Analysis of testicular tissue and sperm parameters in men with testicular germ cell tumour

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13. PragueONCO
27.01.2022

Project contribution

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This work was supported by NU20-03-0030, IBT RVO 86652036, and BIOCEV (CZ.1.05/1.1.00/02.0109).

Aims of the project

1. Assessment of tumor progression and sperm quality parameters such as acrosome integrity, DNA integrity, motility and apoptosis.

2. Epigenetic profiling and analysis of sperm and testicular germ cell epigenome.

3. Assessment of testicular and sperm mitochondrial activity.

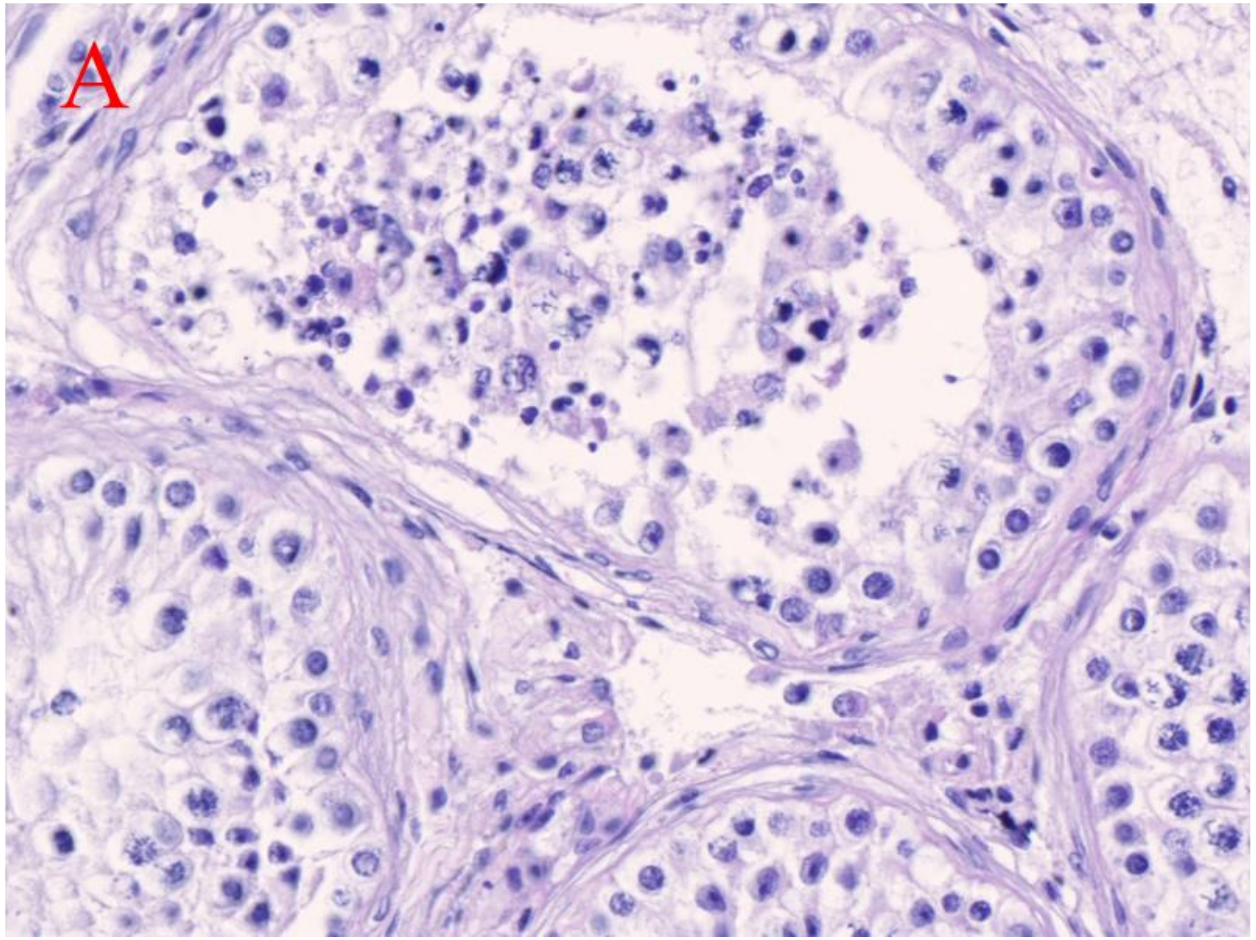
4. Multi-parametric correlation of TGCT sperm pathology assessment.

5. Proposing new methods for TGCT testicular tissue diagnostics.

6. Proposing a set of diagnostic markers for sperm quality diagnostics of TGCT patients' to be used in assisted reproduction.

Preliminary data
05/2020 - 12/2021

Pathophysiology assessment of testicular tissue



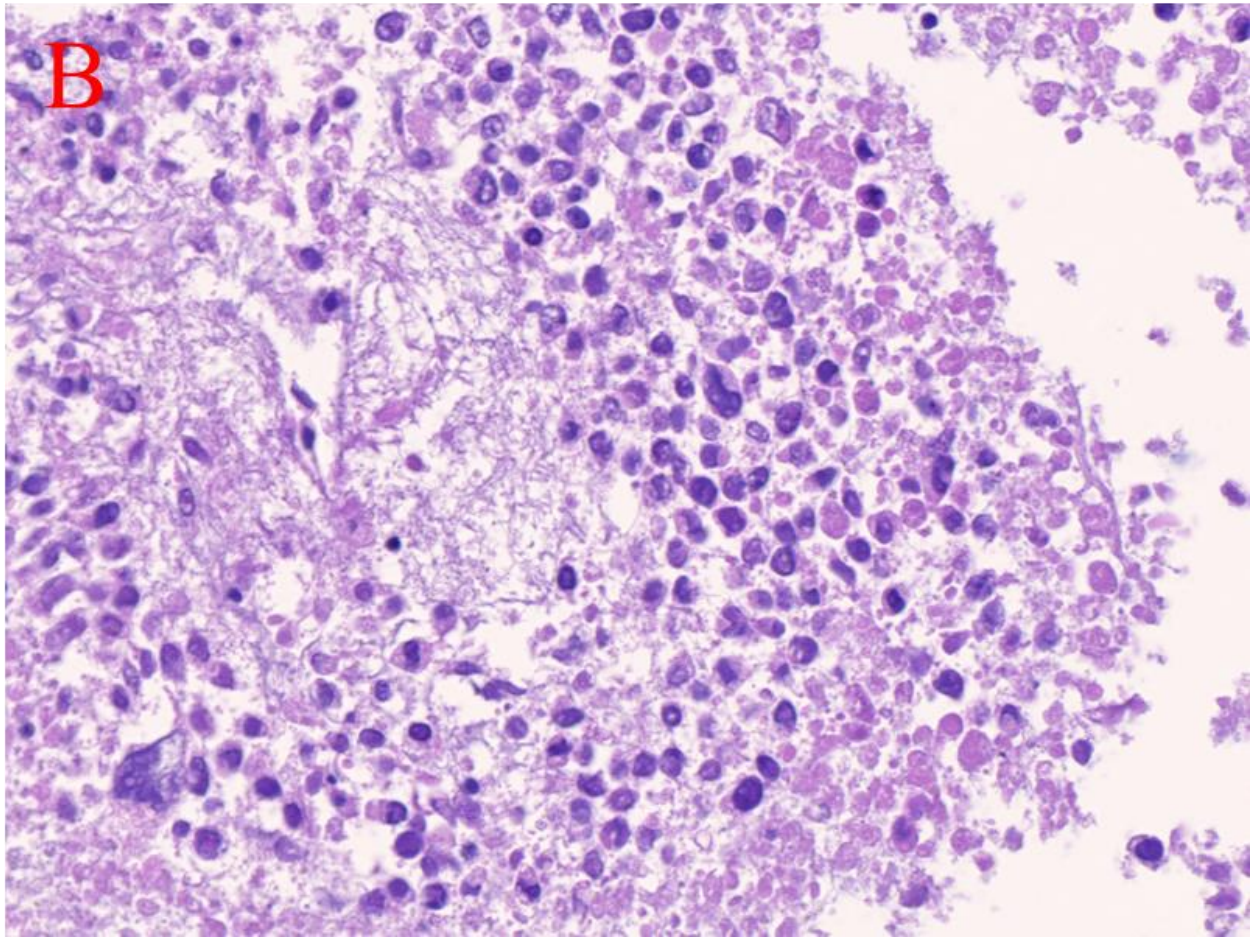


Figure 1: Testicular tissue, Hematoxylin-Eosin labelling. (A) Non-tumor tissue with distinct cell borders, clear cytoplasm, nuclei with prominent nucleoli. (B) TGCT tissue shows seminoma tumor pattern with indistinct cell borders and basophilic cytoplasm, coagulative type of necrosis and granulomatous inflammation. The tumor tissue contains invasive follicular lymphoma and multinucleated syncytiotrophoblast. Most of the histopathology observation points to seminoma tumors and were of similar histology (n = 11).

Assessment of Tumor Severity using Ki67 proliferation marker

- Ki67, a marker of cell proliferation, is a non-histone nuclear protein expressed throughout the active phase of cell cycle, except G0 and early G1.

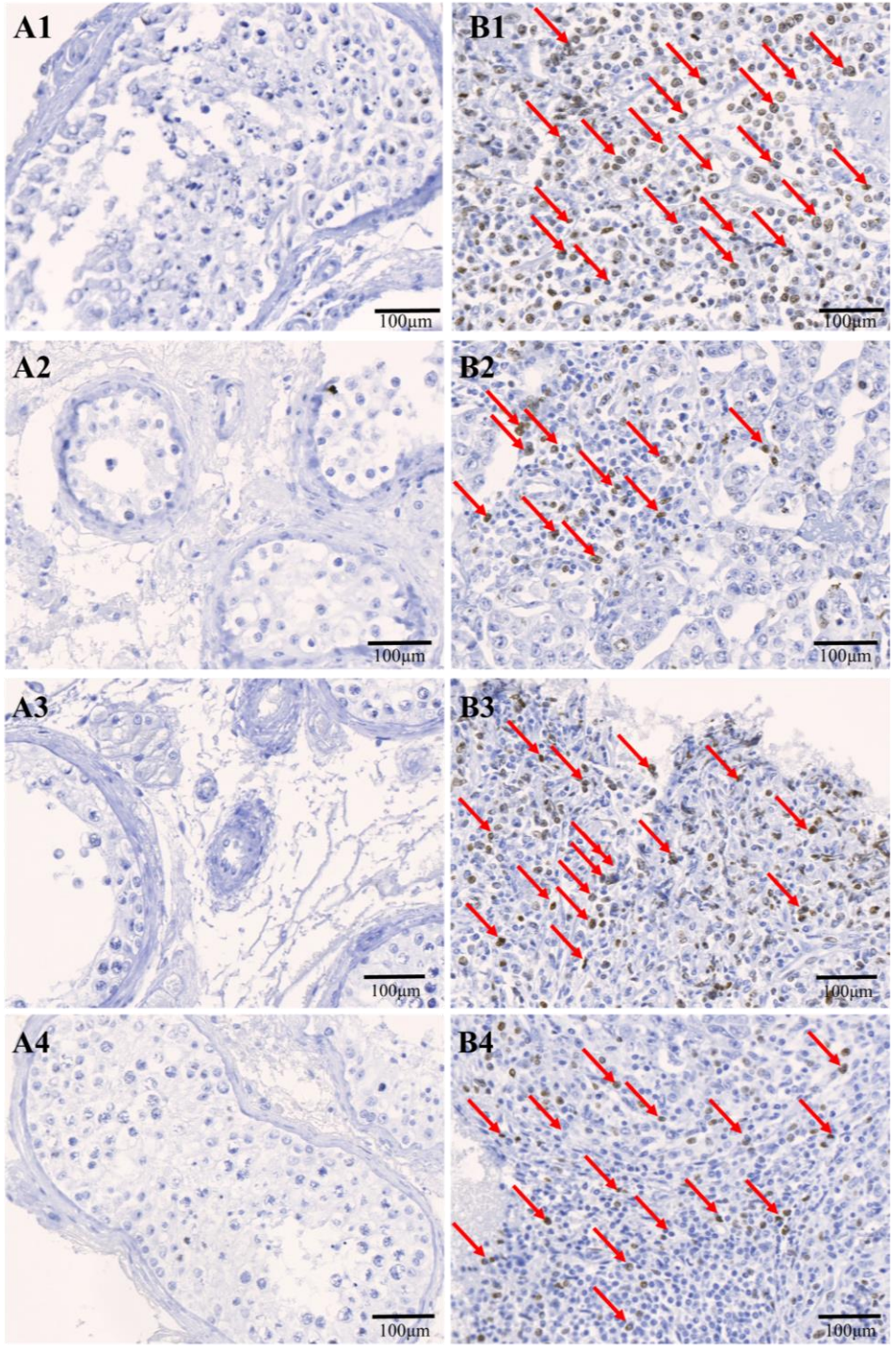
- Ki-67 is an important and reliable predictive and prognostic marker in breast cancer and lung cancer, its crucial role was suggested in TGCT assessment for severity detection and fast diagnosis.

- The Ki-67 percentage score is defined as the percentage of positively stained tumor cells among the total number of malignant cells assessed.

- The Ki-67 proliferation index is assessed by point counting 500 to 1000 cells and is reported as percentage of positive cells.

- In breast cancer, division rate less than 10% is low, 10-20% borderline, and more than 20% is high proliferation index.

- In TGCT no standard cut off values are set, so far 8 patients' tissues were analyzed, and they mostly show higher than 10% proliferation index.



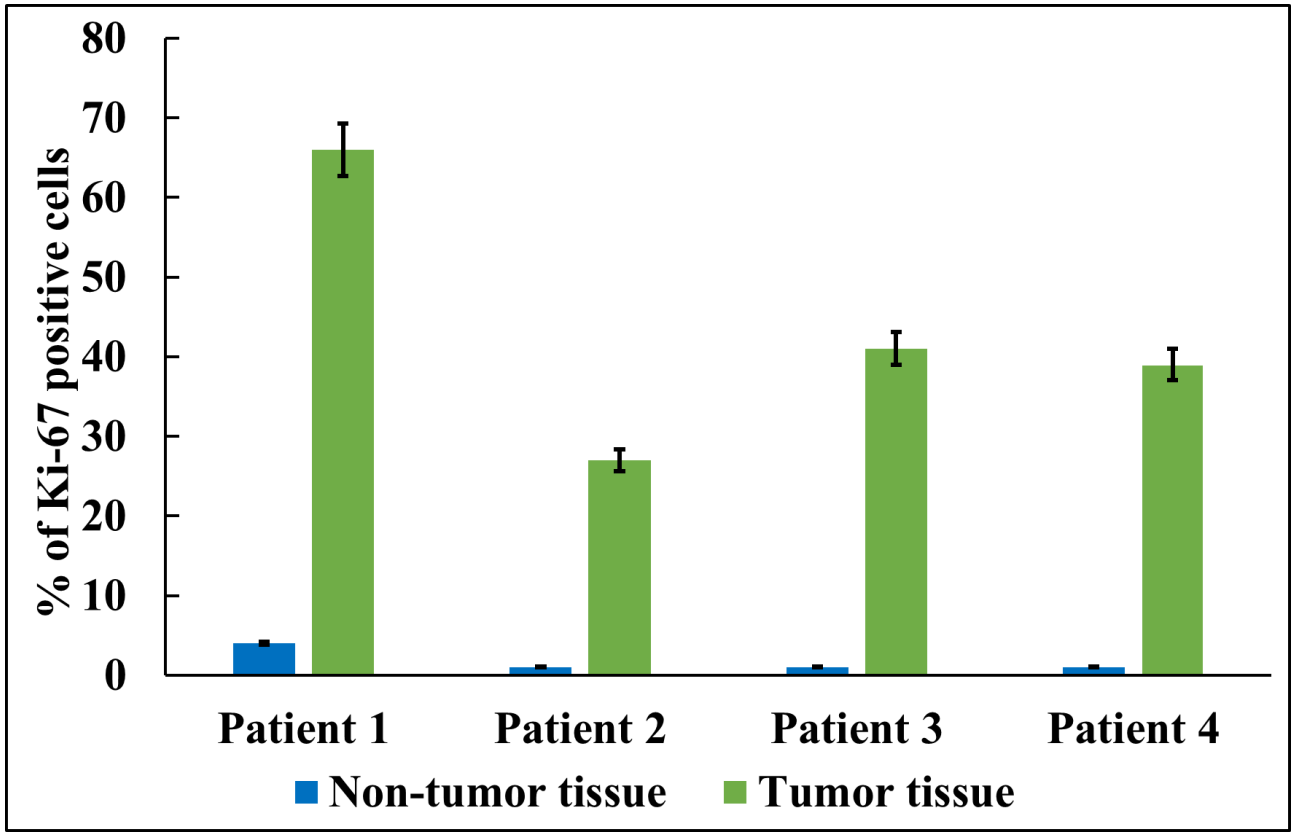
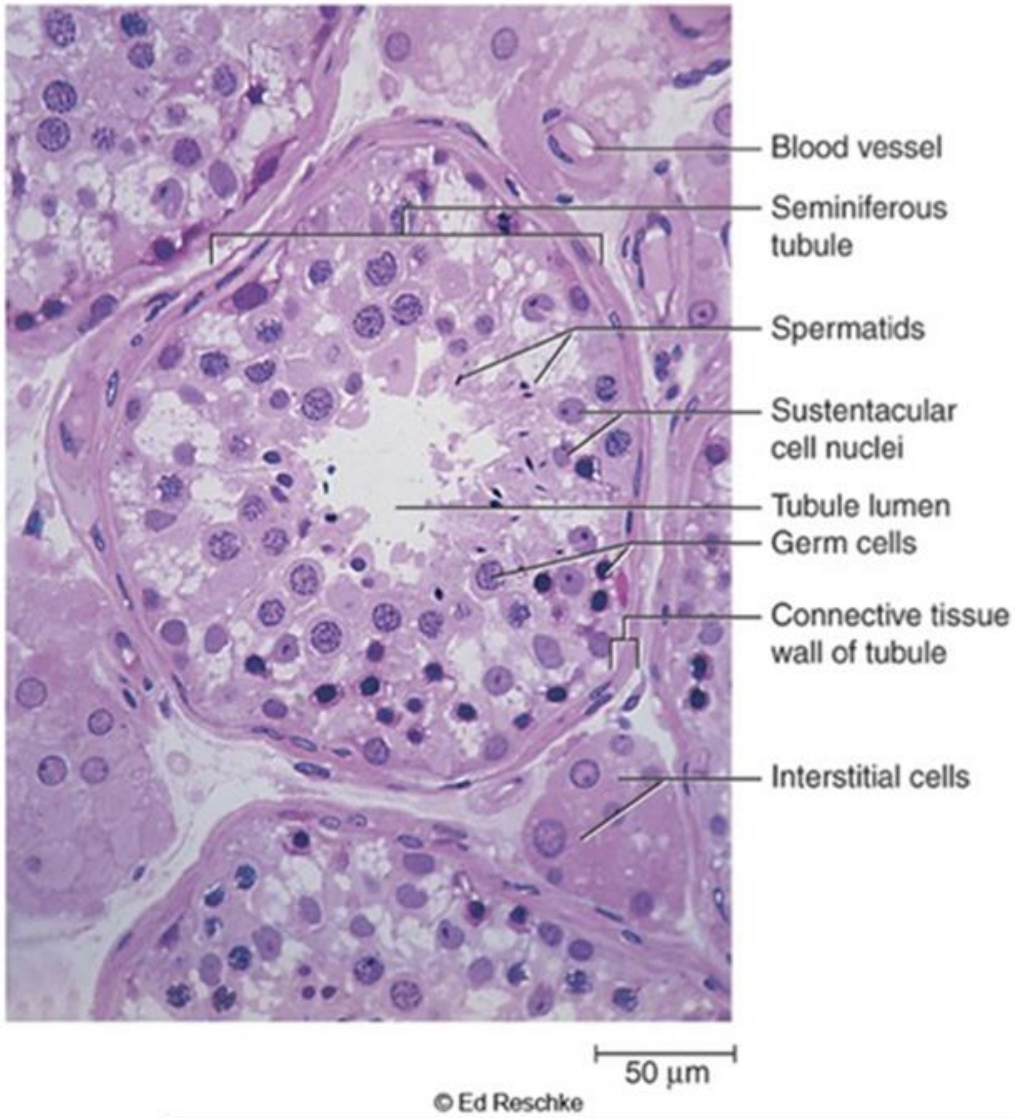


Figure 2: Ki-67 signal detection in non-tumor (A1-A4) and tumor (B1-B4) tissue of 4 TGCT patients and data graphical presentation.
(A1-A4) Non-tumor tissue without any Ki-67 signals.
(B1-B4) Tumor tissue with positive Ki-67 signal.
(B1) cells > 60% - extremely high proliferation rate.
(B2) cells > 18% - medium proliferation rate.
(B3) cells > 40% - high proliferation rate
(B4) cells > 35% - high proliferation rate

Human Seminiferous tissue



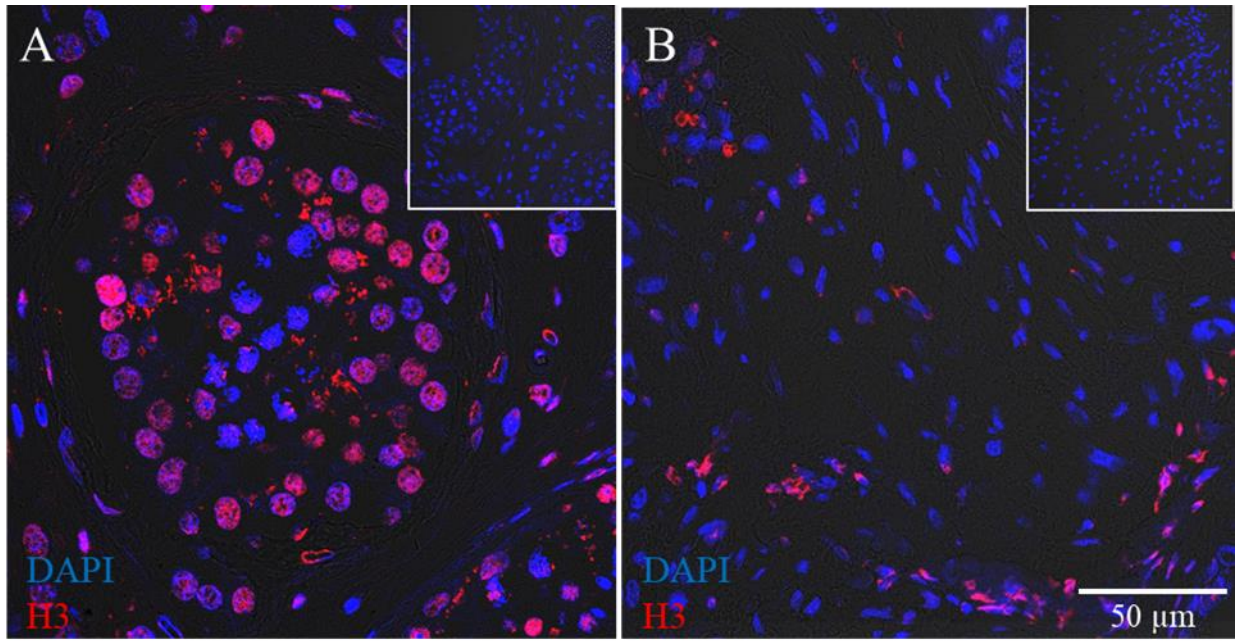


Figure 3: Detection of histone H3 in non-tumor (A) and tumor (B) testicular tissue. Both samples are from a TGCT patient. No morphological structures were found in the tumor tissue, seminiferous tubules are disrupted (B). Negative controls without primary antibody are shown in the corner.

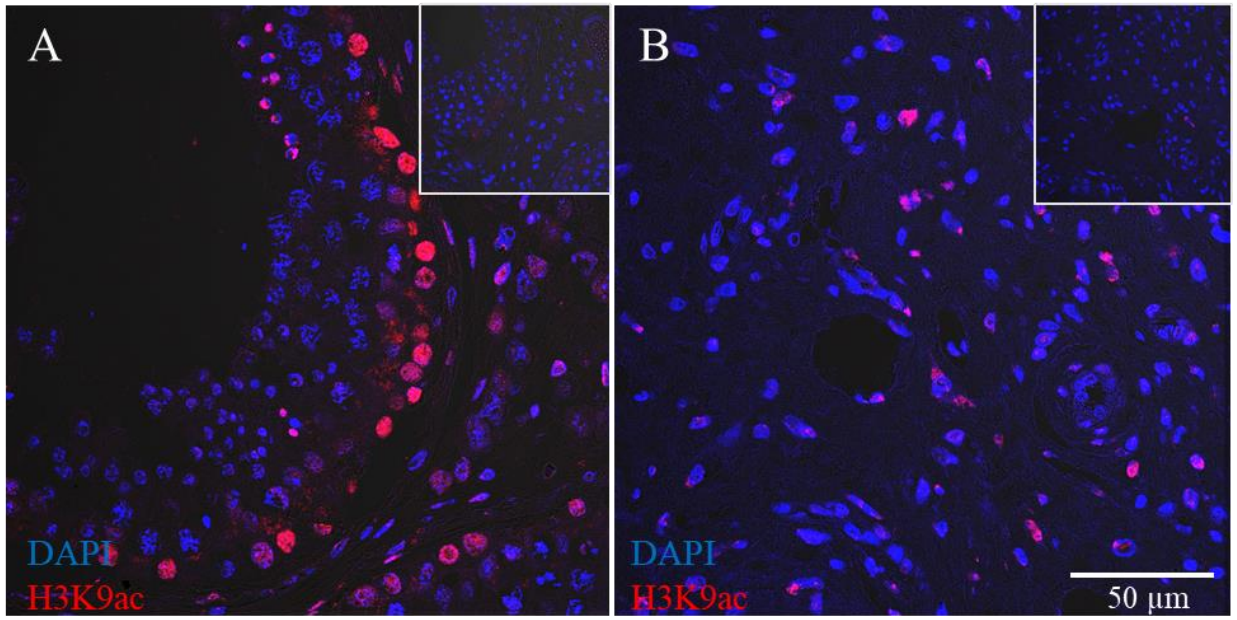


Figure 4: Detection of acetylated histone H3 (H3K9ac) in non-tumor (A) and tumor (B) testicular tissue. Both samples are from a TGCT patient. No morphological structures were found in the tumor tissue, seminiferous tubules are disrupted (B). Negative controls without primary antibody are shown in the corner.

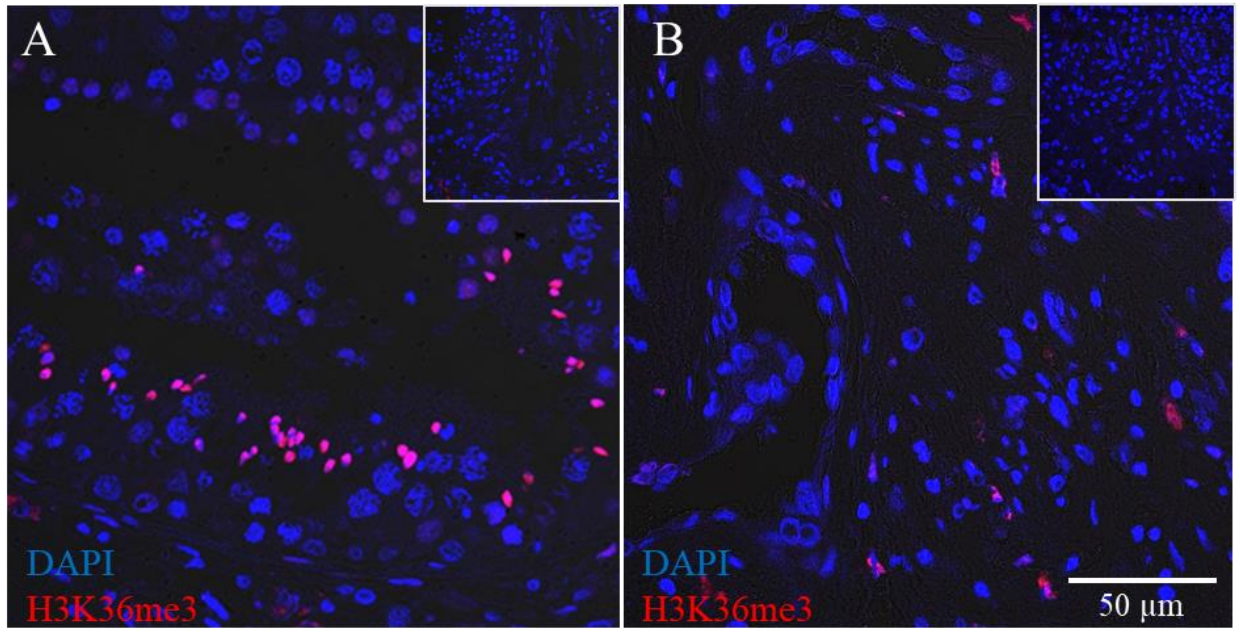


Figure 5: Detection of methylated histone H3 (H3K36me3) in non-tumor (A) and tumor (B) testicular tissue. Both samples are from a TGCT patient. No morphological structures were found in the tumor tissue, seminiferous tubules are disrupted (B). Negative controls without primary antibody are shown in the corner.

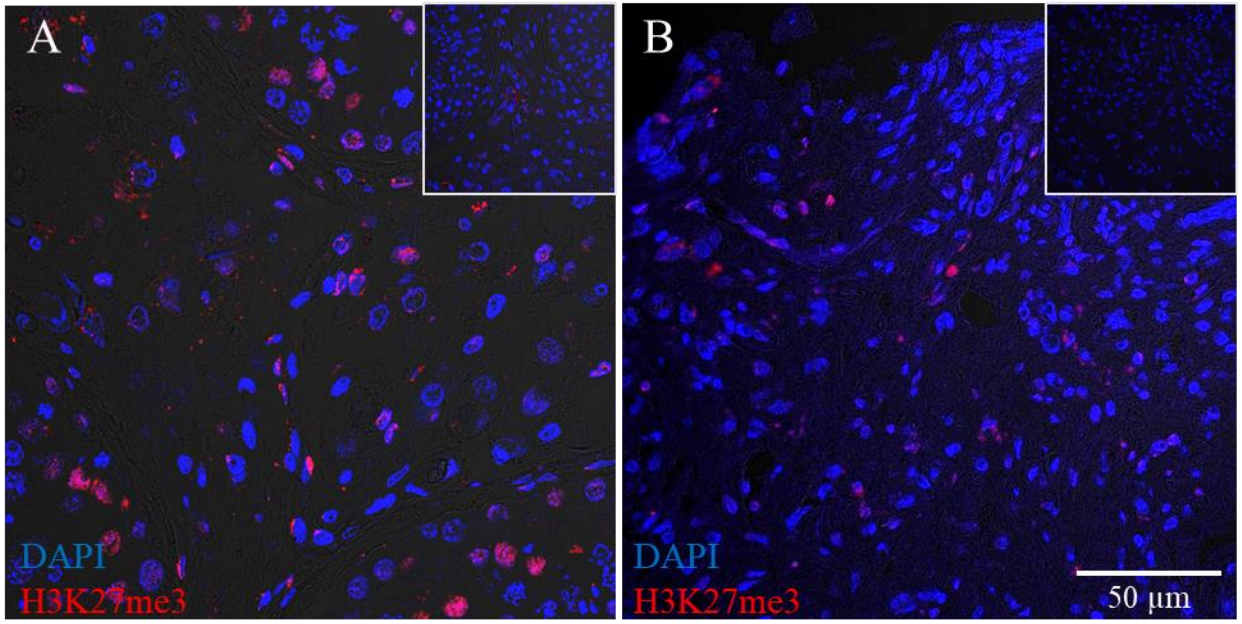


Figure 6: Detection of methylated histone H3 (H3K27me3) in non-tumor (A) and tumor (B) testicular tissue. Both samples are from a TGCT patient. No morphological structures were found in the tumor tissue, seminiferous tubules are disrupted (B). Negative controls without primary antibody are shown in the corner.

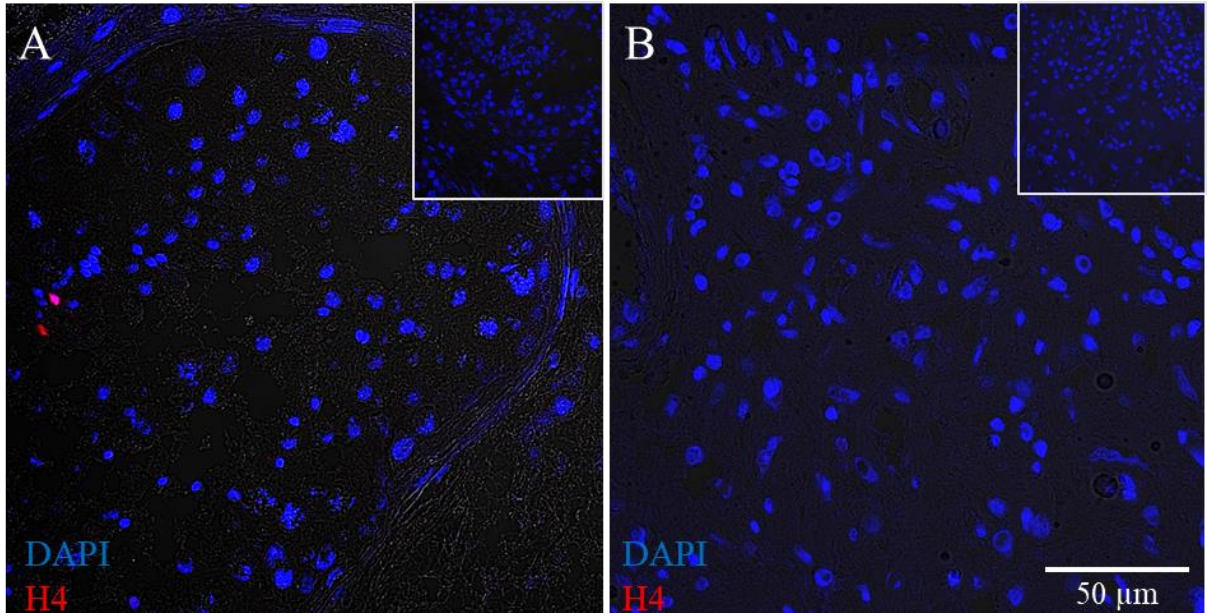


Figure 7: Detection of methylated histone H4 in non-tumor (A) and tumor (B) testicular tissue. Both samples are from a TGCT patient. No morphological structures were found in the tumor tissue, seminiferous tubules are disrupted (B). Negative controls without primary antibody are shown in the corner.

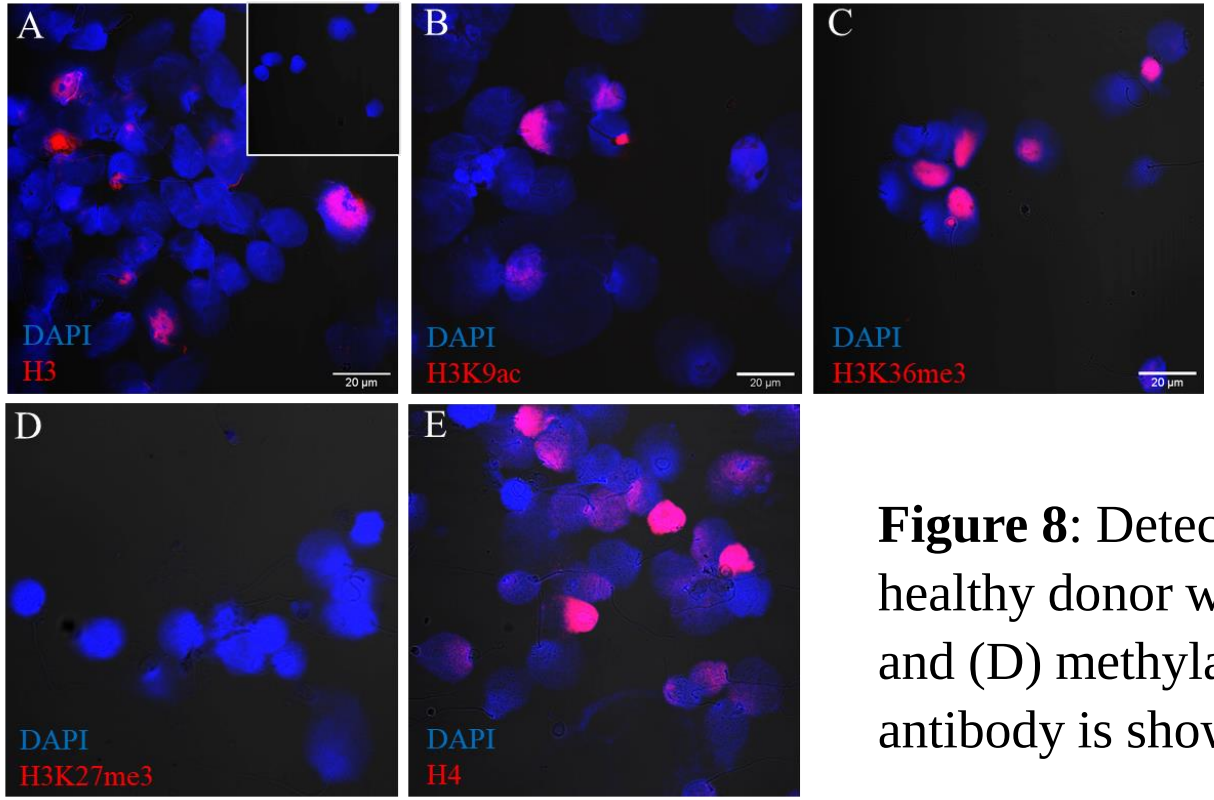


Figure 8: Detection of epigenetic markers in decondensated sperm of one healthy donor with normospermia. (A) histone H3; (B) acetylated H3; (C) and (D) methylated H3; (E) histone H4. Negative control without primary antibody is shown in the corner.

Sperm Quality Comparison

Table 1. Spermiogram, Normozoospermia, WHO 2021 vs TGCT sperm.

Spermiogram	Total sperm count in ejaculate	Ejaculate volume	Sperm concentration	Total motility	Progressive motility	Sperm morphology (normal)
Normospermic (WHO 2021)	39 - 928 mil	1.5 - 7.6 mL	15 – 259 mil/mL	40 - 81 %	32 - 75 %	4 - 48 %
TGCT patients	76.4 mil	3.1 mL	28.1 mil/mL	47.8 %	35.9 %	7.7 %

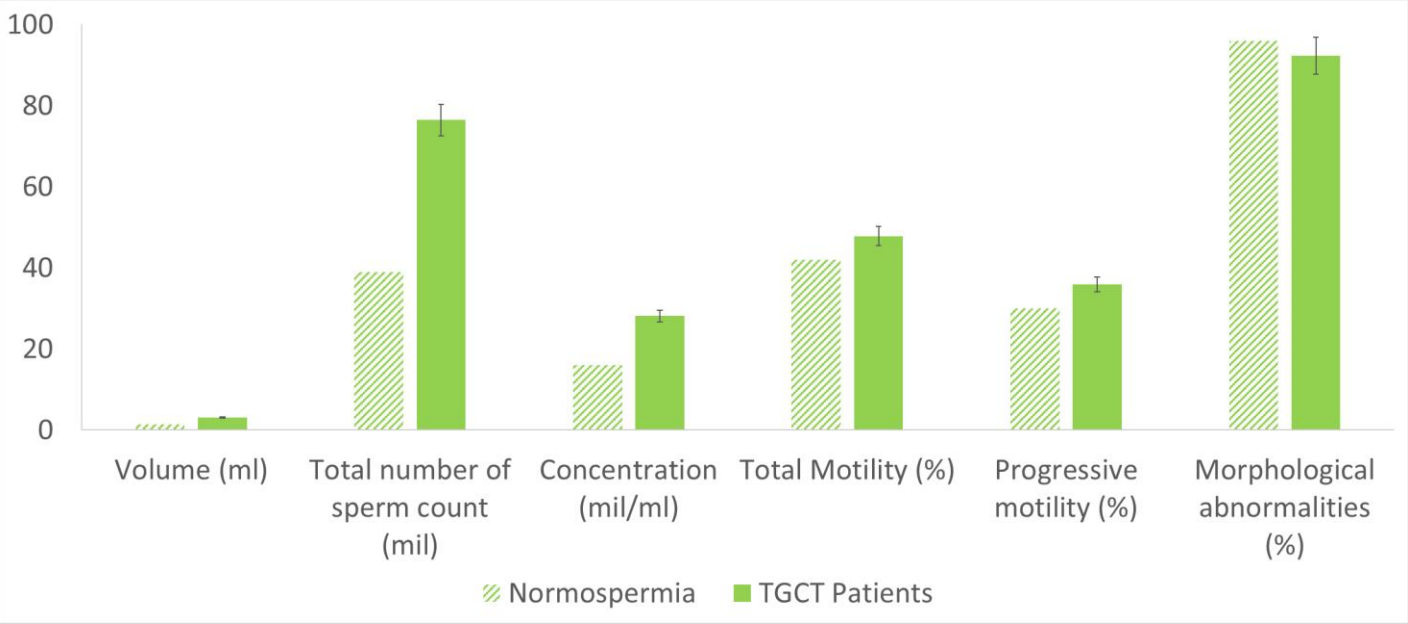


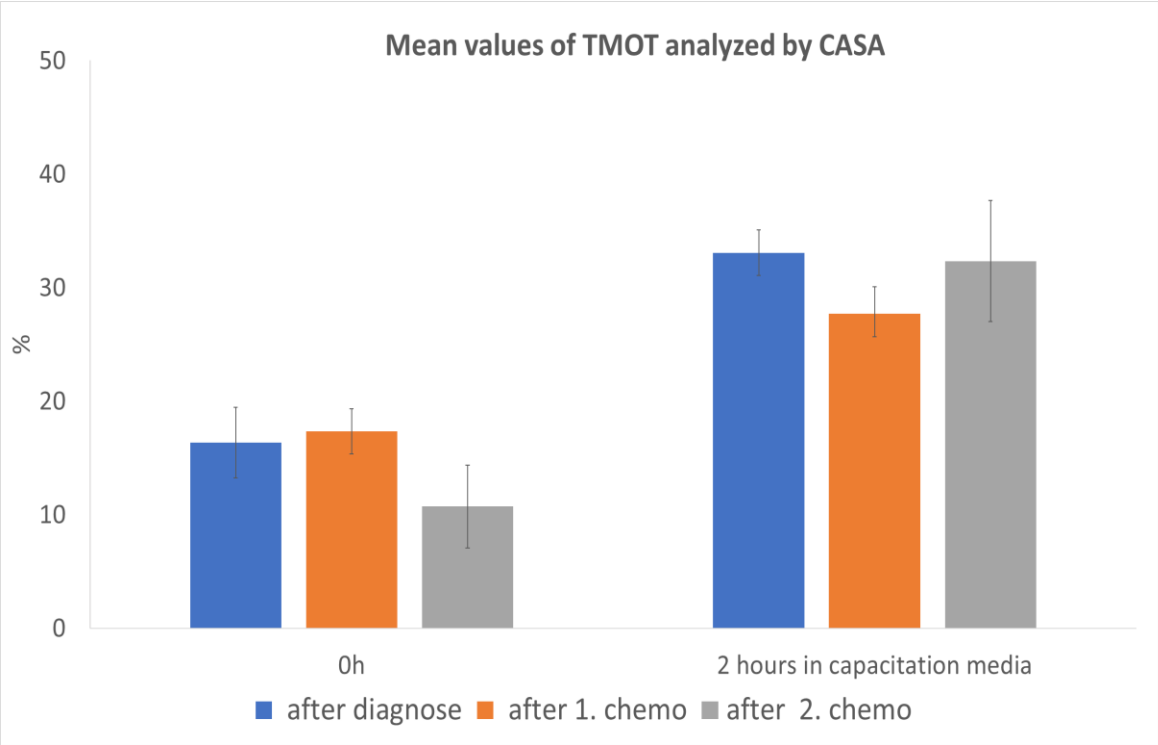
Figure 9: Average spermiogram parameters of TGCT patient; > 92 % sperm display morphological abnormalities.

Table 2. TGCT patients' spermiogram parameters.

Patient Number	Volume (ml)	Total number of sperm count (mil)	Concentration (mil/ml)	Total Motility (%)	Progressive motility (%)	Morphological abnormalities (%)
1	3.9	117	30	33.3	16.7	90
2	3.5	29.1	8.3	27.7	24.1	98
3	2	140	70	54.3	40	90
4	4.1	59.9	14.6	31.5	24.7	90
5	3	105	35	34.3	25.7	90
6	1.7	188.7	111	48.6	36.9	90
7	2	2.6	1.3	53.8	7.7	99
8	1.4	32.2	23	65.2	52.2	95
9	4	228	57	26.3	17.5	97
10	1.6	5.1	3.2	53.1	37.5	97
11	2.3	39.1	17	29.4	23.5	97
12	5.3	106	20	60	50	90
13	2.4	1.7	0.7	0	0	99
14	7.1	92.3	13	46.2	38.5	90
15	1.2	0.2	0.2	0	0	100
16	3	90	30	60	60	90
17	1.8	163.8	91	59	59	80
18	3.5	248.5	71	43.7	32.4	90
19	2.2	61.6	28	64.3	46.4	90
20	2.5	142.5	57	63.2	49.1	90
21	2.5	122.5	49	69.4	59.2	90
22	1.5	18	12	58.3	25	97
23	1.2	13.2	11	45.5	36.4	90
24	8.2	48.4	5.9	22	13.6	97
25	1.7	76.5	45	53.3	40	90
26	2.5	60	24	54.2	41.7	85
27	3.5	157.5	45	86.7	80	85
28	3.1	145.7	47	83	76.6	97
29	0.5	29.5	59	64.4	55.9	85
30	6.1	170.8	28	60.7	53.6	90
31	4.2	29.4	7	28.6	17.1	98
32	6.1	61	10	30	30	97
33	1.5	16.5	11	54.5	36.4	90
34	3.2	4.8	1.5	40	13.3	98
35	2.2	0.2	0.1	0	0	100
36	3	33	11	81.8	72.7	85
37	4.5	6.3	1.4	64.3	14.3	97
38	3.2	57.6	18	66.7	55.6	85

Sperm motility analyzed by CASA

A



B

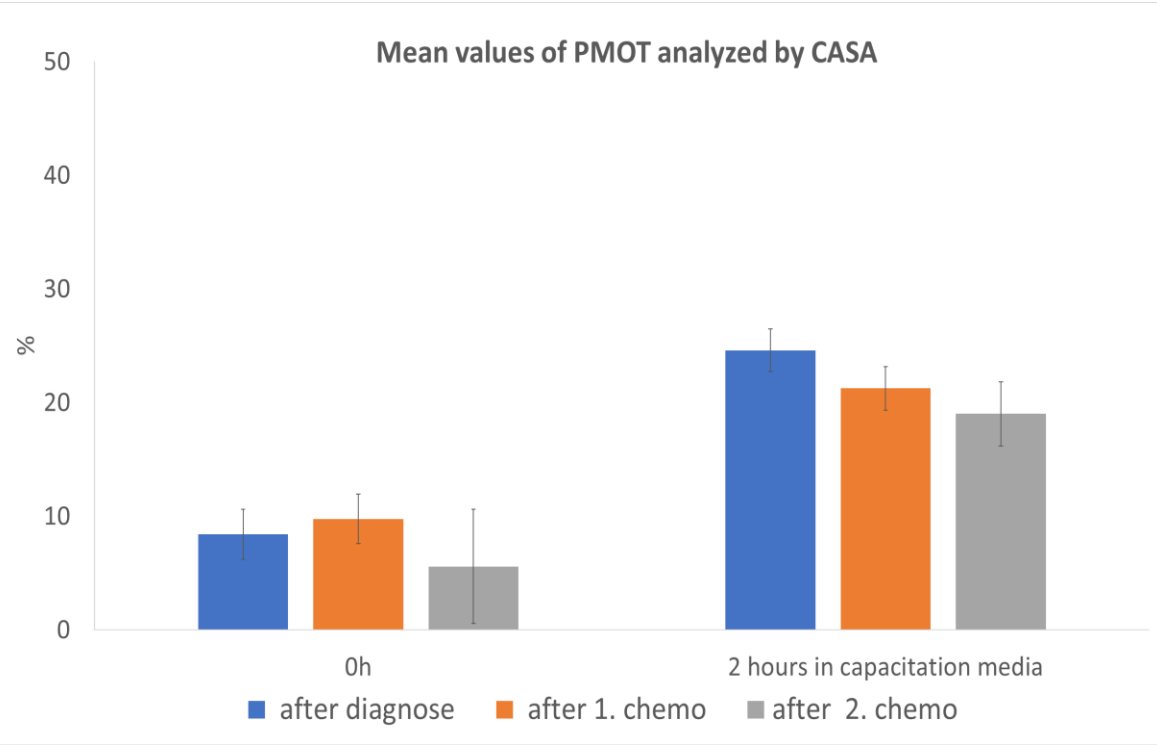


Figure 10: Mean values of sperm motility at initial time (0 h) and after capacitation *in vitro* (2 h). (A) total motility (TMOT), (B) progressive motility (PMOT).
TGCT patients: diagnosed (n=15); 1. chemotherapy (n=12), 2. chemotherapy (n=7). Error bars represent ±SEM.

Sperm kinematic parameters analysed by CASA

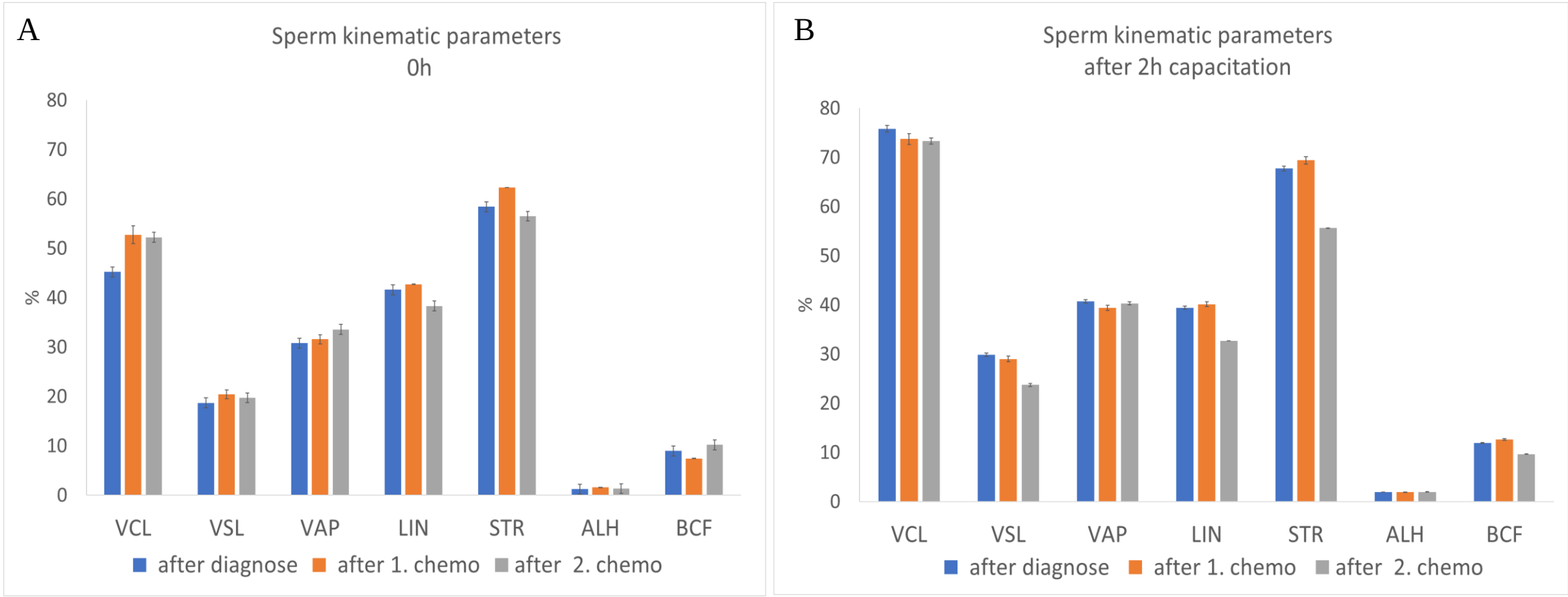


Figure 11: Mean values of sperm kinematic parameters at initial time (0 h) and after capacitation *in vitro* (2 h). TGCT patients: diagnosed (n=15); 1. chemotherapy (n=12), 2. chemotherapy (n=7). Error bars represent ±SEM. VCL – curvilinear velocity path, VSL – straight line velocity, VAP – average velocity path, LIN – linearity, STR – straightness, ALH – lateral head displacement, BCF – beat cross frequency

Sperm acrosome damage visualization

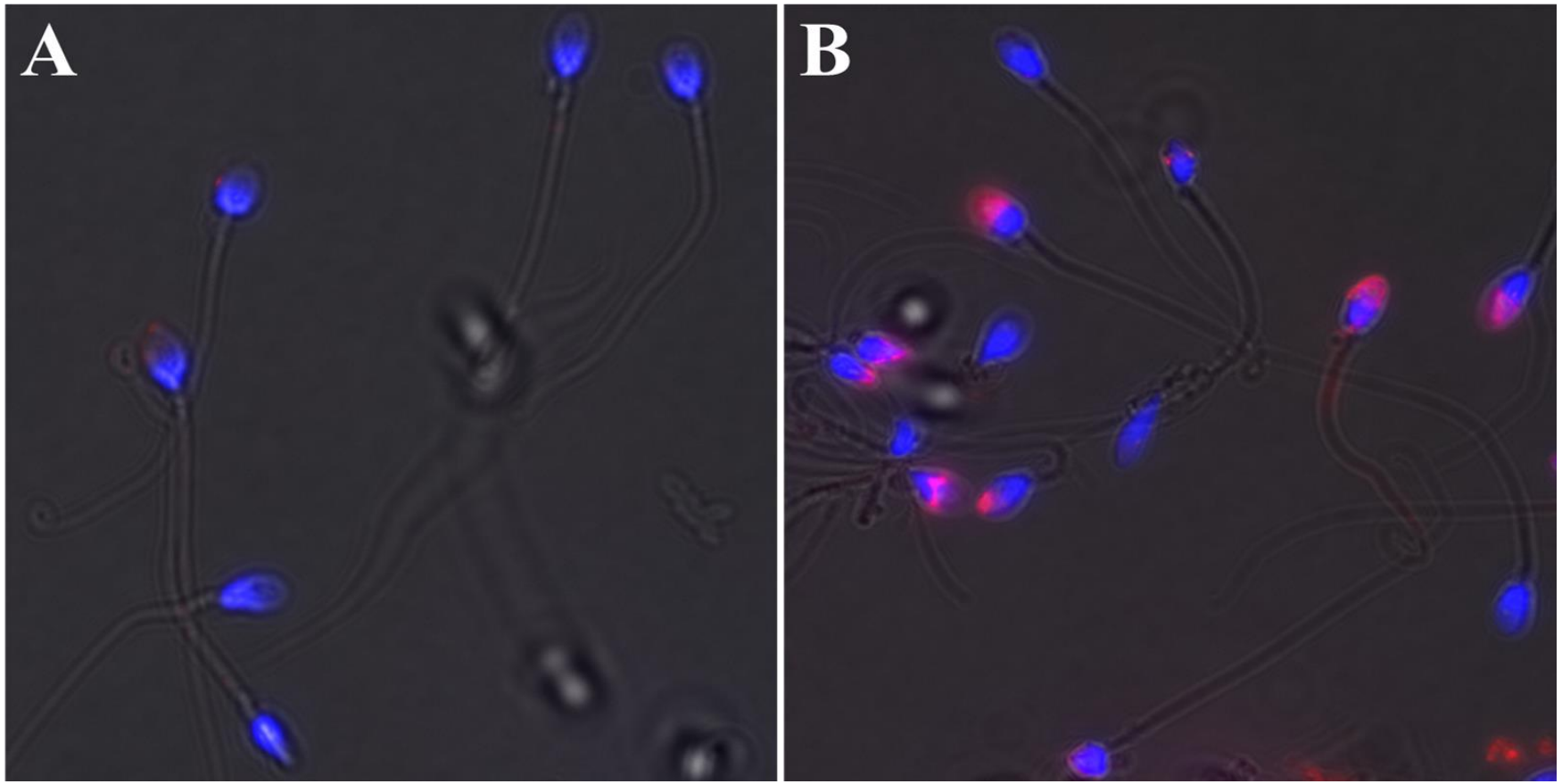


Figure 12: Acrosome integrity assessment (A) Control sperm from healthy donor, majority of sperms display intact acrosome. (B) Sperm from TGCT patient with damaged acrosomes labeled by PNA (red). Nucleus labeled by DAPI (blue), fixed in PFA.

Sperm Damage quantification

Table 3. sperm acrosome assessment (%) healthy donors (A) vs TGCT patients (B), ±SEM.

Control	Control			Patient	Patient		
n	Intact %	SEM	Damaged %	n	Intact %	SEM	Damaged %
9	61.1	0.35	38.9	9	5.9	0.13	94.1

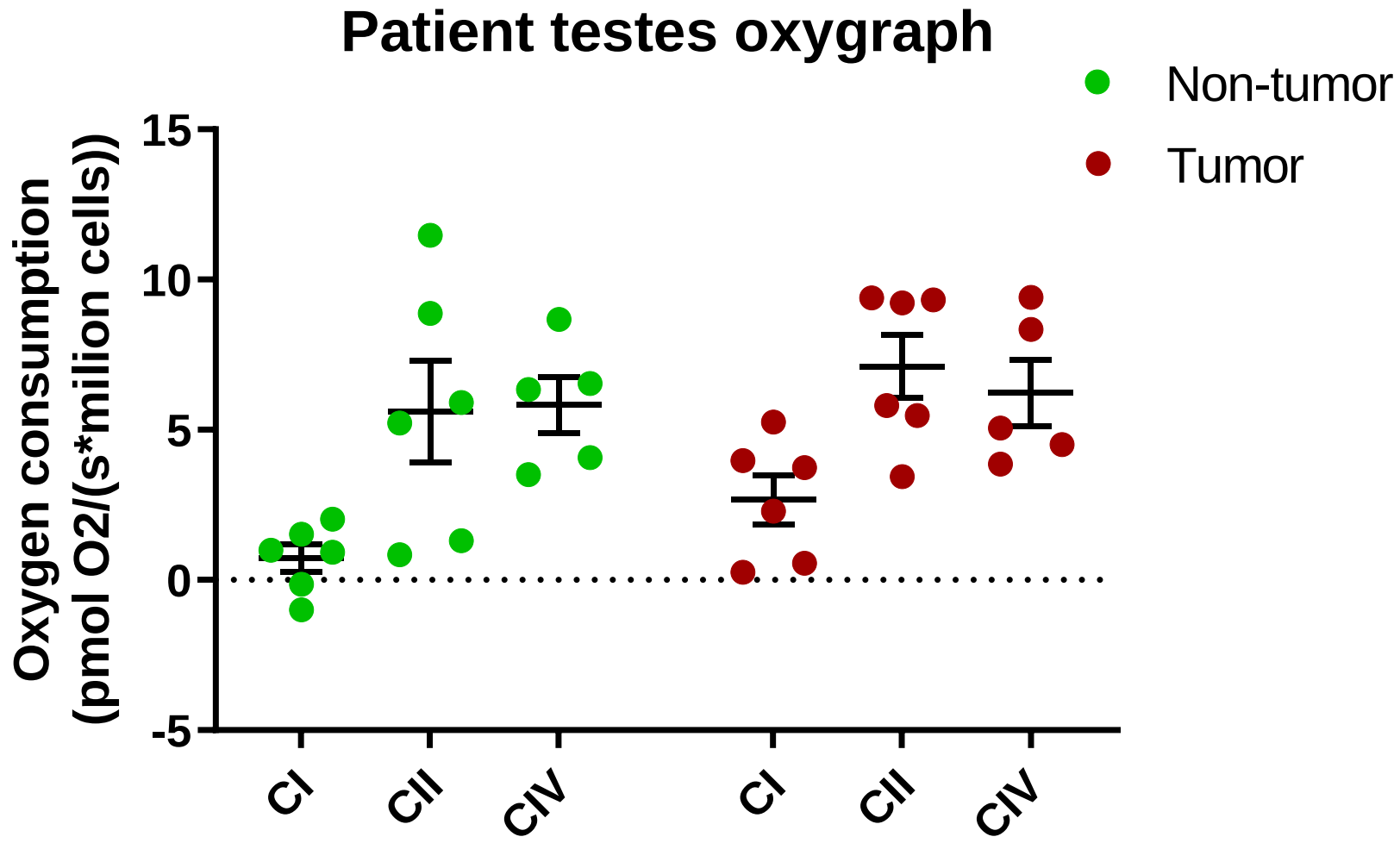


Figure 13: Oxygen consumption of testicular tissue measured by Oroboros O2k-FluoRespirometer (n = 6). Statistical analysis done by paired T-test where p = 0.1079.

Metabolic Two Photon FLIM analysis of TGCT tissue

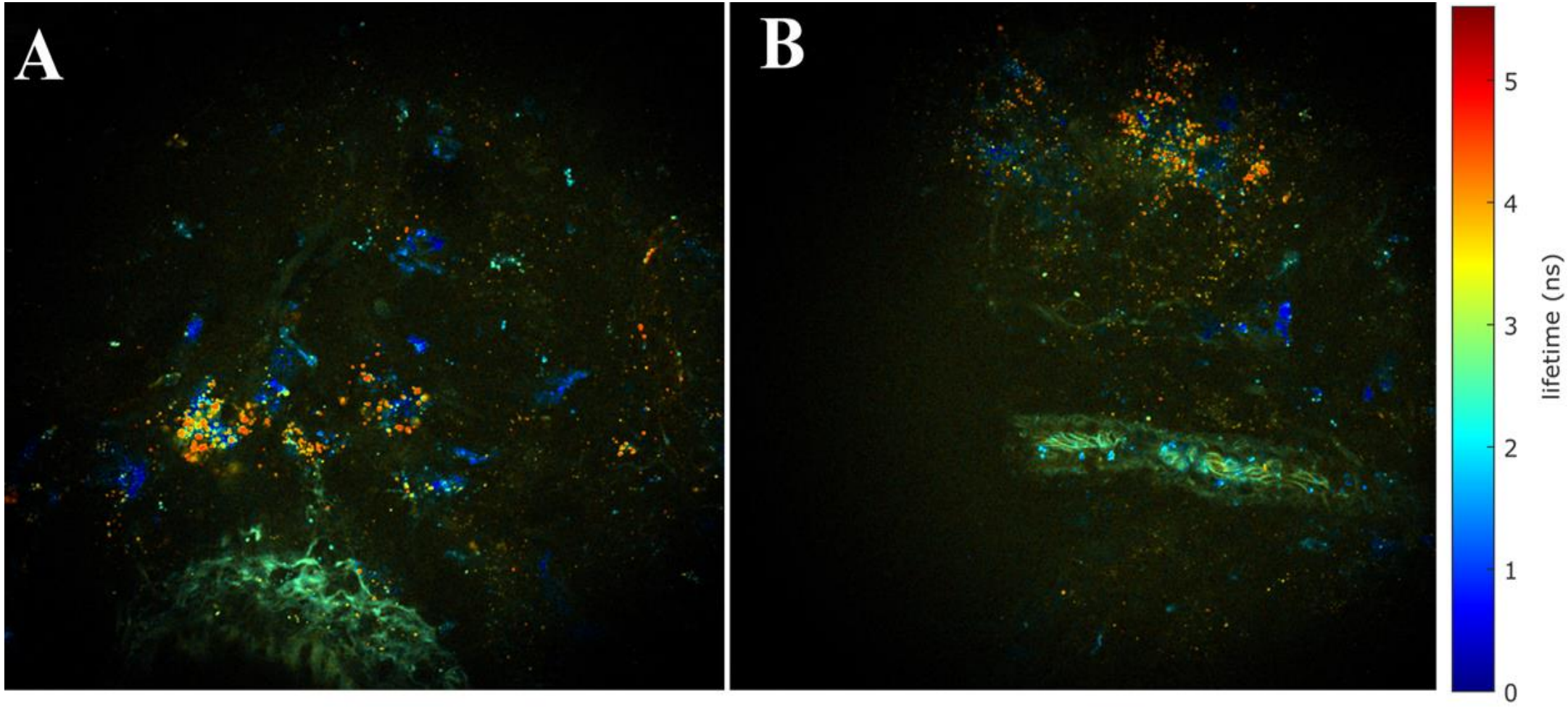


Figure 14: Characterization of intracellular amount of NADH and lifetime in human male testicular germ cell tumor (TGCT), 5 unique lifetimes in range between 0-5.6 ns. ((A) Non-tumor tissue show mostly two different patterns of NADH, one unbound from any enzyme with short lifetime, (B) TGCT tissue show NADH-enzyme bound form with longer lifetime.

Metabolic Two Photon FLIM analysis of TGCT Sperm

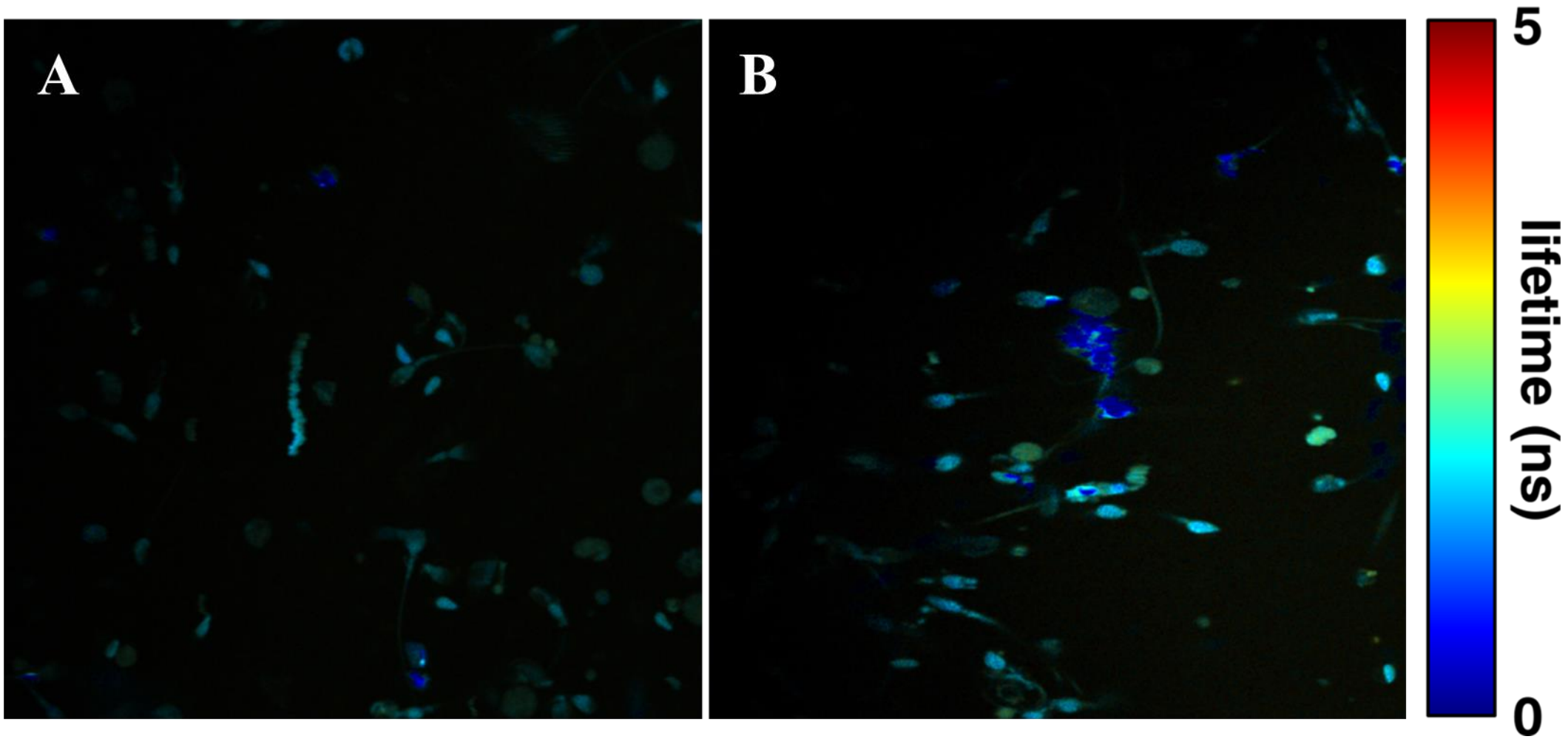
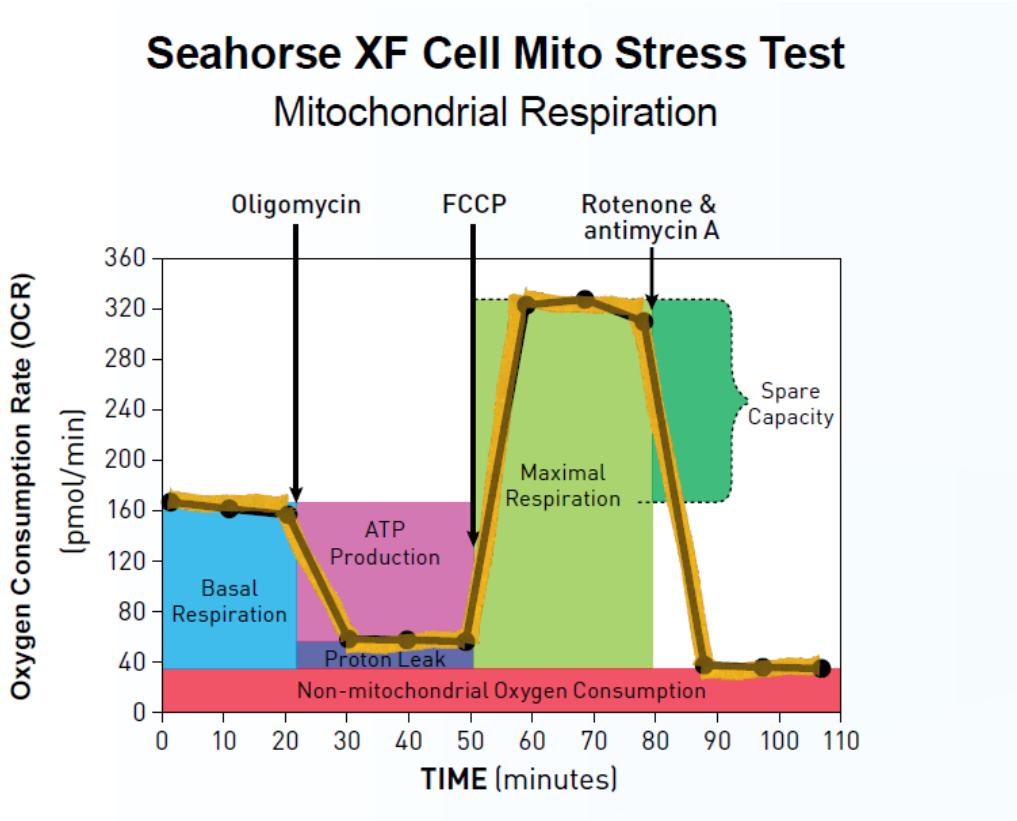


Figure 15: Characterization of intracellular amount of NADH and lifetime in human sperm where 5 unique lifetimes in range between 0-5.6 ns. (A) normospermic sperms show mostly two different patterns of NADH, one unbound from any enzyme with short lifetime, (B) TGCT affected sperms show NADH-enzyme bound form with longer lifetime.

Example of Ideal results according to Agilent

OCR

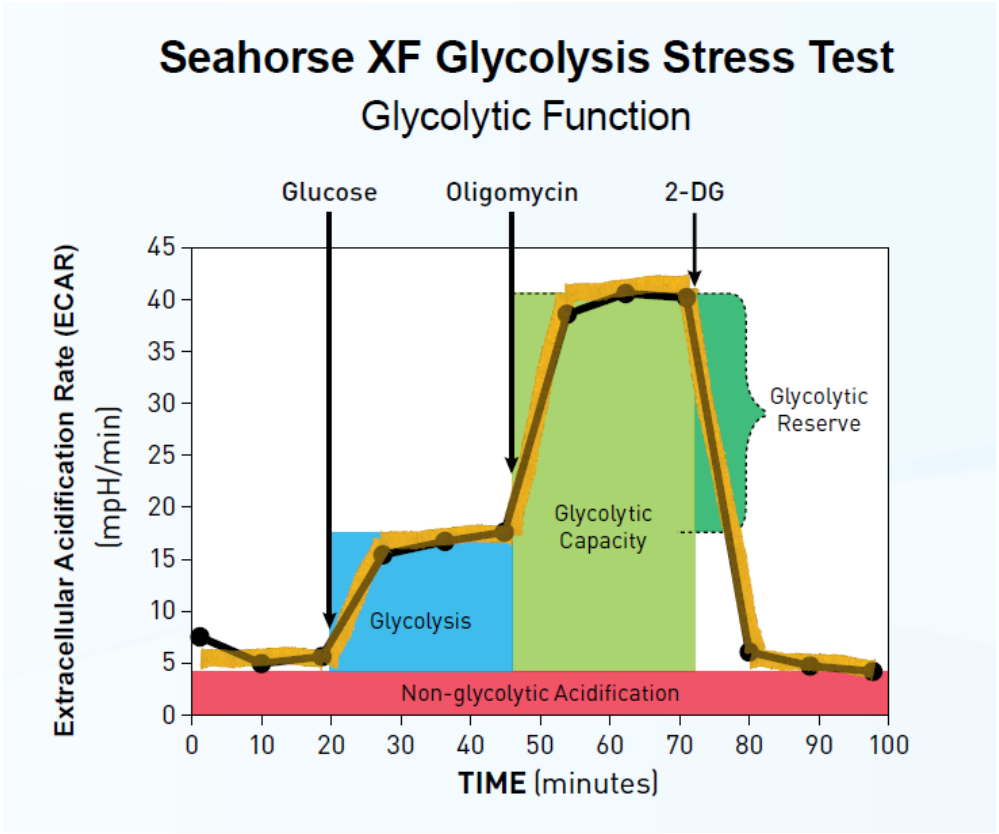
Oxygen Consumption Rate (OXPHOS)



The Seahorse XF Cell Mito Stress Test measures the key parameters of mitochondrial function: basal respiration, ATP production, proton leak, maximal respiration, and spare respiratory capacity.

ECAR

ExtraCellular Acidification Rate (GLYCOLYSIS)



The Seahorse XF Glycolysis Stress Test reports three key parameters of glycolytic function: glycolysis, glycolytic capacity, and glycolytic reserve.

Energetic metabolism of cryopreserved sperm analyzed by Seahorse XFp

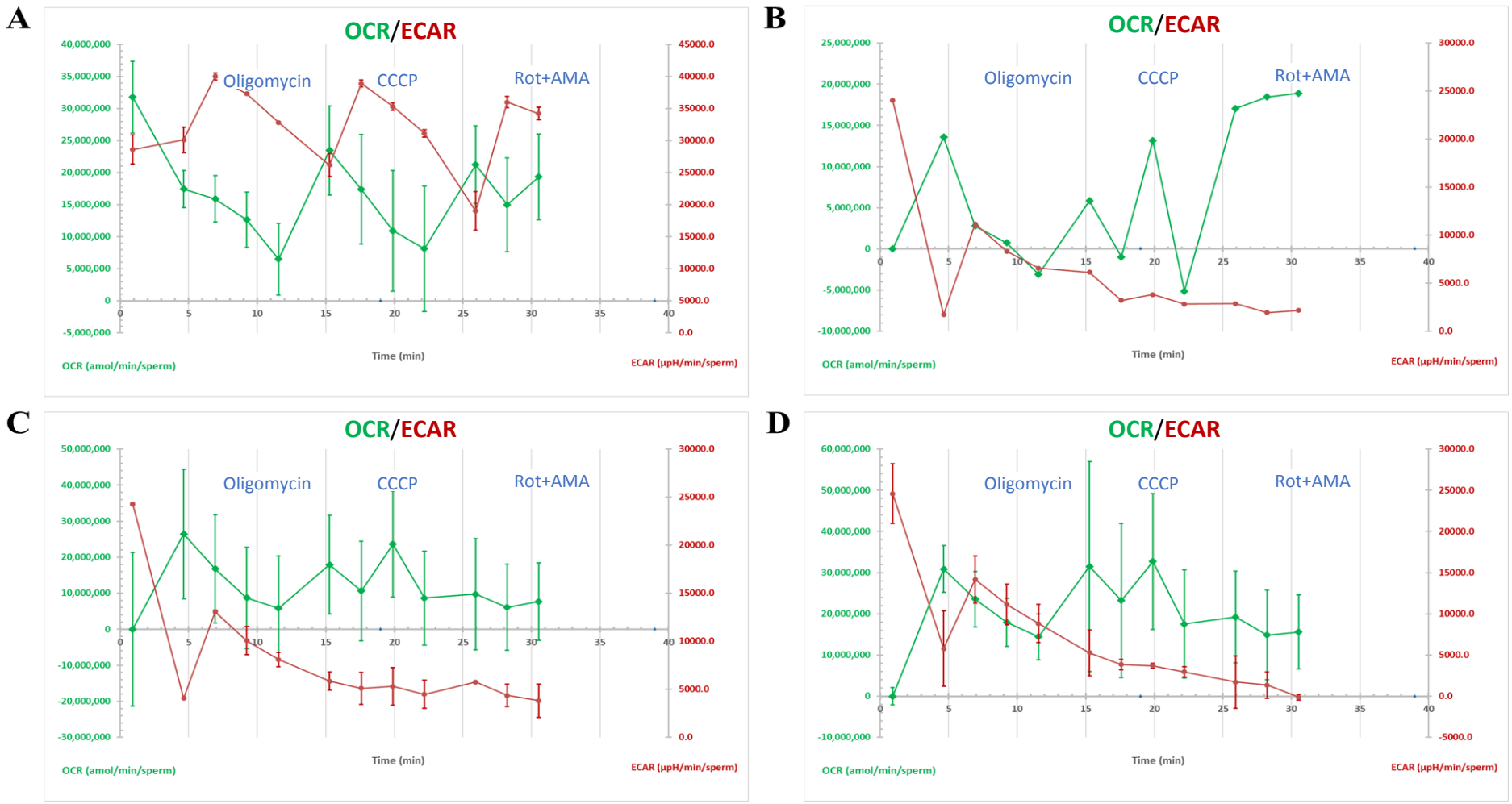


Figure 16. (A) Normospermic sample, (A,B,C) TGCT effected sperm from (B) Patient 1, (C) Patient 2, (D) Patient 3. Normospermic samples exert higher ECAR and lower OCR values, whereas TGCT pathological sperm exerts opposite phenomenon i.e. OCR is higher than ECAR. OCR is the oxygen consumption rate (OXPHOS), and ECAR extracellular acidification rate (GLYCOLYSIS) and performed in parallel.

Sperm viability and respiration activity monitored by ATP production rate

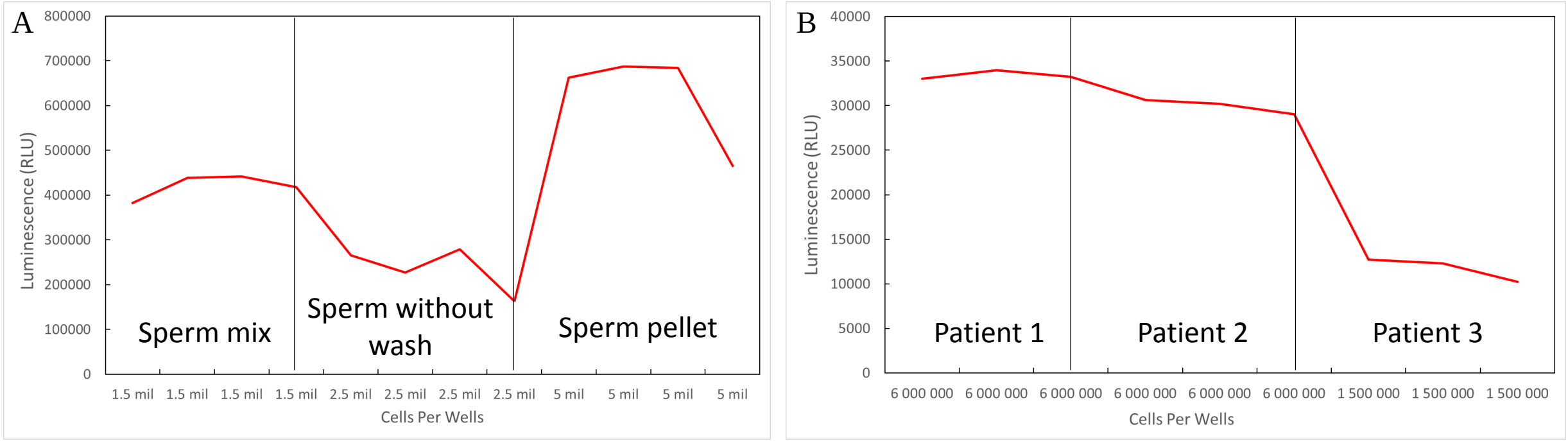


Figure 17: (A) Normospermic sample shows luminescence value indicating higher ATP production, whereas (B) TGCT affected patients' sperm (n=3) showed ATP production rate 20 times lower; RLU – relative light units.

Summary

- Ki67 proliferation marker is a promising method to be used for assessment of severity of TGCT.
- Histone modification assessment in testicular tissue and sperm shows differences between tumor and non-tumor tissue, and further study and correlation analysis is needed.
- Total and progressive sperm motility by CASA points to the best sperm quality in the sample prior to 1^{st} and 2^{nd} chemotherapy.
- Sperm acrosome assessment is crucial for prediction of optimal IVF method, if acrosomes are severely damaged, ICSI is the best (only) choice, but acrosome damage corelates with DNA damage and should not be ignored.
- Altered mitochondrial activity was detected in TGCT tissue and sperm compared to non-tumor tissue and sperm of healthy donors.
- Potential use of mitochondrial markers as tools to diagnose sperm reproductive parameters of TGCT affected men will be considered.

Acknowledgement

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Dalibor Pánek, Imaging methods core facility at BIOCEV

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Thank you for your attention