence of LOC607874 has been shown both in the canine ES of old dogs and in the EF with poor sperm motility, this protein might be considered in the future to be a poor epididymal semen quality marker.

Most regions of the canine epididymis show mRNA expression for WAPdcp [56]. Considering that it possesses serine-type endopeptidase inhibitor activity [57], it might be suggested to act as a serine-protease inhibitor specific for canine epididymis. This protein was found in the canine ES membrane [58] and in the EF, which might suggest that it was bound to the sperm plasma membrane or released from spermatozoa to the EF. WAPdcp was found in ES of very young or old dogs [31].

According to the above-mentioned information, we could hypothesize that BOLL, LOC606925, LOC607874, and WAPdcp might be released from dead or impaired spermatozoa to the EF milieu and, due to this fact, were found to be characteristic of lower-quality epididymal semen.

The study showed that 11 proteins were present in both types of dogs EF (GSM and PSM): AFP, ALB, CES5A, CRISP2, ELSPBP1, GPX5, LCNL1, LTF, NPC2, PLS3, and PTGDS. Four of them were selected based on statistical analysis, which showed content variability depending on the motility group. The content of ALB, CRISP2, LCNL1, and PTGDS was higher in the EF surrounding ES with good motility. The presence of ALB, CRISP2, and PTGDS in the canine cauda EF was confirmed by WB analysis. However, statistical differences between analyzed groups were shown only for ALB and PTGDS.

CRISP2 is a cysteine-rich secretory protein (CRISP) family member found in the authors' previous study of the canine ES [31]. CRISP2 was identified in spermatids [59,60] and in the ejaculated sperm acrosome and sperm tail [61–64]. A decrease in CRISP2 amounts in the sperm is associated with male infertility [65–67]. This fact corresponds with the authors' recent study in which this protein content in the canine ES cells was lower in very young and senile dogs [31].

LCNL1 is a member of the lipocalin family. These proteins transport or store small molecules, such as vitamins, hormones, and secondary metabolites [68]. LCN proteins are hypothesized to be important for sperm maturation [69]. LCNL1 was expressed in different regions of the epididymis [70]. Epididymal fluid LCNL1 seems to be somehow important for ES sperm motility, but the exact mechanism needs to be investigated.

Albumins are low-molecular weight proteins (about 65 kDa) [71], which are secreted by the testes, epididymides, and prostate. Studies have shown that albumins are implicated in the fertilization-associated events, such as sperm capacitation [72] and the zona pellucida penetration [73]. In one of our previous studies, we have shown that the seminal plasma albumin (ALB) is highly correlated with the motility parameters of stallion spermatozoa [74]. Moreover, albumins have been shown to regulate the number of bivalent cations and possess antioxidant properties [75,76]. It could be suggested that the binding of albumins to the ES plasma membrane might play a protective role in sperm motility [74]. It is likely that the albumins, analyzed in the current study, could have a potential effect on the ES motility by another mechanism in which zinc ions might play a significant role. It has been demonstrated that albumins have a strong affinity for zinc ions, thus reducing their concentrations in spermatozoa [74]. Such a mechanism has been shown to enhance sperm motility [74], and we suggest that a similar mechanism could be responsible for improved ES motility, as shown in the current study.

The presence of PTGDS (prostaglandin-H2 D-isomerase, also known as prostaglandin-D2-synthase, or lipocalin-type prostaglandin-D-synthase [77]) was found in the EF and seminal plasma of rams [26] and cats [78]. It is an enzyme that converts the cyclooxygenase product of prostaglandin H2 (PGH2) to prostaglandin D2 (PGD2). It binds small

non-substrate lipophilic molecules such as retinoids [79], which affect the permeability of the plasma membrane, resulting in a greater input of ions from the outside [80]. This phenomenon may be related to the regulation of access of Ca^{2+} ions to the sperm cell, which might be related to sperm motility [1,81]. Intracellular retinol content was shown to positively correlate with sperm motility in humans [82]. A positive correlation between PTGDS content in human sperm and the cells progressive motility was shown [83]. Additionally, PPI networks have revealed that PTGDS is interconnected with several other essential proteins involved in sperm metabolism such as CRISP2, LCNL1, and SERPING1. However, the exact function of PTGDS in ES metabolism is unknown and requires further study.

A broad-range proteomic study revealed that ALB and PTGDS present with greater abundance in EF surrounding sperm with good motility, with high probability are important for the good ES motility.

As immature spermatozoa pass through the epididymis tracts, there is a process of mixing between the EF and the ES, allowing for contact between the substances present in them. The influence of the analyzed proteins on ES mobility should be considered in two aspects. Firstly, the degree of release of proteins from the ES during their maturation or from damaged or immature spermatozoa is important. Secondly, the ES motility might be influenced by secretory proteins produced by the epididymal tissue, which will coat ES and provide changes in the ES surface properties, biochemistry, and metabolism. This metabolically complex environment of the sperm functioning in the epididymis is still a mystery to scientists.

4. Materials and Methods

4.1. Chemicals and Media

All chemicals of the highest purity were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

4.2. Animals

Twenty-three mixed-breed dogs aged from 12 to 132 months (average 72 months) were used in the study. The weight of the dogs was from 9 to 30 kg (mean 19.5 kg). The dogs were kept in the Shelter for Homeless Animals in Tomaryny (Poland) in the same environmental conditions and were fed the standard diet. The dogs were in a program to prevent animal homelessness and promote adoption and were presented for a routine orchiectomy by a qualified veterinary doctor.

4.3. Cauda Epididymal Semen Collection

The experimental dogs' testicles, with the epididymis after removal, were put in sterile plastic bags in a solution of 0.9% NaCl and then placed in a thermobox at a temperature of 4 °C. The tissues were transported within 1 h to the laboratory of the Department of Animal Biochemistry and Biotechnology (University of Warmia and Mazury in Olsztyn, Poland). The gonads were carefully washed with DPBS (Dulbecco's Phosphate-Buffered Saline, Gibco, Grand Island, NY, USA). A sterile scalpel was used to carefully remove the cauda epididymal tissue from the testicle to avoid damage to the blood vessels. An automatic pipette was used to aspirate the effluent of the epididymal semen, according to Ramos Angrimani et al. (2017) [84], with modification. The samples obtained from the right and left epididymis of the same animal were pooled. Following analyses were performed separately for each dog cauda epididymal semen: cauda epididymal spermatozoa quality assessment, cauda epididymal fluid isolation, preliminary sample preparation for proteomic analysis, and nanoUPLC-Q-TOF/MS analysis.

4.4. Cauda Epididymal Spermatozoa Quality Assessment

The ES concentration was determined using a Bürker chamber under a light microscope (Olympus BX41TF, Tokyo, Japan).

A computer-assisted semen analysis (CASA-system, HTM-IVOS 12.3, Hamilton Thorne Biosciences, MA, USA) was used to assess the ES motion parameters. The software settings were described in an earlier study [58]. The procedure was described previously by Mogielnicka-Brzozowska et al. (2020) [78]. The following sperm motion parameters were analyzed in each epididymal semen sample: total motility (TMOT, %), progressive motility (PMOT, %), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), the amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity coefficient (LIN, %).

After an ES motion parameter assessment, the sperm samples were divided into two experimental groups: good sperm motility (GSM) (n = 13) and poor sperm motility (PSM) (n = 10). The GSM group included the sperm samples showing PMOT $\geq$ 55%, and the PSM group included the sperm samples with PMOT < 55%. Supplementary Table S3 shows the age of individual dogs and sperm count.

4.5. Cauda Epididymal Fluid Isolation

The epididymal semen samples were centrifuged at 800× *g* for 10 min at 4 °C to separate EF from the ES [84]. The supernatants (EF) were immediately collected from above the pellets (ES) and transferred to new Eppendorf tubes and then centrifuged again at 15,000× *g* for 10 min [19]. Then, EF samples were stored for two weeks at −80 °C for further LC-MS analysis.

4.6. Preliminary Sample Preparation for Proteomic Analysis

4.6.1. Total Protein Content Measurement

The total protein content in EF was measured using Bradford Reagent (Sigma-Aldrich/B6916).

4.6.2. Sample Preparation for NanoUPLC-Q-TOF/MS Analysis

In order to obtain lipid-free protein extracts, frozen samples were thawed in pre-cooled to −18 °C 800 μL of MTBE:MeOH mixture (3:1). Next, the samples were sonicated in an ice-water-filled sonic bath for 15 min. To pellet the proteins, 400 μL of water:MeOH mixture (3:1) were added to each tube. Next, the Eppendorf tubes were placed in a pre-cooled centrifuge (4 °C) and spun for 7 min at 21,000× *g*. The supernatants were transferred to new tubes and pellets were frozen and kept in −80 °C prior to further analysis.

4.6.3. In-Solution Trypsin Digestion

In-solution protein digestion was processed according to the protocol described by Morelle and Michalski (2007) [85] with some modifications. Protein precipitates were dissolved in 100 μL of 50 mM NH_4HCO_3 (pH 8.0) with 0.1% SDS. Freshly prepared DTT solution was added so that the final concentration of DTT was 20 mM, and the sample was incubated at 60 °C for 1 h. The freshly prepared iodoacetamide solution was then added so that the final concentration was 20 mM, and the sample was incubated for 1 h in the dark. The sample was diluted twice, and 5 μg of trypsin were added. The sample was then left overnight with gentle shaking at 37 °C for complete digestion. After trypsinolysis, 1% formic acid was added to adjust the pH to 3–4, and the sample was desalted on Pierce™ C18 columns (Thermo Fisher Scientific, Waltham, MA, USA), then vacuum dried and resuspended in acetonitrile for further LC-MS analysis.

4.7. NanoUPLC-Q-TOF/MS Analysis

To separate the digested samples, a Waters Acquity liquid chromatography M-Class system (Waters Corp., Milford, MA, USA) equipped with a Peptide BEH C18 analytical column (150 mm × 75 μm; 1.7 μm, Waters Corp.) and a Symmetry C18 precolumn (180 μm × 20 mm; 1.7 μm, Waters Corp.) was performed. Each sample was injected into the pre-column and washed with 99% solvent A (0.1% formic acid in water). Then, peptides were transferred to an analytical column. A mass spectrometry (MS) using Synapt

G2-Si (Waters Corp.) with a nano-electrospray ionization (nESI) source operating under a positive ion mode was performed. Raw chromatography files were analyzed with Byonic software (Protein Metrics, Cupertino, CA, USA). The detected peptides were compared to the SWISSPROT dog proteome (CANLF) downloaded in February 2022. The total intensity was a sum of all the peak intensities over all MS/MS spectra. The protein *p*-value is the likelihood of the peptide-spectrum matches (PSMs) to this protein (or protein group) arising by random chance, according to a simple probabilistic model. A log *p*-value of −3.0 corresponds to a protein *p*-value of 0.001, or one chance in a thousand. A detailed method was described in our previous study [31].

4.8. Bioinformatic Analyses and Imaging of Data

A Venn diagram was constructed using a web tool (http://bioinformatics.psb.uugent.be/webtools/Venn, accessed on 13 October 2022).

The functional enrichment of proteins presented in the dog (*Canis lupus familiaris*) cauda EF were analyzed in both groups (GSM, PSM) in gene ontology (GO) categories: molecular function (MF), biological process (BP), and protein class (PC), with the PANTHER Classification System v. 17.0 online tool (http://pantherdb.org, accessed on 20 October 2022).

ToppCluster software [86] was used to identify the following GO categories: molecular function (MF) and cellular component (CC) enriched by proteins identified in the dog (*Canis lupus familiaris*) cauda EF analyzed in groups (GSM, PSM). Results were summarized in a tabular format and visualized as a relationship network using Cytoscape 3.9.1. [87].

Protein–protein interactions (PPI) were obtained and visualized with the use of the STRING v. 11.5 database (http://string-db.org, accessed on 18 October 2022) [88].

The significance of the dog (*Canis lupus familiaris*) cauda EF proteins was analyzed in groups (GSM, PSM) in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database and the KOBAS 3.0 (http://kobas.cbi.pku.edu, accessed on 6 December 2022) protein functional annotation tool [89]. Annotations were performed with the Canis familiaris database, using the false discovery rate (FDR) of the Benjamini–Hochberg method for the significance threshold. The used threshold was 0.05.

Box plots and GO plots were performed using GraphPad PRISM v. 8.0.1 software (GraphPad Software, San Diego, CA, USA). Heatmaps were performed using OriginPro 2023 (OriginLab Corporation, Northampton, MA, USA).

4.9. Western Blot Analysis

Epididymal fluid samples containing 50 µg of protein were analyzed using 12% SDS-PAGE and then transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Electroblotting was carried out for 1 h at 300 mA, according to the previously described method [90].

The 5% non-fat milk in Tris-buffered saline, TBS (1 M Tris, 5 M NaCl, pH 8.0), containing 0.05% (*v*/*v*) Tween 20, TBST (MP Biomedicals LLC, Santa-Ana, CA, USA) was used for blocking non-specific binding sites. Next, the membranes were incubated with one of the following primary antibodies obtained from Thermo Fisher Scientific: Albumin rabbit polyclonal antibody (1:1000; catalog number PA5-89332;), CRISP2 rabbit polyclonal antibody (1:1000; catalog number PA5-97621), PTGDS rabbit polyclonal antibody (1:1000; catalog number PA1-48023), except Anti-beta Actin antibody (1:2000; catalog number ab8227; Abcam, Cambridge, Great Britain). Despite lengthy searches and correspondence with many manufacturers and suppliers, obtaining an antibody for LCNL1 proved impossible. Primary antibodies were incubated overnight at 4 °C. The membranes were then washed and incubated for 1 h at room temperature with Peroxidase AffiniPure Goat Anti-Rabbit secondary antibody (1:20,000; catalog number 111-035-003; Jackson ImmunoResearch, Baltimore Pike, PA, USA). The ServaLight CL EOS Substrate kit (Serva, Heidelberg, Germany) was used to induce chemiluminescence, and the membranes were then scanned with the ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

The molecular weight standard (PageRuler™ Prestained Protein Ladder, catalog number 26617, Thermo Fisher Scientific) was used to determine the molecular weights of the proteins. The membranes were scanned, and the intensity of the protein bands was quantified using the Image Lab version 5.2 (Bio-Rad Laboratories). The signal was normalized to a stable beta-Actin protein band. Raw Western blot images were attached to Supplementary Materials as Supplementary Figure S1.

4.10. Statistical Analyses

GraphPad PRISM v. 8.0.1 software (GraphPad Software) was used to perform statistical analyses. Epididymal sperm motion parameters were compared between GSM and PSM groups of dogs through a parametric Student's *t*-test, and a non-parametric Mann–Whitney U test was used when data did not meet normality assumptions. Student's *t*-test and the Mann–Whitney U test were also used to compare the abundance of proteins common for GSM and PSM groups of dogs. Spearman's rank coefficient was used to determine the correlations between the relative abundance (MS intensity) of cauda EF proteins and the values of cauda ES motion parameters in dogs. The values were considered to differ significantly at $p < 0.05$.

5. Conclusions

Differences in individual protein expression profiles in the canine EF were established and shown to be associated with ES motility parameters. Complete EF proteome profiling could reveal basic knowledge about protein composition and provide essential information of individual protein participation in mechanisms regulating sperm motility. An understanding of the involvement of particular proteins in the sperm functional properties acquisition crucial for the fertilization process can help pinpoint the causes of male infertility and might be used for the construction of commercial tests suitable for semen quality assessment.

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PUBLIKACJA 2.

OŚWIADCZENIA KANDYDATKI I WSPÓŁAUTORÓW

Załącznik nr 4 do Załącznika nr 1
do Uchwały Nr 362
Senatu Uniwersytetu Warmińsko-Mazurskiego w Olsztynie
z dnia 27 czerwca 2023 roku
wprowadzającej procedurę postępowania
w sprawie nadania stopnia doktora

Olsztyn, 06.05.2026 r.
(miejscowość, data)

Aleksandra Wiktoria Cichowska-Likszo
(imię i nazwisko kandydata)
Zootechnika i rybactwo
(dyscyplina naukowa)

Przewodniczący Rady Naukowej Dyscypliny
Zootechnika i Rybactwo
Uniwersytetu Warmińsko-Mazurskiego w Olsztynie

OŚWIADCZENIE
autora rozprawy doktorskiej

Oświadczam, że w pracy pod tytułem **Cichowska A.W., Wisniewski J., Bromke M.A., Olejnik B., Mogielnicka-Brzozowska M. (2023). Proteome Profiling of Canine Epididymal Fluid: In Search of Protein Markers of Epididymal Sperm Motility, *International Journal of Molecular Sciences*, 24, 14790** mój wkład merytoryczny w jej przygotowanie polegał na: opracowaniu koncepcji i doborze metodologii badań, wykonaniu analiz laboratoryjnych (w tym udziale w analizach NanoUPLC-Q-TOF/MS), walidacji i analizie formalnej danych, opracowaniu bioinformatycznym i statystycznym, interpretacji oraz wizualizacji wyników, przygotowaniu manuskryptu i odpowiedzi na recenzje oraz korekcie manuskryptu po recenzjach.

Cichowska-Likszo A.
(podpis kandydata)

Załącznik nr 5 do Załącznika nr 1
do Uchwały Nr 362
Senatu Uniwersytetu Warmińsko-Mazurskiego w Olsztynie
z dnia 27 czerwca 2023 roku
wprowadzającej procedurę postępowania
w sprawie nadania stopnia doktora

Wrocław, 06.05.2026 r.
(miejscowość, data)

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OŚWIADCZENIE
współautora

Oświadczam, że w pracy pod tytułem **Cichowska A.W., Wisniewski J., Bromke M.A., Olejnik B., Mogielnicka-Brzozowska M. (2023). Proteome Profiling of Canine Epididymal Fluid: In Search of Protein Markers of Epididymal Sperm Motility, *International Journal of Molecular Sciences*, 24, 14790** mój wkład merytoryczny w jej przygotowanie polegał na: pomocy w doborze metodologii i adaptacji oprogramowania do analizy surowych danych po spektrometrii mas, wykonaniu analiz oraz zapewnieniu odczynników i asortymentu laboratoryjnego do NanoUPLC-Q-TOF/MS.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr inż. Aleksandrę Wiktorię Cichowską-Likszo jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydatki mgr inż. Aleksandry Wiktorii Cichowskiej-Likszo polegający na opracowaniu koncepcji i doborze metodologii badań, wykonaniu analiz laboratoryjnych (w tym udziale w analizach NanoUPLC-Q-TOF/MS), walidacji i analizie formalnej danych, opracowaniu bioinformatycznym i statystycznym, interpretacji oraz wizualizacji wyników, przygotowaniu manuskryptu i odpowiedzi na recenzje oraz korekcie manuskryptu po recenzjach.

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podpis

Załącznik nr 5 do Załącznika nr 1
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Senatu Uniwersytetu Warmińsko-Mazurskiego w Olsztynie
z dnia 27 czerwca 2023 roku
wprowadzającej procedurę postępowania
w sprawie nadania stopnia doktora

Wrocław, 06.05.2026 r.
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Uniwersytet Medyczny we Wrocławiu
Katedra ... Biochemii
dr ... Bromke

.....
podpis

Załącznik nr 5 do Załącznika nr 1
do Uchwały Nr 362
Senatu Uniwersytetu Warmińsko-Mazurskiego w Olsztynie
z dnia 27 czerwca 2023 roku
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w sprawie nadania stopnia doktora

Wrocław, 06.05.2026 r.
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OŚWIADCZENIE
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Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr inż. Aleksandrę Wiktorię Cichowską-Likszo jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

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Beata Olejnik
.....
podpis

Załącznik nr 5 do Załącznika nr 1
do Uchwały Nr 362
Senatu Uniwersytetu Warmińsko-Mazurskiego w Olsztynie
z dnia 27 czerwca 2023 roku
wprowadzającej procedurę postępowania
w sprawie nadania stopnia doktora

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OŚWIADCZENIE
współautora

Oświadczam, że w pracy pod tytułem **Cichowska A.W., Wisniewski J., Bromke M.A., Olejnik B., Mogielnicka-Brzozowska M. (2023). Proteome Profiling of Canine Epididymal Fluid: In Search of Protein Markers of Epididymal Sperm Motility, *International Journal of Molecular Sciences*, 24, 14790** mój wkład merytoryczny w jej przygotowanie polegał na: udziale w opracowaniu koncepcji i doborze metodologii badań, zapewnieniu dostępu do materiału badawczego, odczynników i właściwej aparatury pomiarowej, monitorowaniu przebiegu prac badawczych, kontroli jakości danych badawczych, zabezpieczeniu środków finansowych na wykonanie badań i publikację artykułu w czasopiśmie naukowym, udziale w procesie redakcyjnym – korekcie manuskryptu i udzielaniu odpowiedzi na recenzje.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr inż. Aleksandrę Wiktorię Cichowską-Likszo jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

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Mogiel
.....
podpis

PUBLIKACJA 3.

Cichowska-Likszo, A.W., Likszo, P., Fraser, L., Mlynarz, P., Mogielnicka-Brzozowska, M. (2026). ALB, GPX5 and PTGDS as potential biomarkers for the aging processes of the dog epididymis. *Reproduction, Fertility and Development*, *38*, RD25105. https://doi.org/10.1071/RD25105.

RESEARCH ARTICLE
https://doi.org/10.1071/RD25105

Reproduction, Fertility and Development

ALB, GPX5 and PTGDS as potential biomarkers for the aging processes of the dog epididymis

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ABSTRACT

Context. Alterations in the reproductive tract related to aging processes are reflected by changes in biochemical components such as proteins. **Aims.** This study aimed (1) to investigate changes in canine epididymal fluid protein abundance in relation to age, and (2) to select potential protein markers of canine reproductive tract aging. **Methods.** Twenty-three mixed-breed dogs were used in the study. The dogs were divided into the following three experimental groups: ExG1 (1–3 years old); ExG2 (3.5–6 years old); and ExG3 (6.5–11 years old). Cauda epididymal fluid was collected from dissected tissues and centrifuged to separate the epididymal fluid from the epididymal sperm. Chromatography coupled with mass spectrometry (nanoUPLC-Q-TOF/MS) was used to analyze the proteins in the epididymal fluid. **Key results.** The proteomic profiling uncovered 121, 122, and 56 unique proteins in ExG1, ExG2, and ExG3 respectively, with 48 proteins being common across all groups. Gene ontology analysis showed similar main molecular functions for all three ExGs; however, the percentage of particular proteins in each ExG differed. There were three proteins that showed significant ($P < 0.05$) changes in relative abundance among the ExGs, including albumin (ALB), epididymal secretory glutathione peroxidase (GPX5), and prostaglandin-H2 D-isomerase (PTGDS). **Conclusions.** The findings suggest that aging in dogs may influence epididymal fluid protein composition, particularly affecting ALB, GPX5, and PTGDS, which could play roles in oxidative and ion metabolic regulation. **Implications.** These findings suggest that ALB, GPX5, and PTGDS may serve as biomarkers for epididymal aging. Their detection could enhance the evaluation of epididymal semen quality, aiding reproductive medicine practices in dogs of different ages.

Keywords: aging, biomarkers, canine, epididymal fluid, epididymis, mass spectrometry, proteins, proteome.

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Introduction

Senescence is defined as the natural, dynamic, progressive and irreversible process that is dependent on multiple physiological and molecular factors (Frungieri *et al.* 2018). The reproductive system is also affected by aging processes. Changes in reproductive function associated with aging are well-known in humans (Gunes *et al.* 2016), but less is known about the aging processes in animals. In the male reproductive tract, age-dependent changes have been mostly examined for sperm. Studies have shown that semen characteristics, both epididymal and ejaculated, change with age in stallions (Greiser *et al.* 2020), sheep (Lymberopoulos *et al.* 2010), dogs (Bhanmeechao *et al.* 2018; Zmudzinska *et al.* 2022), and boars (Tsakmakidis *et al.* 2012; Czubaszek *et al.* 2020; Hensel *et al.* 2023). The milieu surrounding spermatozoa plays an essential role in maintaining the reproductive ability of the cells (Robaire *et al.* 2006; Dacheux and Dacheux 2014; De Souza *et al.* 2017; Gervasi and Visconti 2017; Björkgren and Sipilä 2019; James *et al.* 2020), highlighting the importance of measuring changes in the biochemical composition of this fluid during the aging process. Epididymal fluid is certainly the most complex, continuously modified milieu of the body (Dacheux and Dacheux 2014). The biochemical composition of the epididymal fluid is the consequence of the absorptive and secretory

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activities of the epididymal epithelium (Dacheux and Dacheux 2002), which is characterized by high protein synthesis and secretion activity (Dacheux *et al.* 2009). These epididymal proteins come into contact with the sperm and might influence the functional features of the reproductive cells. To establish the protein composition of such a complex epididymal microenvironment, large-scale proteomic approaches must be applied. Advanced mass spectrometry associated with detailed bioinformatic analyses could provide new knowledge about the potential mechanisms of individual protein action and their role in the aging of the epididymis. This approach may allow the identification of individual proteins as biomarkers of epididymal senescence.

Despite the documented impact of aging processes on the dog epididymal spermatozoa (Bhanmeechao *et al.* 2018; Zmudzinska *et al.* 2022), elucidation of the changes in the protein composition of the epididymal fluid surrounding the epididymal sperm during sexual maturation and senescence and their possible impact on the epididymal sperm is highly important.

Considering that senescence processes are also reflected in changes in the functioning of the male reproductive system, including the epididymis, we hypothesized that the composition of the epididymal fluid proteome would differ depending on the dog's age and that it would be possible to select the exact proteins the abundance of which would correlate with the age of the individual. The aims of this study were (1) to investigate changes in canine epididymal fluid protein abundance in relation to age, and (2) to select potential protein biomarkers of canine reproductive tract aging.

Materials and methods

Reagents

Unless otherwise stated, the reagents used in the study were purchased from the Sigma Chemical Company (St Louis, MO, USA).

Animals and cauda epididymal semen collection

The study involved 23 mixed-breed dogs grouped into three experimental groups (ExGs) according to the study of Ortega-Pacheco *et al.* (2006), with modifications. These groups including the following: young dogs, aged 1–3 years old (2.19 ± 0.27, mean ± s.e.m.), $n = 8$ (ExG1); adult dogs, aged 3.5–6 years old (5.31 ± 0.31), $n = 8$ (ExG2); and old dogs, aged 6.5–11 years old (8.64 ± 0.68), $n = 7$ (ExG3). The dog weights in ExGs were as follows: ExG1, 9.0–25.3 kg (15.23 ± 1.70, mean ± s.e.m.); ExG2, 14.3–29.0 kg (19.98 ± 2.20); and ExG3, 18.1–30.0 kg (25.13 ± 1.79). All dogs were housed under the same environmental conditions at the shelter for homeless animals in Tomaryny, Poland, and were provided a standard diet. The dogs were routinely castrated by a licensed veterinary surgeon.

After orchiectomy, the experimental-dog testes, along with the epididymis, were placed in sterile plastic bags containing 0.9% NaCl solution and the bags were placed in a thermobox at a temperature of 4°C. The gonads were transported within an hour to the laboratory of the Department of Animal Biochemistry and Biotechnology, University of Warmia and Mazury in Olsztyn, Poland. The tissues were rinsed with Dulbecco's phosphate-buffered saline (DPBS, Gibco, Grand Island, NY, USA). The cauda epididymis was separated from the testis with a sterile scalpel. Cauda epididymal semen was aspirated from the tissue with an automatic pipette, following the protocol of Ramos Angrimani *et al.* (2017), with modifications. Samples from the right and left epididymides of each animal were combined.

Motility and motion parameters of the epididymal spermatozoa

The parameters of epididymal sperm motion were assessed using computer-assisted semen analysis (CASA-system, HTM-IVOS 12.3, Hamilton Thorne Biosciences, MA, USA). The procedure and software settings have been described previously (Mogielnicka-Brzozowska *et al.* 2020; Zmudzinska *et al.* 2022). For each epididymal sperm sample, the following sperm motion parameters were assessed: total motility (TMOT, %), progressive motility (PMOT, %), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), the amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity coefficient (LIN, %). The subpopulations of spermatozoa showing rapid (RAPID, %), medium (MEDIUM, %), slow (SLOW, %) movement, and also static (STATIC, %) cells were distinguished in the analysis.

Initial semen processing: acquisition of cauda epididymal fluid

To separate the epididymal fluid from the epididymal sperm, the epididymal semen samples were centrifuged at 800*g* for 10 min at 4°C (Ramos Angrimani *et al.* 2017). The supernatants (epididymal fluid) were immediately collected from above the sperm pellets, transferred to new Eppendorf tubes, and then centrifuged again at 15,000*g* for 10 min at 4°C (Dacheux *et al.* 2006). Protease and phosphatase inhibitor cocktail was added to each sample before storage at −80°C for further analysis.

The cauda epididymal fluid preparation for proteomic analysis

The detailed procedure used for cauda epididymal fluid preparation and the protocol used for in-solution trypsin digestion have been described previously (Cichowska *et al.* 2023).

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The Bradford reagent was used to determine the total protein content in the epididymal fluid samples. Frozen epididymal fluid samples were thawed in 800 μL of an methyl tert-butyl ether–methanol mixture (3:1) precooled to −18°C. The samples were then sonicated in an ice-water bath for 15 min. To precipitate the proteins, 400 μL of a water–methanol mixture (3:1) was added to each sample. The samples were subsequently centrifuged at 21,000*g* for 7 min at 4°C. The obtained supernatants were transferred to new tubes, and the pellets were frozen and stored at −80°C for further lipidomic analysis. In-solution protein digestion followed the method outlined by Morelle and Michalski (2007), with minor adjustments (according to the section 'PROCEDURE', point 'B In-solution declygosylation after reduction and alkylation of glycoproteins'). In brief, protein precipitates were dissolved in 100 μL of 50 mM NH_4HCO_3 (pH 8.0) with 0.1% sodium dodecyl suflate (SDS). Dithiothreitol (DTT) solution was added (final concentration of DTT was 20 mM), and the samples were incubated at 60°C for 1 h. Next, iodoacetamide solution was added (final concentration was 20 mM), and the samples were incubated for 1 h in the dark. The samples were diluted twice, and digested overnight at 37°C with 5 μg of trypsin under gentle shaking. Then, 1% formic acid was added (to final pH of 3–4), and the samples were desalted on Pierce™ C18 columns (Thermo Fisher Scientific, Waltham, MA, USA), vacuum dried and resuspended in acetonitrile for further liquid chromatography–mass spectrometry (LC–MS) analysis.

Protein identification and relative quantification by nano ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (nanoUPLC-Q-TOF/MS)

The digested samples were separated using a Waters Acquity liquid chromatography M-Class system (Waters Corporation, Milford, MA, USA), which was equipped with a Peptide BEH C18 analytical column (150 mm × 75 μm; 1.7 μm, Waters Corporation) and a Symmetry C18 precolumn (180 μm × 20 mm; 1.7 μm, Waters Corporation). Mass spectrometry analysis was performed using Synapt G2-Si (Waters Corporation) with a nanoelectrospray ionization source operating in positive ion mode. The detailed procedure has been described in our earlier study (Zmudzinska *et al.* 2022).

The raw post-LC–MS data were analyzed using Byonic software (ver. 4.4, Protein Metrics, Cupertino, CA, USA). The settings for peak picking and identification have been previously described in detail (Zmudzinska *et al.* 2022). The discovered peptides were matched against the SWISSPROT dog proteome (CANLF), which was downloaded in February 2022. To reduce the risk of false identifications, comparisons were made with common contaminants and decoys generated through reverse amino acid sequencing *in silico*–cleavage peptide models. The total intensity was calculated by summing all peak intensities across all MS/MS spectra. The protein *P*-value indicates the probability that the peptide spectrum matches for a specific protein (or protein group) occurred by random chance, on the basis of a simple probabilistic model. A log *P*-value of −3.0 corresponds to a protein *P*-value of 0.001, indicating a one in a thousand likelihood of such a match occurring by chance.

Validation of abundances of selected proteins by western blot technique

Western blotting (WB) analysis was performed as previously described (Towbin *et al.* 1979; Cichowska *et al.* 2023). In brief, epididymal fluid samples containing equal amounts of protein (50 μg) were loaded onto 12% SDS polyacrylamide gels and electrophoresed. The separated proteins were subsequently transferred from the gels to Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked, and then incubated overnight at 4°C with one of the following primary antibodies: albumin rabbit polyclonal antibody (1:1000; Catalog number PA5-89332, Thermo Fisher Scientific, Waltham, MA, USA), GPX5 rabbit polyclonal antibody (1:500; Catalog number PA5-102342, Thermo Fisher Scientific), PTGDS rabbit polyclonal antibody (1:1000; Catalog number PA1-48023, Thermo Fisher Scientific), and anti-beta actin antibody (1:2000; Catalog number ab8227; Abcam, Cambridge, UK). The membranes were subsequently washed and incubated for 1 h at room temperature with peroxidase AffiniPure goat anti-rabbit secondary antibody (1:20,000; Catalog number 111-035-003; Jackson Immuno Research, Baltimore Pike, PA, USA). Chemiluminescence was generated by using a ServaLight CL EOS substrate kit (Serva, Heidelberg, Germany). The membranes were then scanned with the ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, Hercules, California, USA) and quantified using the Image Lab (ver. 5.2, Bio-Rad Laboratories). The signal was normalized to a stable beta-actin protein band. A molecular weight standard (PageRuler™ Prestained Protein Ladder, Catalog number 26617, Thermo Fisher Scientific) was used to assess the molecular weights of the proteins.

Proteomic data interpretation and visualization

Venn diagrams were used to identify the number of proteins common and unique for each ExG (https://www.bioinformatics.org/gvenn, accessed 19 November 2024). The PANTHER Classification System (ver. 18.0, online tool, http://pantherdb.org, accessed 24 June 2024) was used to analyze the functional enrichment of the proteins present in the dog (*Canis lupus familiaris*) cauda epididymal fluid divided into three ExGs in gene ontology (GO) categories: molecular function (MF) and biological process (BP). STRING (ver. 12.0, http://string-db.org, accessed 10 February 2025) (Szklarczyk *et al.* 2023) was used to obtain and visualize protein–protein interactions.

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Box plots, GO plots, heatmaps and western blot plots were generated using GraphPad PRISM (ver. 10.4.2 software, GraphPad Software, San Diego, CA, USA). To visualize functions of selected biomarker proteins identified in this study, a BioRender was used (https://biorender.com, accessed February 2025).

Statistics

GraphPad PRISM (ver. 10.4.2) software was used to perform the statistical analyses. A parametric ANOVA or non-parametric Kruskal–Wallis test was used to analyze the abundance of the epididymal fluid proteins in each ExG. Additionally, to detect differences in the abundance of the epididymal fluid proteins among the ExGs, Tukey's multiple comparison test was used. The values were considered to differ significantly when $P < 0.05$. The results are shown as means ± s.e.m. Spearman's rank coefficient was used to determine the correlations between the relative abundances (MS intensity – mass spectrometry intensity) of selected cauda epididymal fluid proteins and the values of cauda epididymal sperm motility and motion parameters in dogs. Values were considered significant at $P < 0.05$.

Ethics approval and consent to participate

All animal studies were conducted following the guidance of Directive 63/2010/EU and the Journal of Laws of the Republic of Poland (2017) regarding the protection of animals that are used for scientific or educational purposes. In accordance with the Polish Laboratory Animal Science Association, the authors have permission to conduct the animal experiments (Numbers 1432/2015; 1508/2015). An exemption letter was obtained from the Local Ethics Committee for Animal Experimentation, Olsztyn, Poland (LKE/01/2022). Additionally, the authors received consent from the director of the Shelter for Homeless Animals in Tomaryny, Poland.

Results

Proteomic profiling of the epididymal fluid using nanoUPLC-Q-TOF/MS

Proteomic profiling of dog epididymal fluid by using nanoUPLC-Q-TOF/MS and UniProt-supported identification discovered 121 unique proteins (UPs) in ExG1, 122 UPs in ExG2 and 56 UPs in ExG3. For all three groups, 48 proteins were common. There were 16 common proteins (CPs) shared between ExG1 and ExG2, 16 between ExG2 and ExG3, and 12 between ExG1 and ExG3 (Fig. 1*a*). The complete list of proteins identified in each ExG, along with details, has been included in the Supplementary Tables S1–S3. Cauda epididymal fluid proteins of dogs according to their presence in ExGs have been included in Table S5.

Considering those proteins that occurred in over 50% of the animals in each group, five UPs were identified for ExG1, one for ExG2 and one for ExG3. There were no CPs found between ExG1 and ExG2. Eight proteins were common to ExG2 and ExG3. Four proteins were common to ExG1 and ExG3. The following seven CPs were identified for all three ExGs: albumin (ALB), carboxylesterase 5A (CES5A), epididymal secretory glutathione peroxidase (GPX5), lactotransferrin (LTF), NPC intracellular cholesterol transporter 2 (NPC2), plastin 3 (PLS3) and prostaglandin-H2 D-isomerase (PTGDS) (Fig. 1*b*).

Protein relative abundance analysis

On the basis of MS intensity measurements, a semiquantitative analysis of CP abundance was performed. When the MS intensities of the CPs in the research groups (ExG1, ExG2 and ExG3) were compared, statistically significant differences were found for three of them. The ALB abundance was the highest in ExG2, but a statistically significant difference in abundance was detected between ExG1 and

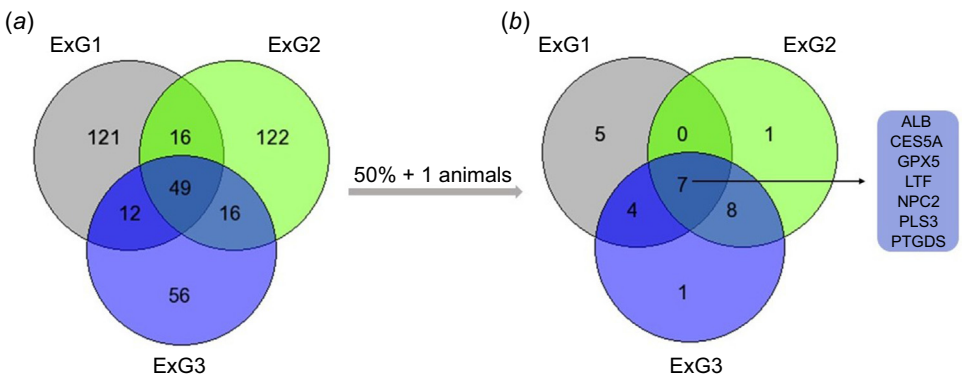


Fig. 1. Venn diagrams showing the number of unique proteins (UPs) and common proteins (CPs) in the cauda epididymal fluid of dogs. The ExG1, ExG2, ExG3 are experimental groups. (*a*) Total number (100%) of identified proteins; and (*b*) number of proteins after the statistical threshold was met when protein was present in over 50% of the animals in the group.

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ExG2 (321.7 ± 63.67 × 10^5 vs 1065.0 ± 241.6 × 10^5; mean ± s.e.m.; P = 0.014). The highest abundance of GPX5 was observed in ExG2, but a statistically significant difference in abundance was found between ExG1 and ExG2 (41.04 ± 5.99 × 10^5 vs 903.3 ± 298.4 × 10^5; P = 0.006). Additionally, the PTGDS abundance was the highest in ExG2; however, statistical analysis showed significant differences in abundance between ExG1 and ExG2 (229.7 ± 46.59 × 10^5 vs 920.2 ± 259.4 × 10^5; P = 0.023) (Fig. 2). For all three proteins showing significant differences in abundance, a Kruskal–Wallis test was performed. No significant differences among groups were found when the MS intensities of CES5A, LTF, NPC2, and PLS3 were compared (Fig. 2).

Western blot

The WB analysis showed differences in the abundance of the selected CPs in the dog epididymal fluid among the ExGs (Fig. 3). ANOVA results showed significant differences among ExGs regarding the ALB content (F = 35.24; P < 0.001). ALB was significantly more abundant in ExG2 than in ExG1 (P < 0.001) and in ExG3 (P = 0.047). Additionally, a significant difference in the ALB abundance was detected between ExG1 and ExG3 (P = 0.003) (Fig. 3*a*). ANOVA results showed significant differences among ExGs regarding the GPX5 content (F = 14.18; P < 0.001). The GPX5 abundance was the highest in ExG3, and statistically significant differences in the GPX5 abundance were shown between ExG2 and ExG3 (P < 0.001), and between ExG1 and ExG3 (P = 0.010) (Fig. 3*b*). ANOVA results showed significant differences among ExGs regarding the PTGDS content (F = 11.93; P = 0.002). The PTGDS abundance was significantly greater in ExG2 than in ExG1 (P = 0.002). Additionally, a significant difference in the PTGDS abundance was detected between ExG1 and ExG3 (P = 0.024) (Fig. 3*c*). For all three proteins, a parametric ANOVA was performed.

Gene ontology analysis of proteins

A gene ontology (GO) analysis of the proteins found in ExG1, ExG2 and ExG3 showed similar main molecular functions (MFs) and biological processes (BPs). On the basis of the GO enrichment results, the predominant molecular functions of the proteins in ExG1 were catalytic activity (45%), binding (33%) and antioxidant activity (22%) (Fig. 4, MF). The main molecular functions of ExG2 and ExG3 were binding (47% and 50% respectively) and catalytic activity (29% and 27% respectively). For the proteins found in ExG1, the most dominant biological processes were localization (41%), metabolic process (17%), response to stimulus (12%) and cellular process (12%). For the proteins found in ExG2, the most dominant biological process was cellular process

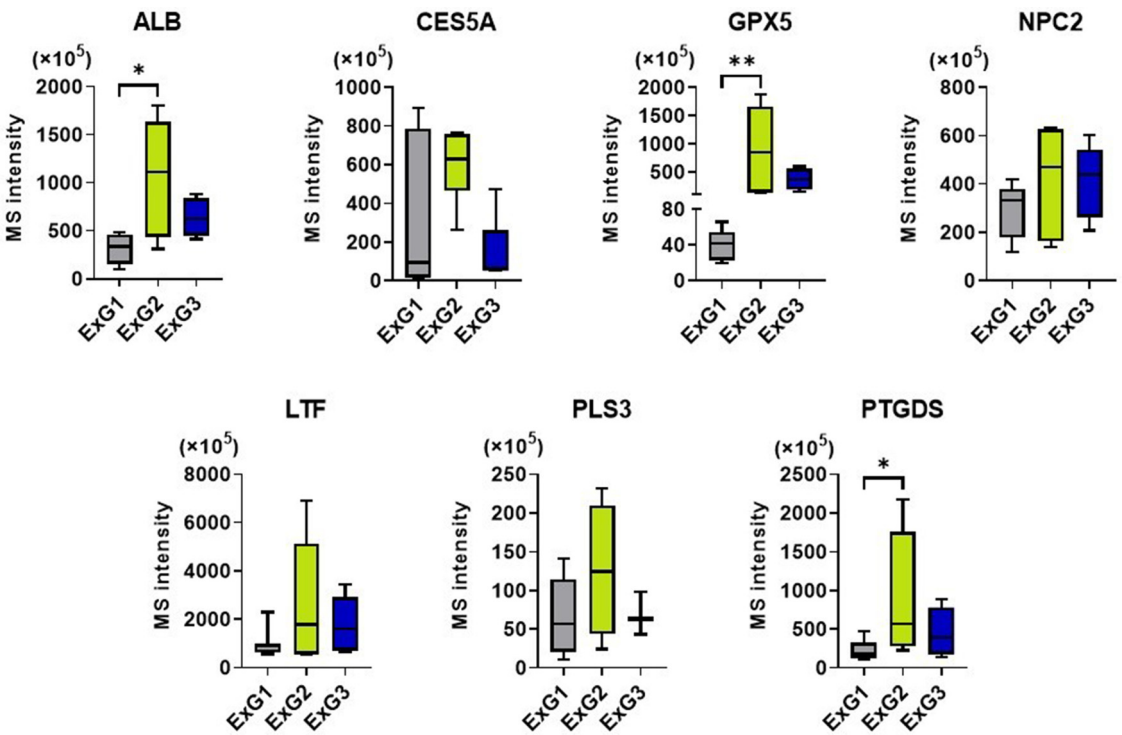


Fig. 2. Box plots representing the abundance (MS intensity) distribution of the common proteins (CPs) in the cauda epididymal fluid of the dogs in different experimental groups (ExG1, ExG2, ExG3). ALB, albumin; CES5A, carboxylesterase 5A; GPX5, epididymal secretory glutathione peroxidase; LTF, lactotransferrin; NPC2, NPC intracellular cholesterol transporter 2; PLS3, plastin 3; PTGDS, prostaglandin-H2 D-isomerase. *P < 0.05; **P < 0.01.

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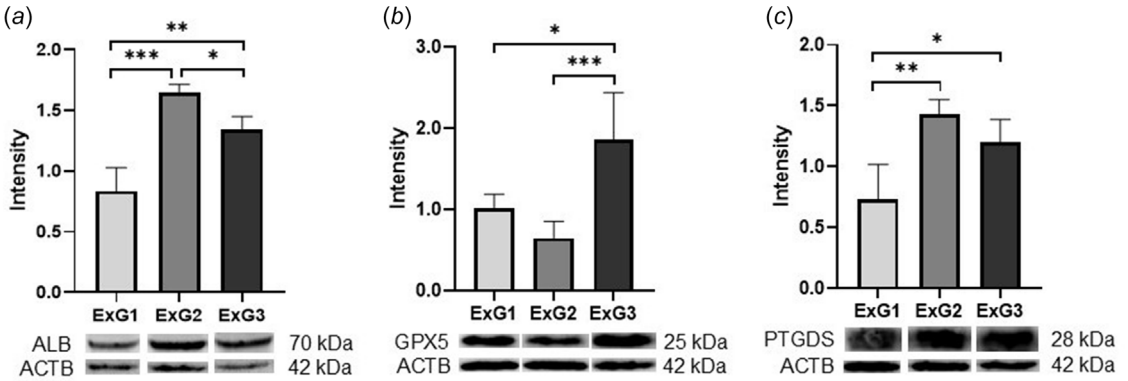


Fig. 3. Validation of differences in the abundances of the cauda epididymal fluid proteins of the dogs in different experimental groups (ExG1, ExG2, ExG3): (*a*) albumin (ALB); (*b*) epididymal secretory glutathione peroxidase (GPX5); and (*c*) prostaglandin-H2 D-isomerase (PTGDS). Each column indicates the normalized protein abundance (mean ± s.e.m.) based on three replicates. The protein abundance represents the band intensity, which significantly differed among the experimental groups (ExG1, ExG2, and ExG3). The intensity was normalized to that of a stable beta actin (ACTB) protein band. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$.

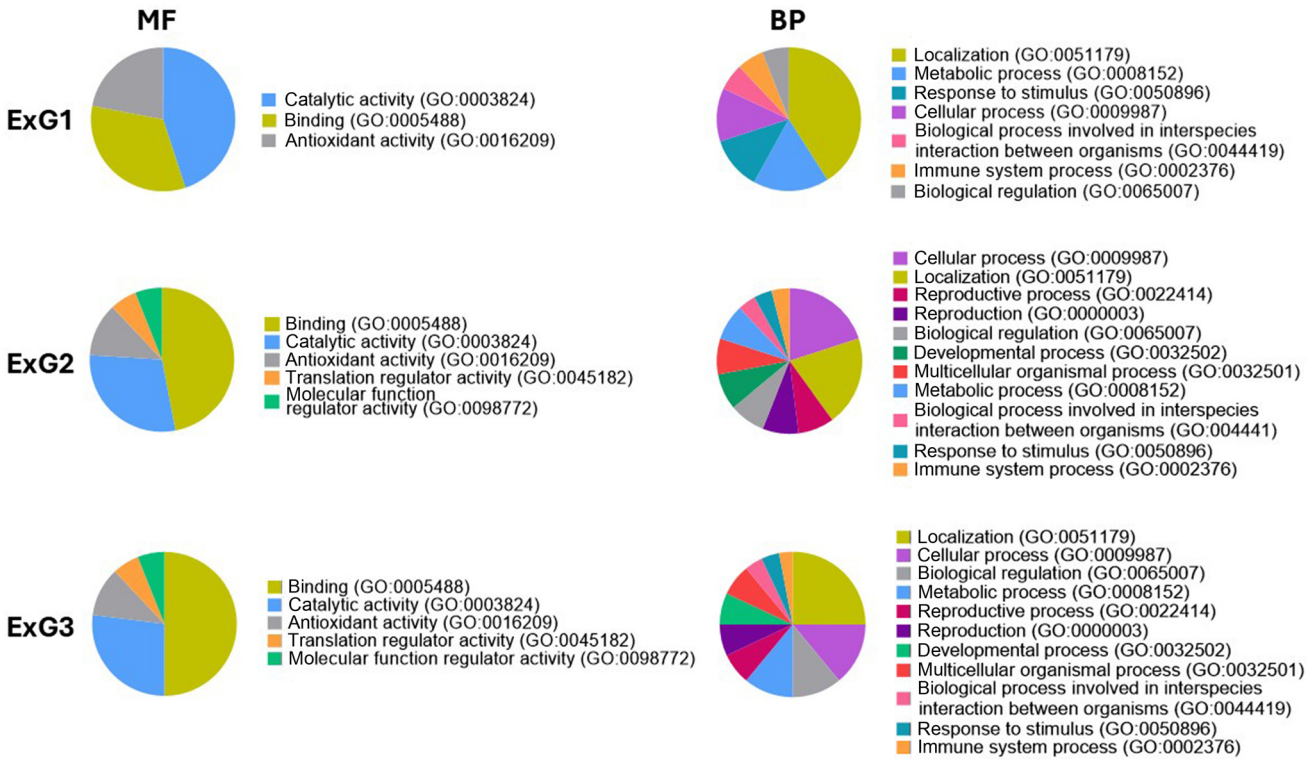


Fig. 4. GO enrichment of the proteins present in the cauda epididymal fluid of the dogs in the three experimental groups (ExG1, ExG2, and ExG3). The following GO categories were distinguished: molecular function (MF) and biological process (BP).

(20%) and localization (20%) (Fig. 4, BP). For the proteins identified in ExG3, the main biological processes were localization (25%), cellular process (14%), biological regulation (11%) and metabolic process (11%) (Fig. 4, BP). The percentage of proteins in all GO terms is included in Table S4.

Protein–protein interaction networks

An interaction network analysis of the GPX5 and PTGDS was performed using STRING. This analysis showed that GPX5 interacts mostly with other members of the GPX family (Fig. 5*a*). PTGDS strongly interacts with different prostaglandins.

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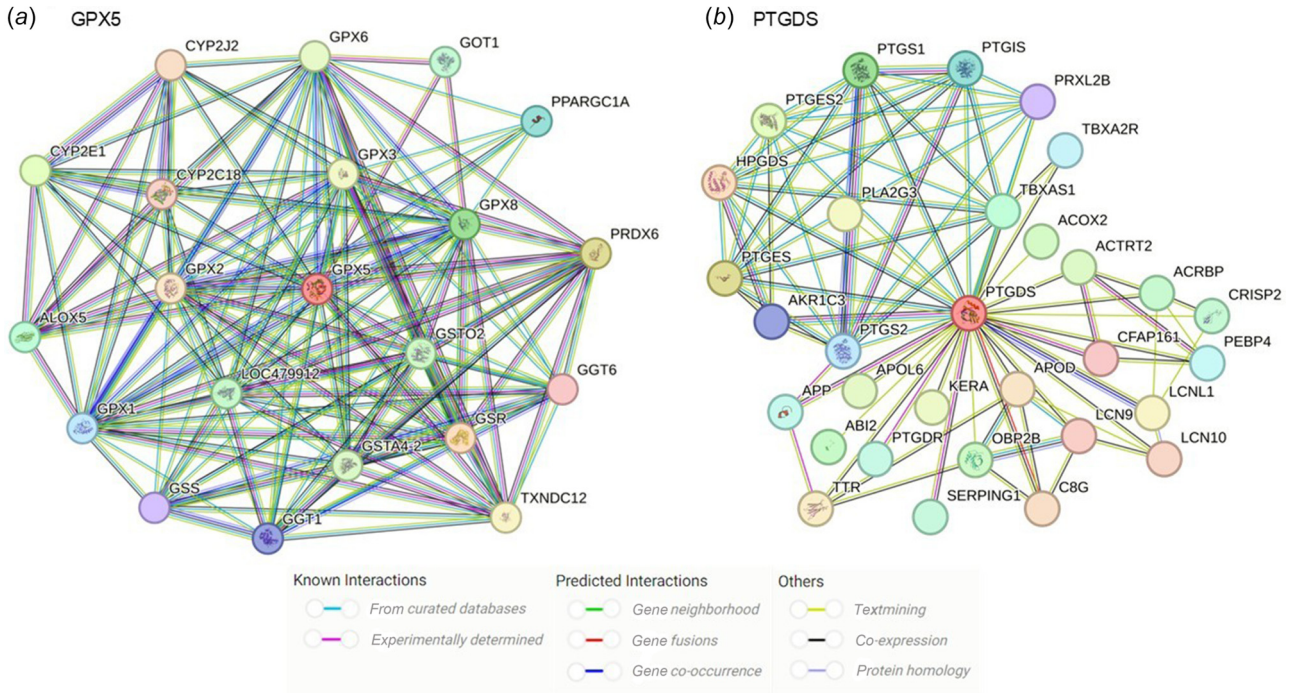


Fig. 5. Protein–protein interaction (PPI) networks for (*a*) epididymal secretory glutathione peroxidase (GPX5), and (*b*) prostaglandin-H2 D-isomerase (PTGDS) in dogs (*Canis lupus familiaris*). The nodes represent proteins, and the edges represent the number of protein–protein interactions. The edges indicate both functional and physical protein–protein associations. The minimum required interaction score was set at medium confidence (0.400). Names of connected proteins in the first (*a*) PPI network: ALOX5, arachidonate 5-lipoxygenase; CYP2C18, cytochrome P450 2C21; CYP2E1, cytochrome P450 2E1; CYP2J2, cytochrome P450 family 2 subfamily J member 2; GGT1, uncharacterized protein; GGT6, gamma-glutamyltransferase 6; GOT1, aspartate aminotransferase; GPX1, glutathione peroxidase; GPX2, glutathione peroxidase; GPX3, glutathione peroxidase; GPX6, glutathione peroxidase; GPX8, glutathione peroxidase; GSR, glutathione reductase; GSS, glutathione synthetase; GSTA4-2, uncharacterized protein; GSTO2, glutathione S-transferase omega 2; LOC479912, uncharacterized protein; PPARGC1A, PPARG coactivator 1 alpha; PRDX6, peroxiredoxin 6; TXNDC12, thioredoxin domain containing 12. Names of connected proteins in the second (*b*) PPI network: ABI2, abl interactor 2; ACOX2, acyl-coenzyme A oxidase; ACRBP, acrosin binding protein; ACTRT2, actin related protein T2; AKR1C3, aldo_ket_red domain-containing protein; APOD, apolipoprotein D; APOL6, apolipoprotein L6; APP, gamma-secretase C-terminal fragment 57; C8G, complement C8 gamma chain; CFAP161, cilia and flagella associated protein 161; CRISP2, cysteine rich secretory protein 2; HPGDS, hematopoietic prostaglandin D synthase; KERA, keratocan; LCN9, lipocalin 9; LCN10, uncharacterized protein; LCNL1, lipocln_cytosolic_FA-bd_dom domain-containing protein; OBP2B, major allergen Can f 1; PEBP4, phosphatidylethanolamine binding protein 4; PLA2G3, phospholipase A2 group III; PRXL2B, peroxiredoxin like 2B; PTGDR, prostaglandin D2 receptor; PTGES, prostaglandin E synthase; PTGES2, prostaglandin E synthase 2; PTGIS, prostaglandin I2 synthase; PTGS1, prostaglandin G/H synthase 1; PTGS2, prostaglandin-endoperoxide synthase 2; SERPING1, serpin family G member 1; TBXA2R, thromboxane A2 receptor; TBXAS1, thromboxane A synthase 1; TTR, transthyretin/thyroid hormone-binding protein.

Because PTGDS belongs to the lipocalin family and is referred to as an enzymatic lipocalin, it has been shown to be associated with numerous members of this family (LCNL1, LCN9, and LCN10). Additionally, its connection with acrosin binding protein (ACRBP) seems interesting (Fig. 5*b*).

Correlations between the relative abundance of marker proteins in epididymal fluid and epididymal sperm motion parameters

To present the associations between the content (MS intensity) of selected cauda epididymal fluid proteins and the epididymal sperm motion parameters of dogs in different ExGs, Spearman's rank correlation coefficient was used.

In ExG1 correlations were shown only for GPX5. A significant positive correlation was detected between the GPX5 content and the percentage of sperm with RAPID movement ($r = 0.68$; $P = 0.044$) and a significant negative correlation was detected between the GPX5 content and the percentage of sperm with STATIC movement ($r = -0.76$; $P = 0.035$) (Fig. 6*a*). In adult dogs (ExG2), ALB content was negatively correlated with the percentage of sperm with SLOW ($r = -0.98$; $P = 0.033$) movement and positively correlated with the percentage of sperm with STATIC ($r = 0.87$; $P = 0.047$) movement (Fig. 6*b*). Additionally, a negative correlation was shown for the GPX5 content and sperm TMOT percentage ($r = -0.93$; $P = 0.033$) (Fig. 6*b*).

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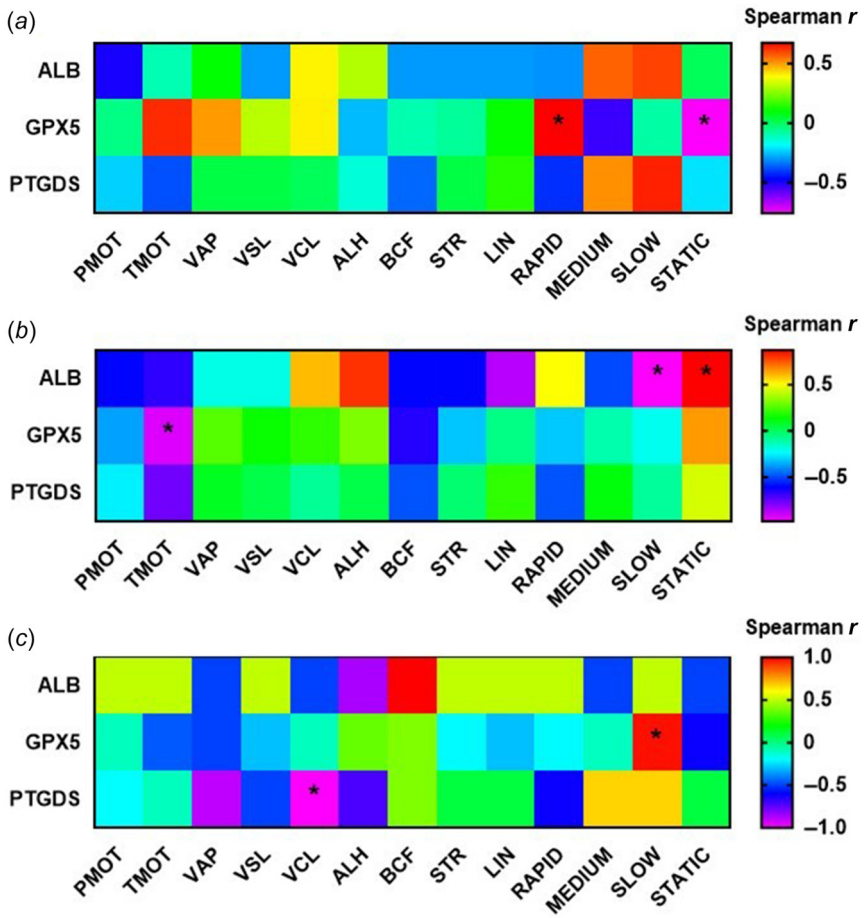


Fig. 6. Heatmaps showing Spearman's rank correlation coefficients between the abundance (MS intensity) of the selected cauda epididymal fluid proteins and the values of the epididymal spermatozoa motion parameters in different experimental groups (ExGs) of dogs: (*a*) ExG1; (*b*) ExG2; and (*c*) ExG3. *$P < 0.05$ (for correlation). ALB, albumin; GPX5, epididymal secretory glutathione peroxidase; and PTGDS, prostaglandin-H2 D-isomerase. The sperm motility and motion parameter abbreviations are explained in section 'Motility and motion parameters of the epididymal spermatozoa' of the Materials and methods.

In the group of oldest dogs (ExG3), a positive correlation was detected between the GPX5 content and percentage of sperm with SLOW movement ($r = 0.97$; $P = 0.035$) and a negative correlation was detected between the PTGDS content and the sperm velocity parameter VCL ($r = -1.00$; $P = 0.017$) (Fig. 6*c*).

Discussion

Aging is a natural, unavoidable process that is characterized by a reduction in coherence, physiological efficiency and the ability to respond to the environmental stress that occurs in organisms over time (Sampson *et al.* 2007; Das and Destouni 2023). It leads to impaired functioning of cells, tissues, and physiological systems. The aging of the male reproductive tract is caused by multifactorial changes at the molecular, cellular and tissue levels, including the accumulation of damage to DNA, protein, and lipid structures (Sampson *et al.* 2007). The hallmarks of reproductive system aging are shown in Fig. 7. It is worth examining changes caused by the senescence of reproductive system organs such as the epididymis at the molecular level when proteins are considered. The proteome analysis of dog semen at the stage of its main

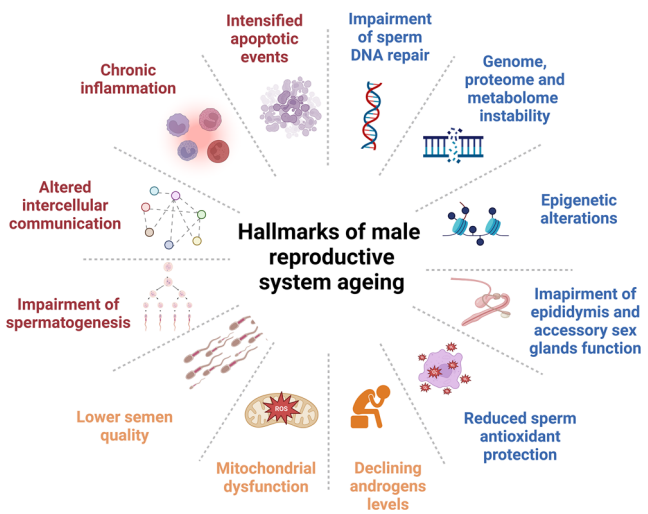


Fig. 7. Hallmarks of male reproductive system aging. Created with BioRender.com.

component formation and its influence on epididymal sperm maturation in the context of aging processes seems important for scientists and for dog breeders. In our previous studies, we demonstrated a relationship between dog age and dog sperm

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motility in terms of the content of marker proteins present in both epididymal sperm (Zmudzinska *et al*. 2022) and epididymal fluid (Cichowska *et al*. 2023). In this study, we focused on changes in the epididymal fluid proteome associated with epididymal aging. Detailed proteome profiling of canine epididymal fluid showed the following three epididymal fluid proteins as potential markers of dog epididymis aging: ALB, GPX5 and PTGDS. The biochemical functions of these proteins and their participation in reproductive processes are described below.

Albumins (ALBs) are low molecular-weight proteins (65 kDa), that are secreted by the testes, epididymides, and prostate, and are key components of canine seminal plasma (Peña *et al*. 2006; Shan *et al*. 2021). ALBs are described as components of canine epididymal sperm (Zmudzinska *et al*. 2022). Seminal plasma ALBs are implicated in fertilization-associated events, such as sperm capacitation (Macías-García *et al*. 2015) and zona pellucida penetration (Arroteia *et al*. 2014). The ALB content in animal seminal plasma was shown to be correlated with the motility parameters of spermatozoa (Mogielnicka-Brzozowska *et al*. 2019). The binding of seminal plasma or epididymal fluid ALB to the sperm plasma membrane of epididymal or ejaculated spermatozoa plays a protective role and influences sperm motility (Mogielnicka-Brzozowska *et al*. 2019; Cichowska *et al*. 2023; Mogielnicka-Brzozowska and Cichowska 2024). The addition of bovine serum albumin to ejaculated canine spermatozoa improves sperm capacitation and acrosome reaction (Araujo *et al*. 2022) and preserves viability and sperm functions (Risopatrón *et al*. 2002). ALBs also possess a strong ability to bind zinc ions, thus reducing their amount in sperms cells and enhancing sperm motility (Mogielnicka-Brzozowska *et al*. 2019). The protein also functions by binding bivalent cations to regulate their amount in reproductive fluids (Lu *et al*. 2008). ALBs exert their protective effects on the structures involved in sperm motility and sperm membrane integrity because of their antioxidant properties. They can absorb lipid peroxides from reproductive fluids, which may positively influence sperm physiology (Roche *et al*. 2008). Considering the abovementioned features of ALBs and the results of these studies, we propose these proteins as potential biomarkers of epididymal semen aging, because the course of the aging process is often associated with disorders in oxidative processes in cells. To our knowledge, the high amount of ALBs in the epididymal fluid may indicate a decent quality of the epididymal semen. Measuring the ALB content in the epididymal fluid provides additional opportunities to confirm semen quality in addition to providing the standard assessment of the sperm motion parameters. There are sperm-related biochemical events regulated by ALBs, but the exact molecular mechanism of their action is not known.

Western blot analysis showed that the concentration of ALB was low in the epididymal fluid of young and old dogs, and it was highest in adult dogs. We speculate that the high amount of ALB in the epididymal fluid surrounding the epididymal sperm is necessary for optimal epididymal sperm protection. ALB plays a pivotal role in antioxidant protection via lipid peroxide absorption and reduction in the amount of Zn^{2+} in sperm, which may increase epididymal sperm motility and viability. This protective action may occur at a low level in the epididymal sperm of old dogs, which may have lower reproductive ability.

The next protein with potential as a canine epididymal fluid aging biomarker is epididymal secretory glutathione peroxidase (GPX5). It is a well-known antioxidant protein that is highly expressed in epididymal tissue and is secreted into the epididymal lumen (Aitken 2009). GPX has been detected in the seminal plasma of bulls, boars and dogs (Neagu *et al*. 2011; Waheed *et al*. 2013; Barranco *et al*. 2016) and in boar and canine epididymal sperm (Barranco *et al*. 2016; Zmudzinska *et al*. 2022). It is found in canine epididymal fluid regardless of the epididymal sperm motion parameters (Cichowska *et al*. 2023). This protein also participates in important metabolic pathways in feline seminal plasma (Mogielnicka-Brzozowska *et al*. 2020). Its key role is to protect sperm from lipid peroxidation (Aitken 2009). The antioxidant enzyme activities of mammalian spermatozoa include those of co-working superoxide dismutase (SOD) and GPX (Alvarez and Storey 1992). The hydrogen peroxide produced by SOD is eliminated by either GPX or catalase to avoid cell damage. The sperm mostly relies on GPX activity to eliminate hydrogen peroxide generated intracellularly (Buffone *et al*. 2012). Both SOD and GPX play significant roles in protecting mammalian sperm against oxygen radical-induced damage leading to motility loss (Buffone *et al*. 2012). A study on dogs showed GPX activity in sperm-rich and post-spermatic fractions (Prete *et al*. 2018). Sperm viability and DNA integrity may be protected by the addition of combination of GPX and SOD to dog semen (Prete *et al*. 2018). The addition of GPX to the extender allows the preservation of semen quality during cold storage in dogs (Thuwanut *et al*. 2011). Protein–protein interaction analysis showed that GPX5 has strong functional connections with other members of GPX family such as GPX1, GPX2, GPX3, GPX6 and GPX8. The results of this study showed that GPX5 content in the epididymal fluid of canines may be an indicator of aging processes in epididymal tissue.

Western blot analysis showed that the GPX5 concentration in the epididymal fluid was lowest in adult dogs and very high in old dogs. However, the results from MS intensity measurement were slightly different and highest GPX5 amount was shown in the adult group. It is difficult to explain this difference. LC–MS and WB protein amount measurement methods are different in their basis and usually WB is used to confirm results from LC–MS. Differences between results obtained by LC–MS and WB analyses are expected, and can be attributed to both methodological and biological factors. LC–MS quantifies peptide-level signals and can distinguish proteoforms and post-translational modifications, whereas WB detection depends on epitope accessibility and antibody

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specificity. Thus, both methods probe distinct molecular features of the same protein, which may naturally lead to divergent outcomes (Kiernan *et al.* 2002; Gauthier *et al.* 2015; Yang and Herold 2017; Stevens and Pukala 2020). In the old dogs, oxidative processes might be the most intense, so high GPX5 activity in epididymal fluid would be desirable to remove excess free radicals that may damage epididymal sperm. The antioxidative activity of this enzyme is needed for the reduction of hydrogen and lipid peroxides preserving sperm DNA integrity and membrane stability (Chabory *et al.* 2009). From the other side, also in the adult dogs high activity of GPX5 in epididymal fluid would be desirable to protect sperm from peroxides excess. Future studies are needed to fully explain this finding.

The third protein showing potential as a biomarker of dog semen aging is prostaglandin-H2 D-isomerase (PTGDS). It is also known as prostaglandin-D2-synthase (PTGDS) or lipocalin-type prostaglandin-D-synthase. PTGDS is found in the epididymal fluid of rams (Van Tilburg *et al.* 2021) and seminal plasma of cats (Mogielnicka-Brzozowska *et al.* 2020). This enzyme converts the cyclooxygenase product of prostaglandin H2 (PGH2) to prostaglandin D2 (PGD2) (Candenas and Chianese 2020). Lipocalins bind to specific cell-surface receptors and form macromolecular complexes (Flower 2000). PTGDS can bind small non-substrate lipophilic molecules such as retinoids. Additionally, it functions as a scavenger for harmful hydrophobic molecules (Zhou *et al.* 2010). PTGDS also influences sperm plasma membrane permeability, leading to alterations in the influx of ions from the exterior. This may be associated with regulating the entry of Ca^{2+} ions into the sperm cell and changes in sperm motility (Zhou *et al.* 2010; Van Tilburg *et al.* 2021). Research has demonstrated a positive link between PTGDS concentrations in human sperm and the progressive motility of the cells (Chen *et al.* 2007). PTGDS is strongly functionally interconnected with various critical proteins implicated in sperm metabolism, including those in the PTG family and LCN family. A proteomic investigation showed a greater prevalence of PTGDS in the epididymal fluid of dogs, which envelops sperm with high motility (Cichowska *et al.* 2023). Nonetheless, the precise role of PTGDS in sperm metabolism remains elusive.

The amount of PTGDS shown by WB and semiquantitative protein analysis was the highest in the epididymal fluid of adult dogs and then it drops slightly in old dogs. It was the lowest in the young dog group. It is difficult to explain

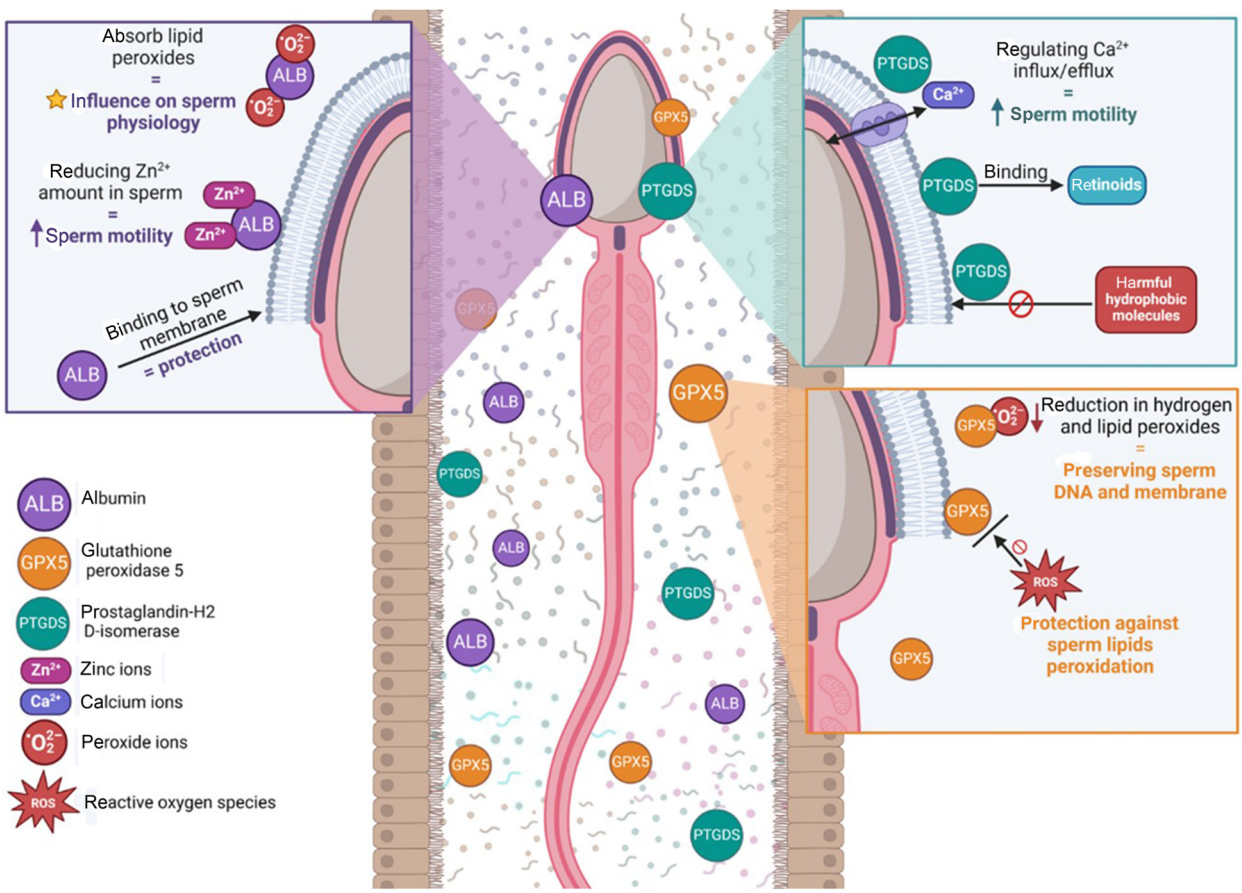


Fig. 8. Canine epididymal fluid biomarker proteins that coat epididymal sperm and provide changes in its surface properties, biochemistry and metabolism. Created with BioRender.com.

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these events, but if we consider the ability of PTGDS to bind harmful hydrophobic molecules, thus protecting the epididymal sperm in this way, regulating Ca^{2+} influx/efflux and enhancing the epididymal sperm motility, we might say that the action of this enzyme must be strongest in epididymal fluid of sexually active males. It may be postulated that young dogs do not have the PTGDS, GPX5 and ALB systems active enough to protect the epididymal sperm. All three proteins strongly bind to canine epididymal sperm and play a role in their protection and metabolism regulation. Potential biomarker protein functions are visualized in Fig. 8.

Aging-induced changes in the dog epididymis may be shown by differences in the content of some biomarker proteins in the epididymal fluid, but to understand the role of epididymal fluid biomarker proteins in epididymal sperm metabolism and maturation, detailed research on each of these proteins is necessary. The authors hope that by using specific antibodies it will be possible in the near future to construct simple diagnostic tests for analyzing the content of the above-mentioned biomarker proteins in canine epididymal fluid. The results of this study provide a potential opportunity to use protein markers in the case of the need to analyze epididymal semen from valuable elderly or very young sires. The use of the above-mentioned markers might be extended in the future to the examination of epididymal semen collected by urethral catheterization or microsurgical procedures (microsurgical epididymal sperm aspiration (MESA) and percutaneous epididymal sperm aspiration (PESA)) (Zambelli *et al.* 2008; Shah 2011; Varesi *et al.* 2013; Kuczmarski *et al.* 2020; Mogheiseh *et al.* 2022). This will allow to determine whether the obtained epididymal semen meets certain basic quality criteria indicating its usefulness in assisted reproductive techniques. This phenomenon may be particularly important for endangered species and valuable breeds.

Study limitations

The study was limited by the sample size available, which may not reflect the biological variability within the wider canine population. The diversity of dogs, as a result of the use of mixed-breeds, may have introduced genetic variability affecting protein expression, potentially confounding age-related effects. Ages of dogs were estimated in some cases because of unknown histories, which may have affected age groupings. The cross-sectional study design involved the collection of samples from different individuals at various ages rather than following the same animals over time, which limits causal interpretation of age-related changes. Although several proteins showed altered abundance, their biological roles in epididymal aging were not experimentally validated. The study is absent of reproductive performance data because it did not correlate proteomic findings with semen quality (except of sperm motility) or fertility outcomes, which would strengthen the interpretation of the biomarkers' relevance.

Conclusions

Proteomic profiling of dog epididymal fluid by using nanoUPLC-Q-TOF/MS uncovereded differences in protein composition, which was dependent on dog age. Albumin, epididymal secretory glutathione peroxidase and prostaglandin-H2 D-isomerase significantly changed in relative abundance in the epididymal fluid of the dogs in the experimental groups. The role of albumin (ALB), epididymal secretory glutathione peroxidase (GPX5) and prostaglandin-H2 D-isomerase (PTGDS) in the aging processes of the epididymis may result from the participation of these proteins in the regulation of oxidative processes and divalent ion metabolism. We suggest that the application of specific antibodies in future studies will facilitate the use of simple diagnostic tests and precisely characterize the biological functions of the analyzed protein biomarkers of dog epididymal fluid.

Supplementary material

Supplementary material is available online.

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Data availability. All data generated or analyzed during this study are included within the article (and its supplementary files).

Conflicts of interest. The authors declare that they have no conflicts of interest.

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Oświadczam, że w pracy pod tytułem **Cichowska-Likszo A.W., Likszo P., Fraser L., Mlynarz P., Mogielnicka-Brzozowska M. (2026). ALB, GPX5 and PTGDS as potential biomarkers for the aging processes of the dog epididymis.** ***Reproduction, Fertility and Development*****, *38*, RD25105** mój wkład merytoryczny w jej przygotowanie polegał na: opracowaniu koncepcji i doborze metodologii badań, wykonaniu analiz laboratoryjnych (w tym udziale w analizach NanoUPLC-Q-TOF/MS), walidacji i analizie formalnej danych, opracowaniu bioinformatycznym i statystycznym, interpretacji oraz wizualizacji wyników, przygotowaniu manuskryptu i odpowiedzi na recenzje oraz korekcie manuskryptu po recenzjach, pełnym udziale w procesie redakcyjnym jako autor korespondencyjny.

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dr Paweł Likszo
(tytuł zawodowy/stopień/tytuł naukowy, imię i nazwisko)

Przewodniczący Rady Naukowej Dyscypliny
Zootechnika i Rybactwo
Uniwersytetu Warmińsko-Mazurskiego w Olsztynie

OŚWIADCZENIE
współautora

Oświadczam, że w pracy pod tytułem **Cichowska-Likszo A.W., Likszo P., Fraser L., Mlynarz P., Mogielnicka-Brzozowska M. (2026). ALB, GPX5 and PTGDS as potential biomarkers for the aging processes of the dog epididymis.** ***Reproduction, Fertility and Development*****, *38*, RD25105** mój wkład merytoryczny w jej przygotowanie polegał na: wsparciu w analizie Western blot – zapewnieniu i obsłudze oprogramowania wykorzystywanego do analizy oraz udziale w walidacji wyników badań.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez mgr inż. Aleksandrę Wiktorię Cichowską-Likszo jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydatki mgr inż. Aleksandry Wiktorii Cichowskiej-Likszo polegający na opracowaniu koncepcji i doborze metodologii badań, wykonaniu analiz laboratoryjnych (w tym udziale w analizach NanoUPLC-Q-TOF/MS), walidacji i analizie formalnej danych, opracowaniu bioinformatycznym i statystycznym, interpretacji oraz wizualizacji wyników, przygotowaniu manuskryptu i odpowiedzi na recenzje oraz korekcie manuskryptu po recenzjach, pełnym udziale w procesie redakcyjnym jako autor korespondencyjny.

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podpis

Załącznik nr 5 do Załącznika nr 1
do Uchwały Nr 362
Senatu Uniwersytetu Warmińsko-Mazurskiego w Olsztynie
z dnia 27 czerwca 2023 roku
wprowadzającej procedurę postępowania
w sprawie nadania stopnia doktora

Olsztyn, 06.05.2026 r.
(miejscowość, data)

prof. dr hab. Leyland Fraser
(tytuł zawodowy/stopień/tytuł naukowy, imię i nazwisko)

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prof. dr hab. Piotr Młynarz
(tytuł zawodowy/stopień/tytuł naukowy, imię i nazwisko)

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pozyskaniu środków finansowych umożliwiających przeprowadzenie analiz NanoUPLC-Q-TOF/MS, w szczególności zakup niezbędnych odczynników i asortymentu laboratoryjnego oraz krytyczną analizę manuskryptu

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(miejscowość, data)

dr hab. inż. Marzena Mogielnicka-Brzozowska, prof. UWM
(tytuł zawodowy/stopień/tytuł naukowy, imię i nazwisko)

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