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Original research

## Cumulative increases in circulating mtDNA as a potential biomarker of brain injury in rugby union: a pilot study

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### ABSTRACT

**Objectives:** Rugby is a high contact sport that can lead to head contact events, especially in forward players, triggering inflammatory processes. Mitochondrial DNA, released during injury, may act as a proinflammatory signal. This study aimed to assess levels of three mitochondrial DNA forms—mitochondrial (Fraction 1), protein bound (Fraction 2), and naked (Fraction 3) – across a rugby season and correlate them with neuroinflammatory markers, blood parameters, and head impact exposure.

**Design:** Observational, longitudinal.

**Methods:** Thirteen male professional rugby players were monitored across two matches in the 2023–24 season. Blood samples were collected before (T0, T2), immediately after (T1, T3) each match, and one month post season (T4). Mitochondrial DNA levels and integrity were quantified, along with neuroinflammatory markers and pro-inflammatory cytokines, hematological parameters, immune receptor expression and monocyte distribution.

**Results:** Mitochondrial DNA levels increased progressively from T0 to T4, particularly in forward players. Post-match, Fraction 2 and 3 mitochondrial DNA levels were elevated, with a peak at T4. Interleukin-6 and interleukin-8 rose after both matches, while tumor necrosis factor- $\alpha$  increased only at T3. Neurofilament light chain levels spiked post-match but normalized afterward. Baseline mitochondrial DNA correlated with several hematological and metabolic markers, and immune cell subsets. Global Positioning System data linked mitochondrial DNA levels with high powered actions and contact intensity, especially in forward players.

**Conclusions:** Repeated head impacts in rugby lead to sustained mitochondrial DNA elevation, suggesting its potential as an early biomarker of cell damage and neuroinflammation. This may aid in preventing sports-related neurodegeneration.

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### Practical implications

- There is a progressive increase in circulating mtDNA over the course of a competitive season in contact sports, suggesting a cumulative effect of repeated head impacts.
- MtDNA can be released in response to head trauma, depending on positional playing role, and acts as inflammatory molecules. Depending

on its circulating form, it can change after each match and over the season. Depending on its circulating form, it may change over the course of the season and may undergo greater fragmentation after each match due to the trauma suffered.

- There are significant correlations between mtDNA levels and inflammatory markers such as IL-6, IL-8, TNF- $\alpha$ , and neurofilament light chain (NF-L), a well-known marker of axonal damage, reinforcing its potential role as a marker of neurotrauma.
- There are correlations between mtDNA levels and GPS-tracked high-intensity actions, demonstrating a relationship between physical exertion, contact frequency, and neuroinflammatory responses.

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## 1. Introduction

Concussion is a complex pathophysiologic process affecting the brain that causes neuropathological disturbances.<sup>1</sup> The Zurich Consensus (2012) differentiated concussion from traumatic brain injury (TBI), whereas the American Academy of Neurology (AAN) (2013) categorized concussion as a mild TBI (mTBI), defining it as a biomechanically induced brain function alteration, typically impairing memory and orientation, with or without loss of consciousness (LOC).<sup>2,3</sup> The 2023 CISC consensus statement on concussion in sport updated the definition of concussion to “traumatic brain injury caused by a direct blow to the head, neck or body resulting in an impulsive force being transmitted to the brain that occurs in sports and exercise-related activities.”<sup>4</sup>

Concussions are typically caused by acceleration-deceleration forces, producing rotational and linear brain stress. These forces initiate metabolic cascades, such as glutamate-mediated potassium efflux and calcium influx, resulting in a transient hypermetabolic state.<sup>4</sup> Clinically, mild cases manifest as confusion and disorientation that often resolve spontaneously. Post-concussive symptoms may include headache, dizziness (often disequilibrium), and cognitive deficits in memory and attention.<sup>2</sup>

In adults, contact sports remain one of the leading causes of concussion, with estimates reaching approximately 3.8 million sports- and recreation-related head injuries each year in the United States, although the actual number may be higher due to underreporting.<sup>1,5</sup> Rugby, a high-contact sport with frequent tackles, carries a high risk, especially for forwards who endure more impacts.<sup>6</sup> Concussed athletes are immediately removed from play and monitored until symptoms resolve.<sup>6</sup>

The World Rugby acknowledges a link between repeated head impacts and cognitive impairment, depression, memory loss, and reduced verbal fluency, with effects detectable three months post-injury.<sup>7</sup> Chronic traumatic encephalopathy (CTE), a neurodegenerative disease, is common in athletes exposed to repetitive head trauma.<sup>5</sup> A single moderate/severe brain injury may also trigger neurodegenerative mechanisms, including dementia. Neurodegeneration is associated with dysregulated inflammation in the central nervous system (CNS), disrupting cellular homeostasis.<sup>8</sup> Head injury-induced oxidative stress, largely via reactive oxygen species (ROS), perpetuates chronic neuroinflammation.<sup>9</sup> Key cytokines such as interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), involved in both repair and secondary injury, are produced in the brain and enter systemic circulation after blood-brain barrier (BBB) compromise.<sup>10</sup>

In this context, circulating cell-free mitochondrial DNA (cf-mtDNA) is emerging as a potential marker of inflammation and neuronal injury. Released after trauma, cf-mtDNA mimics bacterial DNA and acts as a damage-associated molecular pattern (DAMP), activating inflammatory pathways like nucleotide-binding domain like receptor 3 (NLRP3) inflammasome, toll-like receptor 9 (TLR9), and GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway, which induce cytokine release.<sup>11,12</sup>

While regular physical activity generally reduces mtDNA release and inflammation, excessive or traumatic exercise can elevate cf-mtDNA levels due to muscle damage and oxidative stress.<sup>10,13</sup> Additionally, neurofilament light chain (NF-L) and glial fibrillary acidic protein (GFAP) are promising biomarkers of axonal and astrocytic injury, respectively.<sup>14</sup> NF-L, a cytoskeletal protein absent in healthy circulation, is released upon axonal damage and correlates with longer recovery.<sup>15</sup> GFAP, expressed in astrocytes and upregulated after injury, is linked to head impact severity.<sup>14</sup> Elevated plasma NF-L and GFAP may thus indicate severe brain injury, particularly with LOC, ataxia, or prolonged symptoms.<sup>16</sup>

This study investigates whether cf-mtDNA, alongside cytokines and neuroinflammatory markers, could serve as an early biomarker of brain injury in rugby players. We explored associations with cf-mtDNA levels and integrity, inflammation profiles, hematological parameters, GPS tracking, and brain injury incidence and severity, aiming to develop preventive strategies for concussed athletes.

## 2. Methods

Thirteen male rugby players, aged 20–30, were recruited from a professional team during the United Rugby Championship season 2023–24. All enrolled athletes have obtained a certificate of competitive activity from the professional sports club. Athletes showing signs of inflammatory diseases or injuries occurring just before or during the study were excluded.

This study was performed in agreement with the Declaration of Helsinki and approved by Ethics Committee of Area Vasta Emilia Nord (protocol number 1104/2021/SPER/AUSLMO - 0028381/23). All the participants signed an informed consent form.

The athletes involved in the study played at least 40 min in each match examined, as shown in Supplementary Table 1. They were subjected to a venous blood sample of 9 mL immediately before (T0) and after (T1) the first match, before (T2) and after (T3) the second match, and about one month after the end of the season (T4) when the athletes can be considered at rest. The two matches analyzed were about two months apart during the playing season and separated by three matches, selected in order to obtain different measurements and monitor the athletes throughout the season. Both matches were played at the home stadium (Parma, Italy); the first match took place in January and the second about two months later. The post-season blood sampling was carried out in July, well after the end of the monitored season and before the start of the new training season. All blood samples collected were processed within 12 h. The head impacts received by each rugby player were counted during the two matches examined, recorded through video review and match observation. The workflow of the study is shown in Supplementary Fig. 1.

Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from venous blood using a density-gradient centrifugation standard method. Viable PBMCs were stored in liquid nitrogen, and plasma was stored at  $-80^{\circ}\text{C}$  until use. Plasma samples (named Fraction 1) were subjected to differential centrifugations. A first centrifugation at 18,000g for 30 min at  $4^{\circ}\text{C}$  was performed to obtain a fraction free from mitochondria, large EVs, and cell debris, named Fraction 2.<sup>17</sup> Subsequently, this fraction underwent ultracentrifugation at 100,000g for 2 h at  $4^{\circ}\text{C}$  to obtain Fraction 3, where naked cf-mtDNA is present, using the Optima MAX-XP instrument and the MLA-150 rotor (Beckman Coulter, Brea, CA, USA).

Fifty-four chemical, hematological and hormonal parameters, reported in Table 1 as mean and standard deviation, were evaluated at the BLU Laboratory of Baggiovara (NOCSAE, Baggiovara, Modena, certification #ISO90012015). Clinical chemistry analytes were measured in the CoreLab on full-automated clinical chemistry platforms, based on state-of-the-art enzyme kinetic techniques, immunoturbidimetric techniques, and colorimetric methods (Chemistry analyzerS Olympus 680 and LX20, Beckman Coulter). Complete blood count with formula was performed with Accucount technology for red and white cells and platelets and VCS technology and triple impedance counting for leukocyte formula and spectrophotometric determination of hemoglobin and reticulocytes (after staining with methylene blue) (Hematology Analyzers DXH 750 and DXH 800, Beckman Coulter). Blood circulating hormones were detected on full automated platforms based on CMIA methods (Chemiluminescent Immunoassay Analyzer: Architect, Abbott Laboratories, Chicago, Illinois, USA; DXI, Beckman Coulter; LiaisonXL, DiaSorin, Saluggia, Italy).

Total DNA was extracted from 200  $\mu\text{L}$  of plasma of all fractions, by using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The amount of mtDNA was quantified by droplet digital PCR (ddPCR) on a Bio-Rad QX200 ddPCR droplet system (Bio-Rad Laboratories, Hercules, CA, USA) following the protocol described by Kilic et al.<sup>18</sup> Droplet reading was performed on a QX200 ddPCR droplet reader and the analysis was performed using QuantaSoft Analysis software (version 1.7.4.0917). Moreover, analysis of cf-mtDNA integrity was conducted by ddPCR on Fractions 1, 2 and 3. A multiplex

**Table 1**  
Hematochemical and hormonal parameters evaluated at T0, T1, T2, T3, and T4. Values are expressed as mean and standard deviation.

	T0	T1	T2	T3	T4
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
White blood cells (*10 <sup>3</sup> /μL)	5.76 ± 1.02	12.07 ± 2.91	5.60 ± 1.36	13.08 ± 3.67	4.88 ± 0.70
Red blood cells (*10 <sup>3</sup> /μL)	5.24 ± 0.23	5.02 ± 0.25	5.07 ± 0.33	5.02 ± 0.30	5.03 ± 0.16
Hemoglobin (g/dL)	15.64 ± 0.70	14.98 ± 0.76	15.13 ± 0.87	14.93 ± 0.80	15.13 ± 0.44
Hematocrit (%)	46.13 ± 2.02	44.17 ± 1.94	45.13 ± 2.24	44.55 ± 2.17	45.36 ± 1.25
MCV (fl.)	88.20 ± 3.60	87.99 ± 3.46	89.25 ± 3.96	88.77 ± 3.49	90.31 ± 3.00
MCH (pg)	29.88 ± 1.29	29.84 ± 1.34	29.93 ± 1.57	29.77 ± 1.27	30.13 ± 1.13
MCHC (g/dL)	33.88 ± 0.53	33.92 ± 0.44	31.64 ± 6.17	33.52 ± 0.51	33.38 ± 0.32
RDW (cv%)	13.33 ± 0.45	13.15 ± 0.48	13.24 ± 0.52	13.17 ± 0.48	13.45 ± 0.45
Platelets (*10 <sup>3</sup> /μL)	220.38 ± 56.84	232.85 ± 47.74	219.60 ± 55.31	230.60 ± 37.29	209.67 ± 47.64
MPV (fl.)	8.77 ± 0.91	8.80 ± 0.81	8.90 ± 0.85	8.99 ± 0.78	8.67 ± 0.68
Neutrophils (%)	52.34 ± 8.97	83.69 ± 4.05	53.88 ± 8.60	82.01 ± 5.15	47.93 ± 6.26
Neutrophils (*10 <sup>3</sup> /μL)	3.05 ± 0.85	10.16 ± 2.69	3.10 ± 1.26	10.86 ± 3.51	2.36 ± 0.55
Lymphocytes (%)	34.65 ± 8.03	9.58 ± 3.16	34.71 ± 7.38	9.83 ± 3.98	39.56 ± 5.55
Lymphocytes (*10 <sup>3</sup> /μL)	1.97 ± 0.53	1.11 ± 0.30	1.87 ± 0.28	1.18 ± 0.23	1.92 ± 0.32
Monocytes (%)	9.99 ± 2.97	6.03 ± 1.18	8.96 ± 1.99	7.55 ± 1.70	9.02 ± 1.94
Monocytes (*10 <sup>3</sup> /μL)	0.57 ± 0.16	0.72 ± 0.22	0.49 ± 0.08	0.97 ± 0.31	0.44 ± 0.08
Eosinophils (%)	2.23 ± 1.23	0.33 ± 0.46	1.74 ± 1.01	0.28 ± 0.31	2.72 ± 1.67
Eosinophils (*10 <sup>3</sup> /μL)	0.13 ± 0.07	0.03 ± 0.04	0.10 ± 0.06	0.03 ± 0.03	0.13 ± 0.08
Basophils (%)	0.82 ± 0.36	0.41 ± 0.16	0.74 ± 0.28	0.36 ± 0.15	0.83 ± 0.41
Basophils (*10 <sup>3</sup> /μL)	0.05 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.02
Reticulocytes (*10 <sup>3</sup> /μL)	0.05 ± 0.02	0.06 ± 0.02	0.05 ± 0.02	0.06 ± 0.02	0.05 ± 0.02
Reticulocytes (%)	0.95 ± 0.36	1.14 ± 0.36	1.06 ± 0.31	1.20 ± 0.34	1.08 ± 0.33
Protein C (%)	99.69 ± 14.16	79.08 ± 16.28	96.60 ± 10.97	88.50 ± 11.90	98.83 ± 19.26
Protein S (%)	94.54 ± 11.35	90.00 ± 12.21	91.80 ± 9.03	95.80 ± 11.64	86.83 ± 4.26
GH (ng/mL)	0.27 ± 0.36	1.91 ± 2.60	1.39 ± 3.94	2.07 ± 2.55	0.08 ± 0.05
IGF1 (ng/mL)	233.24 ± 48.61	250.84 ± 49.58	232.61 ± 42.87	233.09 ± 43.54	227.78 ± 41.11
Glucose (mg/dL)	77.31 ± 17.10	85.23 ± 15.22	82.00 ± 19.76	73.70 ± 11.63	87.75 ± 12.12
Urea (mg/dL)	47.38 ± 8.16	49.08 ± 9.52	44.30 ± 8.73	50.90 ± 10.28	46.83 ± 9.68
Creatinine (mg/dL)	1.16 ± 0.17	1.53 ± 0.25	1.20 ± 0.22	1.71 ± 0.24	1.11 ± 0.12
eGFR (mL/min)	60.00	55.92 ± 4.87	60.00	53.10 ± 5.65	60.00
Uric acid (mg/dL)	5.29 ± 1.08	5.58 ± 1.24	5.34 ± 1.24	6.36 ± 1.20	5.58 ± 1.24
Cholesterol (mg/dL)	193.46 ± 40.07	183.08 ± 35.34	176.90 ± 33.46	182.20 ± 37.46	183.58 ± 29.36
HDL (mg/dL)	57.85 ± 14.76	56.15 ± 11.38	55.10 ± 13.26	57.40 ± 10.95	56.67 ± 14.47
LDL (mg/dL)	121.08 ± 28.14	118.23 ± 29.34	113.20 ± 23.45	112.80 ± 26.12	114.58 ± 22.92
Triglycerides (mg/dL)	81.85 ± 32.84	69.46 ± 18.41	68.50 ± 20.36	64.90 ± 19.74	79.17 ± 30.80
Bilirubin T (mg/dL)	0.86 ± 0.30	0.60 ± 0.12	0.85 ± 0.26	0.71 ± 0.17	0.88 ± 0.23
Bilirubin D (mg/dL)	0.16 ± 0.05	0.13 ± 0.03	0.17 ± 0.05	0.14 ± 0.03	0.18 ± 0.05
Proteins T (g/dL)	7.32 ± 0.31	7.02 ± 0.43	7.21 ± 0.38	7.76 ± 0.30	6.98 ± 0.23
GOT-AST (U/L)	49.31 ± 16.83	46.77 ± 10.94	41.30 ± 10.36	48.80 ± 15.92	49.67 ± 23.88
GPT-ALT (U/L)	42.31 ± 17.25	38.69 ± 13.40	40.80 ± 23.80	39.80 ± 21.66	40.58 ± 44.27
Gamma-GT (U/L)	18.00 ± 5.13	17.77 ± 5.73	15.50 ± 4.20	16.30 ± 4.42	16.58 ± 3.58
CK (U/L)	902.31 ± 589.15	803.54 ± 351.35	603.30 ± 221.85	942.80 ± 575.39	877.67 ± 15.11
Amylase (U/L)	61.00 ± 16.12	76.23 ± 27.24	57.20 ± 18.10	84.60 ± 50.84	58.00 ± 15.11
Sodium (mEq/L)	139.08 ± 1.50	138.46 ± 1.51	138.50 ± 1.84	138.70 ± 1.16	139.67 ± 1.07
Potassium (mEq/L)	4.67 ± 0.42	4.11 ± 0.33	4.20 ± 0.26	3.83 ± 0.16	4.33 ± 0.35
Iron (μg/dL)	121.38 ± 29.92	98.08 ± 18.20	120.60 ± 18.63	84.90 ± 16.20	120.33 ± 34.74
Transferrin (mg/dL)	240.92 ± 22.31	240.46 ± 19.21	234.40 ± 13.66	251.80 ± 15.60	253.92 ± 38.61
% Transferrin saturation	36.46 ± 11.13	29.00 ± 5.37	36.70 ± 6.25	23.60 ± 3.75	34.17 ± 9.70
Ferritin (ng/mL)	157.62 ± 93.35	151.85 ± 74.54	118.00 ± 50.46	127.70 ± 56.14	164.08 ± 95.54
TSH (μU/mL)	1.54 ± 0.41	2.62 ± 0.72	1.25 ± 0.59	3.47 ± 0.72	1.42 ± 0.48
Testosterone (ng/mL)	3.73 ± 0.84	2.73 ± 1.14	4.69 ± 1.39	3.21 ± 1.22	3.98 ± 1.86
Cortisol (μg/dL)	7.06 ± 1.62	17.37 ± 2.99	7.42 ± 2.93	18.17 ± 4.23	13.87 ± 3.13

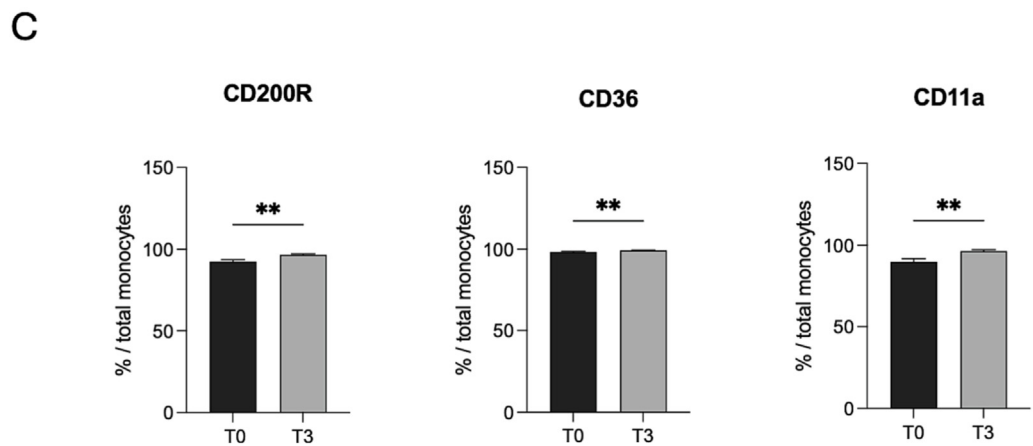
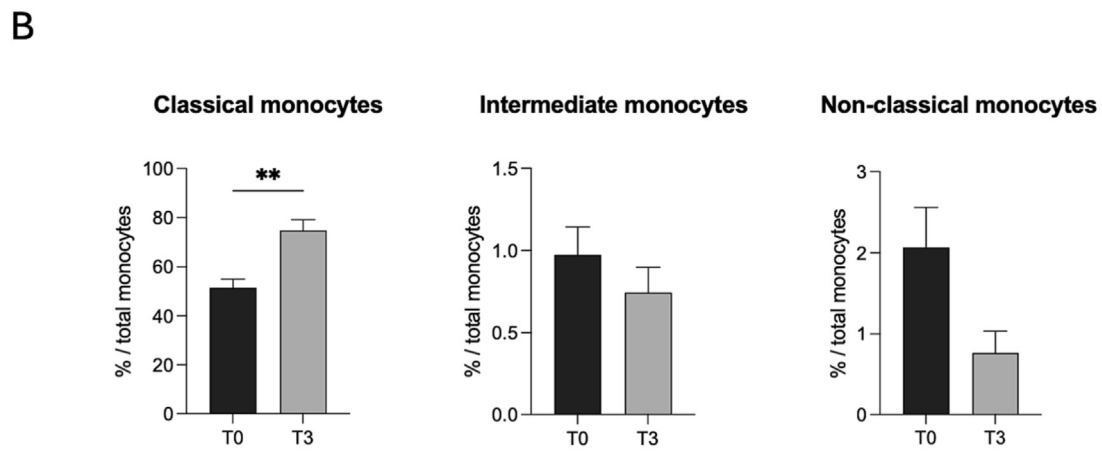
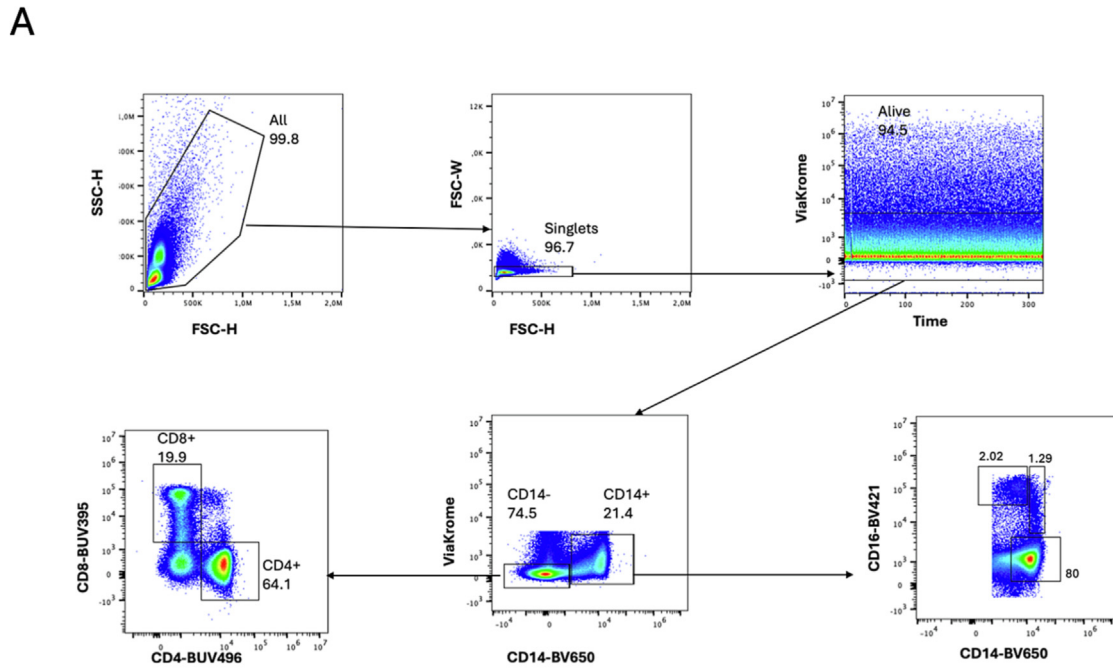
reaction was designed to simultaneously quantify two amplicons located 1000 bp apart on the mitochondrial gene ND2 (Bio-Rad Laboratories, FAM: hg19 chrM:3163–3285; HEX: hg19 chrM:9716–9838) as previously described.<sup>13</sup> The integrity index of cf-mtDNA was estimated by calculating the ratio between the number of single positive droplets, which correspond to fragmented DNA, and double positive droplets, which correspond to intact mtDNA.

Plasmatic inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) and biomarkers NF-L and GFAP were quantified by the ELLA Automated Immunoassay system (Biotechne, San Jose, CA, USA) following the manufacturer's instructions.<sup>19</sup> Data are expressed as pg/mL.

Immunophenotyping of PBMCs was performed by flow cytometry. Thawed PBMCs were washed twice with RPMI 1640 supplemented with 10% fetal bovine serum and 1% each of sodium pyruvate, nonessential amino acids, antibiotics, 0.1 M HEPES, 55 μM β-mercaptoethanol, and 0.02 mg/mL DNase. Cells were then stained with the viability marker ViaKrome 808 IR840 (Beckman Coulter, Brea, CA, USA) for 20 min at

room temperature (RT) in PBS. After washing with FACS buffer (PBS with 2% FBS), they were incubated at 37 °C for 20 min in PBS with a panel of fluorochrome-conjugated monoclonal antibodies (mAbs) targeting CCR7-BV785, CXCR3-PE-Cy5, and CCR4-AF594. Following another FACS buffer wash, cells were stained for 20 min at RT in PBS with mAbs against CD4-BUV496, CD8-BUV395, CD14-BV650, CD16-BV421, CD49d-AF488, CD162-APC750, CD11a-PE-Dazzle, CD45RA-BB700, CD161-PC7, CD36-AF700, HLA-DR-BUV661, and CD200R-PE. After a final FACS wash, cells were permeabilized and stained with TBET-APC (BioLegend). At least 1,000,000 cells per sample were collected. All antibodies were pre-titrated on human PBMCs and used at optimal signal-to-noise concentrations.<sup>20</sup>

For monocyte phenotyping, thawed PBMCs were stained with ViaKrome 808 IR840 and mAbs against CD16-BV421, CD14-BV650, HLA-DR-BUV661, CCR2-BV605, CXCR4-PE, and CCR5-BV421 (BioLegend). Samples were acquired on a CytoFLEX LX flow cytometer (Beckman Coulter) and analyzed with FlowJo 9.9.6 (Ashland, OR, USA) using a



**Fig. 1.** Changes in monocyte subpopulations and marker expression of enrolled rugby players analyzed by cytofluorometric analysis. A. Gating strategy used for cytofluorometric analysis of monocytes. Monocytes were identified according to physical parameters, i.e., forward scatter-height (FSC-H) and side scatter-height (SSC-H). Then, we excluded cell doublets from the analysis, and we identified live monocytes through the fluorophore Viakrome. Finally, we recognized monocyte subpopulations based on CD14 and CD16 expression: classical (CD14 ++, CD16 –), intermediate (CD14 ++, CD16 +), and non-classical (CD14 +, CD16 +) monocytes. B. Histograms showing the percentage of classical, intermediate and non-classical monocytes determined by cytofluorometric analysis before (black columns) and after (gray columns) the two matches analyzed during the season. C. Histograms depicting the percentage of CD200R, CD36 and CD11a expression before (black columns) and after (gray columns) the two matches considered. Data are shown as mean ± SEM from thirteen samples. \*\*p < 0.01.

sequential gating strategy to identify classical, non-classical, and intermediate monocyte subsets (Fig. 1A).<sup>21</sup>

During all matches, rugby players wore a Vector S7 GPS Device (Catapult, London, UK) inside a dedicated, tight-fitting Vector vest. The device is inserted into a pocket located in the upper thoracic region, between the scapulae, which optimizes satellite signal reception and ensures device stability during high-intensity movement and impacts. This placement also minimizes the risk of direct collisions with the device. The upper back is the standardized positioning to guarantee consistent, high-quality performance and biomechanical data acquisition. The GPS records the main parameters related to the number of accelerations and decelerations in space and time, contact involvement, repeated high intensity efforts and the distribution of efforts of each player according to role.

Statistical analysis comparing different experimental conditions was conducted using the Friedman test for repeated measures, ANOVA followed by the Bonferroni means comparison or *t*-test, as appropriate. Potential correlations between cf-mtDNA concentrations, other measured soluble factors, and blows sustained by athletes were analyzed using the Spearman correlation test and linear regression analyses. Prism 9.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analyses. A significance level of  $p \leq 0.05$  was considered statistically significant.

### 3. Results

Thirteen male professional rugby players, aged 22 to 33 years, were monitored across two matches conducted approximately two months apart during the 2023–2024 season. Anthropometric data, including age, height, and weight, were collected for each player, alongside their specific game positions, at the same matches. This demographic and positional information was analyzed to evaluate the potential impact of different playing roles on the physiological responses and the incidence of match-related trauma. Moreover, Supplementary Table 1 shows the number of head impacts received by each player during the two matches examined.

We first analyzed the routinely monitored hematochemical parameters across the season and in post-season measurement (Table 1). Although most values remained within physiological ranges, several changed notably.

Following both matches, there was a consistent decrease in lymphocyte and eosinophil counts, accompanied by increases in white blood cells, reticulocyte percentage, as well as monocyte and neutrophil counts. Red blood cells and RDW dropped significantly only after the first match (T1), whereas a significant reduction in basophils was observed exclusively after the second match (T3). In parallel, hemoglobin and hematocrit levels decreased at T1, while creatinine levels were elevated following both matches. As expected, potassium and iron levels after each match (T1 and T3) were markedly lower than the values recorded before the match (T0 and T2). Hormonal analysis revealed increases in TSH, cortisol, and GH after each match, and along with a significant testosterone decrease, as well as an increase in IGF-1 only at T1.

As head impacts can potentially impact the innate immune response, we performed cytofluorometric analysis to evaluate the expression of key immune receptors on immune cells and to characterize the distribution of monocyte subsets (classical, intermediate, and non-classical). We chose to analyze primarily monocytes, as they can be activated by extracellular mtDNA.<sup>22</sup> As mentioned before, the classification of these subsets was based on a gating strategy outlined in Fig. 1A, with measurements taken before (T0) and after (T3) the two monitored rugby matches.

Among the rugby players enrolled, we observed an increase in the percentage of classical monocytes at T3, while the percentages of intermediate and non-classical monocytes declined (Fig. 1B). Furthermore, we detected elevated percentages of CD200R, CD36, and CD11a

expression after the matches (Fig. 1C), suggesting enhanced monocyte activation and adhesion at T3 compared to baseline (T0). These findings indicate an intensified immune response and potential inflammation associated with concussions.

Cf-mtDNA quantification showed an upward trend in mtDNA concentration following each match. Additionally, mtDNA levels progressively increased throughout the season and remained elevated even one month after its conclusion. The significant increase from T0 to T4 suggests a cumulative effect (Fig. 2A, left panel). Dividing mtDNA levels based on player roles, we observed that forward players, who are more frequently involved in tackles, exhibited a progressive increase in mtDNA levels (Fig. 2A, central panel). Back players also showed an increase in mtDNA, although less markedly (Fig. 2A, right panel).

As in the previous study on boxers,<sup>13</sup> we applied our optimized protocol to differentiate the various forms of mtDNA.<sup>13</sup> Data showed a progressive reduction in the amount of mtDNA following differential centrifugation, with Fraction 3 – where naked mtDNA is present – containing approximately  $10^5$  copies of mtDNA per mL. We detected elevated cf-mtDNA levels after each match, both after the centrifugation at 18,000g (Fraction 2; Fig. 2B, left panel) and the ultracentrifugation (Fraction 3; Fig. 2B, right panel), reaching approximately  $6 \times 10^5$  copies/mL and  $5 \times 10^5$  copies/mL, respectively. Notably, data revealed a progressive increase in mtDNA copies over the course of the season, with mtDNA levels significantly higher at T4 than at T0 in both Fractions 2 and 3.

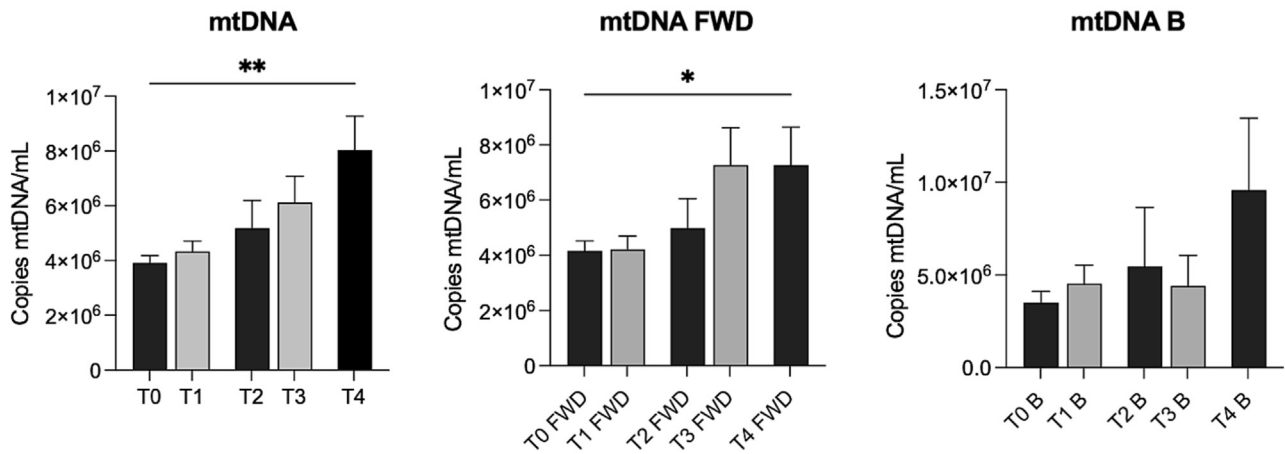
Regarding mtDNA integrity, in Fraction 1 we observed a gradual increase in fragmentation throughout the season, particularly after the second match (T3), across the entire cohort of monitored players (Fig. 2C, left panel). Conversely, no significant differences were observed in mtDNA integrity following centrifugation at 18,000g (Fraction 2; Fig. 2C, central panel) or ultracentrifugation (Fraction 3; Fig. 2C, right panel).

Repeated head injuries trigger a massive release of cytokines and neuroinflammatory markers into circulation, exacerbating the inflammatory response.<sup>13</sup> Consistently, we observed a significant increase in the pro-inflammatory cytokines IL-6 and IL-8 after each match, whereas TNF- $\alpha$  exhibited a statistically significant increase only after the second match (T3) (Fig. 3A).

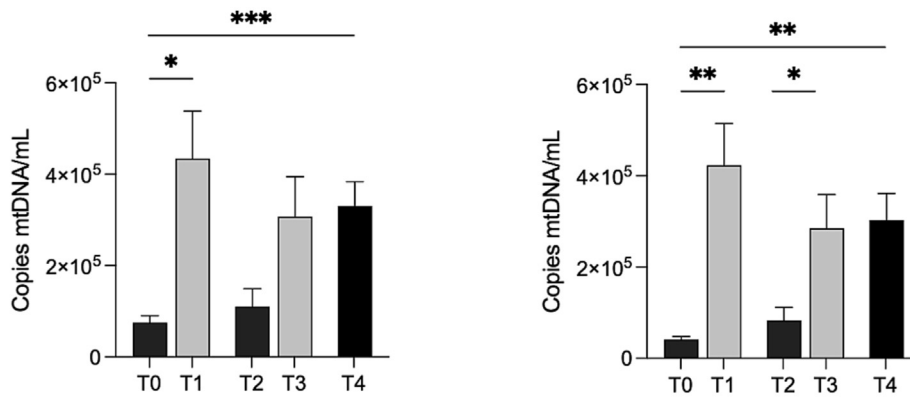
To assess neuroinflammatory processes, we quantified the concentrations of two well-established neuroinflammatory markers, GFAP and NF-L, in the enrolled subjects. GFAP levels increased after each match compared to pre-match values in the total cohort, although the differences did not reach statistical significance (Fig. 3B, upper panels). When stratifying players by role, we observed the same non-significant trend (Fig. 3B, lower panels). Similarly, NF-L levels significantly increased after each match and then returned to pre-match values also in the post-season measurement, as shown in Fig. 3C (upper panels). The changes observed across the five timepoints were statistically significant not only in the total population, but also in forward players' group. Back players exhibited the same trend, though not statistically significant (Fig. 3C, lower panels).

GPS tracking collected during the two rugby matches provided insights into the players' physical exertion and match involvement. Repeated high intensity efforts (RHIEs), contact involvements, total distance covered, and accelerations were recorded for each player. Additionally, the efforts with an intensity greater than 85% and the number of actions with a power greater than 25.5 W were also evaluated. The values were divided into forward and back players to evaluate the different involvement based on the game role. As expected, back players performed significantly more accelerations and sprints during matches, but forwards experienced significantly more physical collisions (Fig. 4A). In both matches the players performed approximately five RHIEs per match, with total contacts ranging from 23 to 26, most of which were under 85% intensity. In agreement with previous observations, forward players are typically involved in more contacts than backs, while backs recorded slightly more RHIEs. Moreover, back

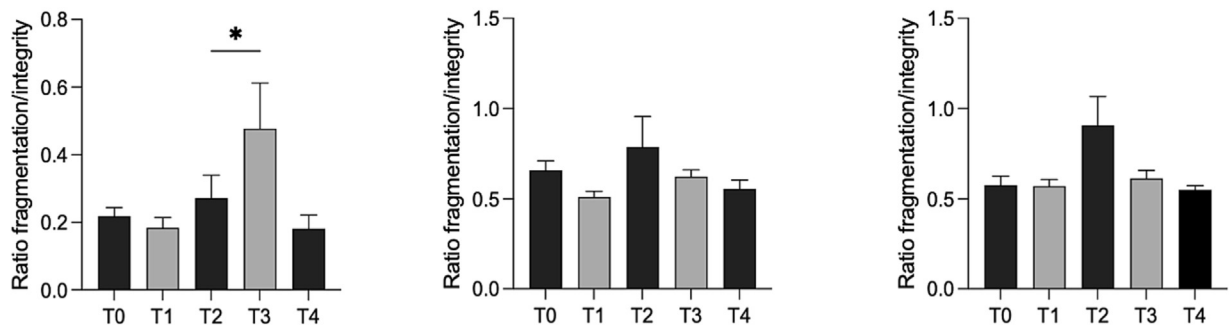
A



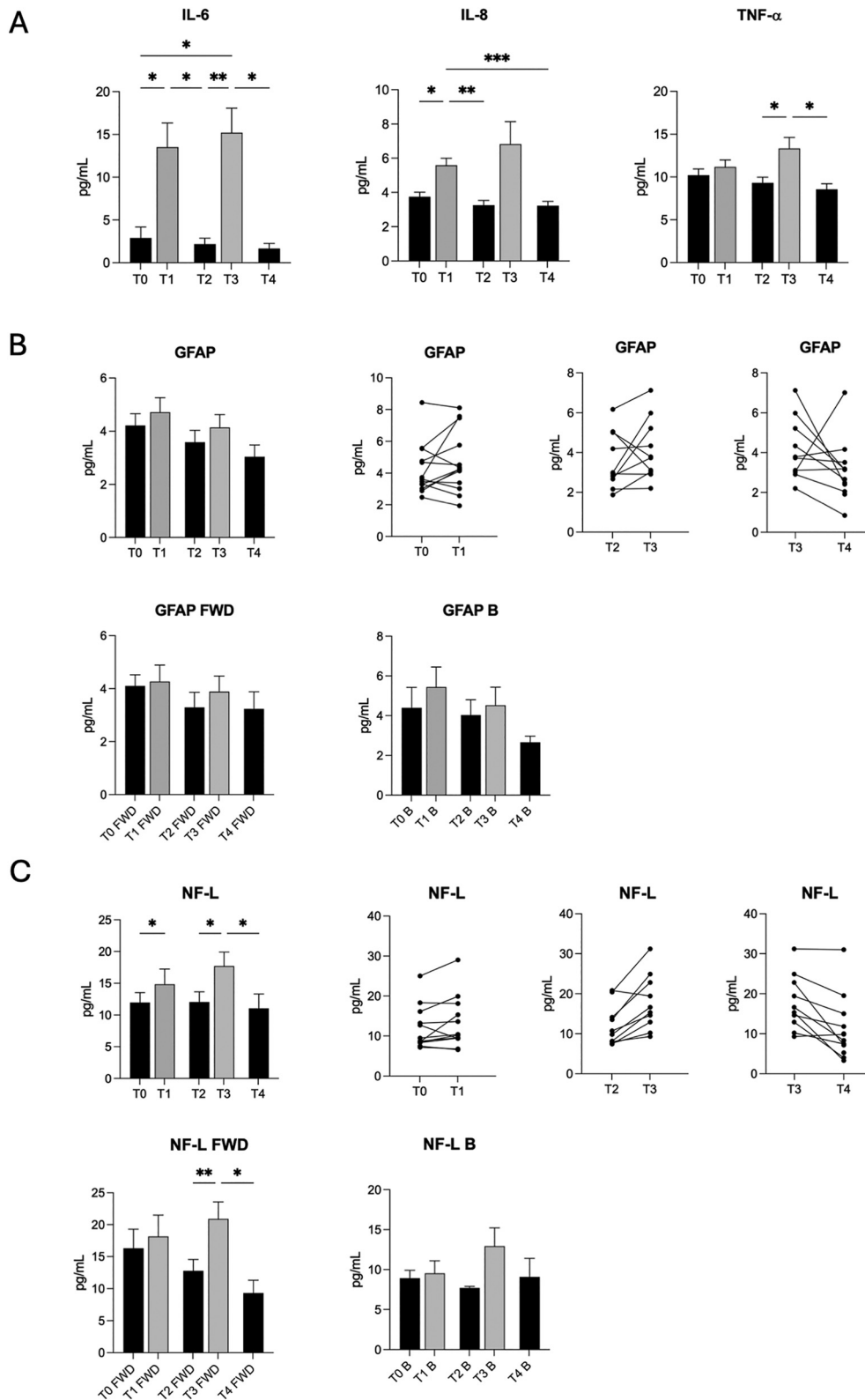
B



C

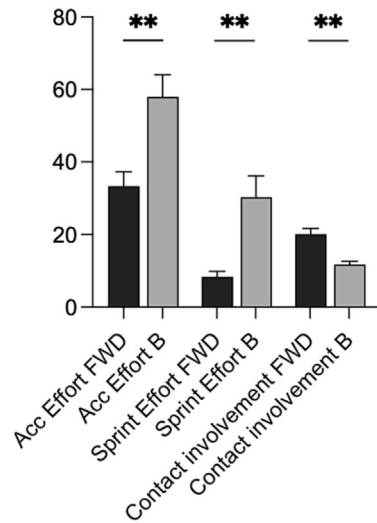


**Fig. 2.** Changes in cf-mtDNA levels from enrolled rugby players along the season. A. Histograms depicting the number of copies of mtDNA per mL in the total cohort of subjects enrolled (left panel) and dividing players according to the role (central and right panel) before (black columns), after (gray columns) each match, and one month after the end of the season (T4, black column). Data are shown as mean  $\pm$  SEM from thirteen samples. \* $p < 0.05$ ; \*\* $p < 0.01$ . B. Histograms depicting the number of copies of mtDNA per mL in Fraction 2 (left panel) and Fraction 3 (right panel) in the total cohort of subjects enrolled before (black columns), after (gray columns) each match, and one month after the end of the season (T4, black column). Data are shown as mean  $\pm$  SEM from thirteen samples. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . C. Histograms showing the fragmentation of mtDNA in Fractions 1 (left panel), 2 (central panel) and 3 (right panel), before (black columns), after (gray columns) each match, and one month after the end of the season (T4, black column), as measured by multiplex ddPCR. The columns represent the ratio of fragmented to intact mtDNA. Data are shown as mean  $\pm$  SEM of the ratio from thirteen samples. \* $p < 0.05$ .

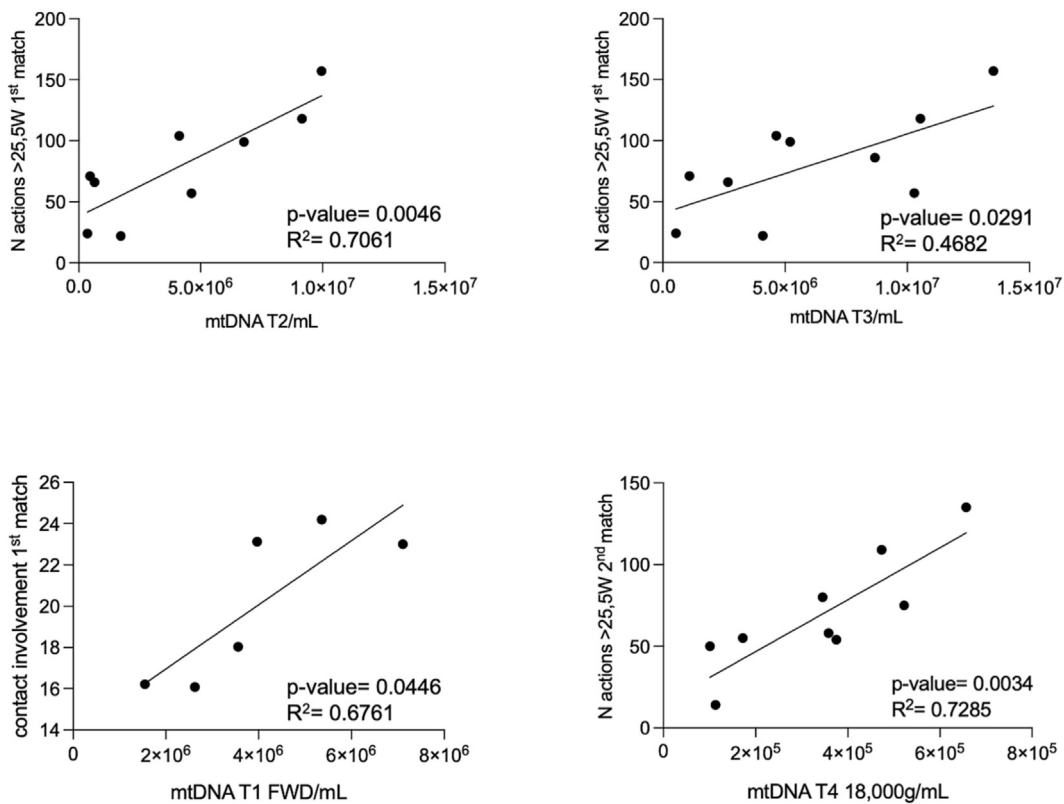


**Fig. 3.** Changes in inflammatory and neuroinflammatory markers in enrolled rugby players along the season. A. Cytokine levels (IL-6, left panel; IL-8, central panel; TNF- $\alpha$ , right panel) measured in Fraction 1, expressed in pg/mL, before (black columns), after (gray columns) each match, and one month in the post-season (T4, black column). Data are shown as mean  $\pm$  SEM from ten samples. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . B. Upper panels: GFAP concentration before (black columns) and after (gray columns) each match and one month in the post-season (T4, black column) in the total cohort of subjects. Symbols and line graphs depicting GFAP levels before (T0, T2) and after (T1, T3) each match, between the last measurement of the season (T3) and the post-season period (T4) in all players. Lower panels: GFAP levels before (black columns) and after (gray columns) each match and one month in the post-season (T4, black column) both in forward and back players (lower line). Data are expressed as pg/mL and are shown as mean  $\pm$  SEM from thirteen samples or from the two groups of samples. C. Upper panels: histogram showing NF-L concentration before (black columns) and after (gray columns) each match and one month in the post-season (T4, black column) in the total cohort of subjects. Symbols and line graphs depicting NF-L levels before (T0, T2) and after (T1, T3) each match, between the last measurement of the season (T3) and the post-season period (T4) in all players (upper line). Lower panels: histograms representing the concentration of NF-L before (black columns) and after (gray columns) each match and one month in the post-season (T4, black column) both in forward and back players. Data are expressed as pg/mL and are shown as mean  $\pm$  SEM from thirteen samples or from the two groups of samples. \* $p < 0.05$ ; \*\* $p < 0.01$ .

A



B



**Fig. 4.** Correlations among mtDNA levels and GPS tracking. A. Histograms showing the number of acceleration efforts, sprint efforts, and contact involvements in forwards (black bars) and backs (gray bars), as recorded by GPS tracking. Data are cumulative from the two monitored matches and are presented as mean  $\pm$  SEM for the two groups.  $**p < 0.01$ . B. Correlation between mtDNA levels before (T2) and after (T3) the second match in the total population and the number of high-powered actions performed during the first match (upper line). Correlation between mtDNA levels in forwards after the first match (T1) and the number of contacts experienced during the same match and correlations between mtDNA levels in the post-season (T4) in Fraction 2 and the number of contacts experienced during the second match (lower line).

players executed significantly more high-powered actions ( $>25.5$  W) and covered greater distances than forwards during matches (Table 2).

Analysis of the correlation between GPS tracking and mtDNA levels revealed that mtDNA concentrations before and after the second

match (T2 and T3) were significantly correlated with the number of high-powered actions performed during the first match, with p-values of 0.0046 and 0.0291 and  $R^2$  of 0.706 and 0.468, respectively, for the total cohort of subjects enrolled (Fig. 4B, upper line). A correlation

**Table 2**

GPS tracking before (T1) and after (T3) the two matches considered in total players and after the stratification between forward players and backs. Values are expressed as mean and standard deviation.

		T1			T3		
		Total	Forward	Back	Total	Forward	Back
RHIEs per bout – Mean	Mean	4.92	4.80	5.13	4.70	4.28	5.66
	SD	0.71	0.86	0.39	0.91	0.67	0.65
Contact involvement total count	Mean	23.85	28.63	16.20	26.30	29.71	18.33
	SD	11.05	10.84	6.50	11.10	11.48	4.73
Total distance	Mean	4771.99	3901.83	6512.32	4578.05	3962.45	6732.63
	SD	1994.01	1865.11	636.11	1646.62	1271.83	220.25
Total acceleration load	Mean	1580.42	1338.16	2064.94	1559.02	1362.64	2246.38
	SD	626.03	609.11	319.11	548.78	445.40	64.59
Efforts > 85%	Mean	0.50	0.63	0.25	0.22	0.29	0
	SD	0.67	0.74	0.50	0.44	0.49	0
Efforts > 90%	Mean	0	0	0	0	0	0
	SD	0	0	0	0	0	0
Efforts > 95%	Mean	0	0	0	0	0	0
	SD	0	0	0	0	0	0
N actions > 25.5 W	Mean	85.75	61.83	134.50	70.00	55.14	122.00
	SD	44.41	27.06	27.74	35.41	21.37	18.38

between mtDNA levels at T1 and total contact involvement experienced during the first match was found only in forward players, who are more frequently involved in tackles and high-impact plays.<sup>6</sup> This relationship was further confirmed through linear regression analysis suggesting that forwards show a stronger correlation between mtDNA levels and match-induced trauma (Fig. 4B, lower line).

Regarding the different forms of cf-mtDNA, the concentration of mtDNA in Fraction 2 one month after the end of the season (T4) was significantly correlated with the number of high-powered actions performed during the second match analyzed, for the total cohort of subjects enrolled (Fig. 4B, lower line).

#### 4. Discussion

Concussions are common in athletes involved in contact sports like football, boxing, and rugby, where repeated head injuries often occur within a single season.<sup>13</sup> High-impact sports such as rugby may cause cellular damage and mtDNA release, which acts as a DAMP and triggers inflammation.<sup>10,23</sup>

This study investigated whether repeated collisions increase cf-mtDNA and examined correlations between brain injury, mtDNA release, inflammation, neuroinflammatory markers, and head impact frequency and intensity.

The main finding of the study was a progressive increase in cf-mtDNA throughout the season, consistent with previous data on non-professional boxers, with levels remaining elevated post-season—suggesting that the rest period considered, which is about one month, is not sufficient to allow mtDNA levels to return to baseline. Repeated head impacts also reduced mtDNA integrity, particularly when released in its naked, unprotected form.

Prior research on cf-mtDNA changes after exercise has been inconsistent. Some found reduced levels following prolonged moderate activity in healthy individuals and professional volleyball players, while others, such as Stawski et al., observed a transient increase after intense exercise.<sup>24,25</sup>

Several factors may explain these discrepancies. First, unlike prior studies focused on single sessions, we analyzed multiple matches across the season. We consistently observed slight, non-significant post-match cf-mtDNA increases, suggesting two overlapping phenomena: acute, transient rises after individual matches and a chronic, progressive accumulation over time. The latter implies that trauma-induced cf-mtDNA release is not resolved by resting periods and may contribute to chronic neurodegenerative disease onset. Due to off-season monitoring limitations, we could not assess whether prolonged recovery normalizes cf-mtDNA levels.

Second, rugby involves frequent impacts including those to the head, unlike the moderate-intensity exercise studied previously, likely causing more extensive cellular damage and mtDNA release. This was confirmed by stratifying players by role: forwards, exposed to more frequent impacts, showed significantly higher mtDNA release. Additionally, cf-mtDNA levels increased with action intensity, particularly at T2 and T3.

High-intensity activity alters hematochemical parameters and typically raises blood glucose to meet energy demands.<sup>18</sup> Our hematochemical and GPS-based analyses confirmed players' health status and excluded other causes for elevated cf-mtDNA. Post-exercise changes in white blood cells, creatinine, red blood cells, hemoglobin, and hematocrit mirrored findings in triathletes and treadmill-based studies.<sup>17,26</sup> Hormonal changes in GH, cortisol, TSH, and IGF-1 aligned with previous results in boxers, reflecting physiological adaptation rather than pathology.<sup>13,27</sup>

GPS tracking, widely used for assessing movement and intensity, supported our findings. The data, useful for rehabilitation, also highlighted rugby dynamic nature—alternating high- and low-intensity periods.<sup>7</sup> Greater mtDNA release in forwards was likely due to their repeated contact exposure, as confirmed by GPS data and prior studies.<sup>7</sup> However, as GPS-recorded collisions alone do not directly measure head acceleration, future studies incorporating direct measurements of head acceleration or clinically verified concussions are needed to evaluate this relationship.

Overall, both exertion intensity and contact frequency contribute to elevated cf-mtDNA levels.

Our prior research showed that mtDNA can be recognized by monocytes via DAMP receptors like TLR9, initiating inflammation. Here, immunophenotyping revealed a post-match increase in total monocytes, especially the classical subset—known for promoting inflammation. This suggests that cf-mtDNA effects may be amplified by a higher number of responsive cells.

To further explore neuroinflammatory effects of repeated head impacts, we measured GFAP and NF-L. GFAP, a 50 kDa filament protein forming astrocytic structure, enters circulation after BBB disruption from neurotrauma.<sup>28</sup> Elevated serum GFAP levels are linked to cognitive decline and dementia.<sup>29</sup> In our study, GFAP increased post-match but not significantly, returning to baseline before the next match and post-season. This trend aligns with reports that GFAP peaks 20–24 h post-injury and declines over 72 h.<sup>30</sup>

NF-L, a widely recognized biomarker of brain injury, is released into circulation following head trauma. In contrast to previous findings in boxers, our study revealed a significant post-match increase in NF-L levels, which subsequently returned to pre-match baseline levels. Notably, we observed a marked reduction of NF-L concentration one month

after the end of the season. This pattern was particularly evident in forwards, who sustain more impacts.

Our results indicate that head impacts can trigger various inflammatory and neuroinflammatory processes. The neuroinflammatory markers GFAP and NF-L may be insufficient for long-term detection as their degradation within a few days post-trauma likely renders them undetectable in circulation. By contrast, cf-mtDNA progressively increased through the season and post-season, particularly in high-contact players. Thus, monitoring cf-mtDNA could offer insight into ongoing inflammation and inform personalized training and recovery strategies.

## 5. Conclusion

This pilot study demonstrates that cumulative exposure to head impacts during a professional rugby season is associated with a progressive and sustained increase in circulating cf-mtDNA, particularly in forward players who experience more frequent and intense impacts. These elevations persisted even one month after the end of the season, indicating that recovery periods may be insufficient for the resolution of trauma-induced molecular alterations.

Our findings also suggest that cf-mtDNA, especially in its unbound form, could serve as a sensitive and long-lasting biomarker of subclinical neuroinflammation and systemic cellular stress in contact sports. Unlike transient markers such as GFAP and NF-L, which return to baseline shortly after injury, cf-mtDNA showed a cumulative trend, potentially reflecting unresolved inflammation or continuous low-grade tissue damage.

These results underscore the need for more individualized monitoring protocols in contact sports. Integrating cf-mtDNA measurements into athlete health surveillance could enable early identification of players at risk for chronic neurodegeneration and guide adjustments in training load or return-to-play timing. Nevertheless, further studies are required to validate cf-mtDNA as a diagnostic and prognostic biomarker across larger cohorts, various sports disciplines, and extended post-season periods.

## CRediT authorship contribution statement

Valentina Selleri: Conceptualization, investigation, methodology, data curation, data analysis, writing – original draft preparation, and writing – review & editing  
 Marco Bazo: Resources and methodology  
 Giorgia Sinigaglia: Investigation and visualization  
 Giada Zanini: Resources and investigation  
 Giulia Micheloni: Resources and visualization  
 Francesca Coppi: Resources, methodology, and visualization  
 Roberta D'Alisera: Resources and investigation  
 Gustavo Savino: Supervision and resources  
 Milena Nasi: Conceptualization, methodology, and writing – review & editing  
 Marcello Pinti: Conceptualization, methodology, writing – original draft preparation, and writing – review & editing.

## Confirmation of ethical compliance

This study has been approved by Ethics Committee of Area Vasta Emilia Nord (protocol number 1104/2021/SPER/AUSLMO-0028381/23).

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## Declaration of interest statement

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jsams.2026.03.024>.

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