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Published Version:
Granata, S., Bernardini, C., Glogowski, P.A., Romito, G., Salaroli, R., Algieri, C., et al. (2024). Mitochondrial bioenergetic dysfunction linked to myxomatous mitral valve degeneration explored by PBMCs metabolism analysis. BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS, 1865(4), 1-4 [10.1016/j.bbabio.2024.149505].

Availability:
This version is available at: https://hdl.handle.net/11585/978934 since: 2024-09-06

Published:

DOI: http://doi.org/10.1016/j.bbabio.2024.149505

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15 October 2026

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The final published version is available online at: **https://doi.org/10.1016/j.bbabio.2024.149505**

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Mitochondrial bioenergetic dysfunction linked to myxomatous mitral valve degeneration explored by PBMCs metabolism analysis

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Abstract

Impaired mitochondria cause an impressive decrease in ATP production becoming a common condition of cardiovascular diseases. Myxomatous mitral valve disease (MMVD) is characterized by mitochondrial dysfunction. By a non-invasive procedure of metabolism analysis on peripheral blood mononuclear cells, we exploit *ex-vivo* studies that directly constitute a translational approach to evaluate the cell bioenergetics. Cell ATP production decreased in the presence of MMVD, whereas glycolysis was unaffected. In MMVD, the mitochondrial activity underwent a significant reduction of basal respiration, maximal respiration, and ATP production. Our results depicted a pathological condition of MMVD characterized by cell metabolism deprived of mitochondrial energy support.

Keywords: Mitochondria; cell metabolism; bioenergetics; ATP production; myxomatous mitral valve disease.

Mitral regurgitation due to mitral valve prolapse (*i.e.*, a displacement of some portion of one or both leaflets of the mitral valve into the left atrium during systole as a consequence of myxomatous degeneration) is a chronic cardiovascular disease that affects approximately 144 million people across the world [1], with an incidence that increases with age. This condition can lead to several complications, primarily stroke and heart failure which are considered to be the leading causes of cardiac morbidity and fatality in affected individuals [2]. Interestingly, there is evidence that the disease process documented in humans shares many similarities with canine myxomatous mitral valve degeneration (MMVD) [3,4]. Thus, the dog can be used to study human disease and improve its knowledge [3,5], in accordance with the concept promoted by the One Health approach[6]. As well as the human disease (which can be classified in stages A, B, C and D) [7], canine MMVD can progress through different stages of disease severity (ACVIM B1, B2, C and D) [8]. Despite the abundant data already available on the clinical, echocardiographic, therapeutic, and prognostic features of human and canine disease at different stages [7,8], little is known to date about the cellular mechanisms and abnormalities expressed by affected subjects, including mitochondrial ones.

Since cardiomyocytes contain an abundant number of mitochondria, cardiovascular diseases (CVDs) are often associated with mitochondrial dysfunction, which results in energy deficits and metabolic abnormalities. However, recent research in the field has focused on the relevance of inflammation and the potential role of mitochondrial impairment in circulating cells, which so far has revealed that human peripheral blood mononuclear cells (PBMCs) mitochondrial disruption might lead to a vicious loop of enduring cardiac dysfunction [9]. So far, only a few studies have focused on PBMCs data variability in MMVD-affected dogs [10,11]. More specifically, Garncarz et al performed the analysis of their PBMCs transcriptomes and observed a significant difference between clusters of cells in dogs with no clinical signs and with clinical signs [10]. Additionally, Druzhaeva and colleagues have found that dogs with advanced stages of MMVD (ACVIM C and D) present an altered ratio of CD3+CD4+ and CD3+CD8+ lymphocytes compared to the ones with ACVIM B2, implying that these lymphocytes might play a role in the pathophysiology of congestive heart failure in dogs [11] or human with MMVD, where lymphocytes and their ratio to other PBMCs changed significantly [3].

Our research aims to investigate whether PBMCs isolated from blood samples of dogs with MMVD own differences in mitochondrial function compared to those derived from healthy dogs, in order to better understand the role of PBMCs in the pathophysiology of MMVD and to potentially identify new therapeutic strategies with a translational approach from bed to bench and “One Health” concept to balance and optimize the health of people and animals.

Dogs subjected to cardiological evaluation requested by the owner were considered in the present study to compose the experimental group. Specifically, the evaluation was needed to investigate for the first time the cardiac status in dogs showing clinical signs suggestive of an underlying heart disease (*e.g.*, a heart murmur) or to check an already known cardiac disorder. In detail, only individuals affected by MMVD were finally included in the experimental group. The diagnosis of MMVD was based on clinical and echocardiographic findings, including a left apical systolic murmur and thickened or prolapsing mitral valve leaflets or both on two-dimensional echocardiography associated with mitral valve regurgitation on color flow Doppler interrogation [8,12]. Disease severity was classified according to the guidelines of the American College of Veterinary Internal Medicine into stages B1, B2, C, and D [8]. The control group consisted of healthy subjects. Specifically, dogs were considered healthy based on an unremarkable clinical history and physical examination as well as on normal routine blood exams (*i.e.*, complete blood count, serum chemistry), echocardiography and surface electrocardiogram. Blood samples were collected from animals enrolled in the present study, approved by the Italian Ministry of Health as dictated by D.Lgs 26/2014 (approval n° 89380; all procedures were performed in compliance with ARRIVE guidelines).

PBMCs isolation was performed as described by Mavropoulou and colleagues [13], with some modifications. Briefly, PBMCs were separated by density gradient centrifugation using Ficoll-Paque (Cytiva Sweden AB, SE-751 84 Uppsala Sweden) at 400*g* for 45 minutes at 20°C. The cells were taken from the surface of the Ficoll-

Paque into DPBS (Gibco™, Thermofisher, USA). PBMCs were washed with 10 mL of DPBS, then centrifuged at 150*g* for 10 minutes, then PBMCs were washed with 5 mL of DPBS and centrifuged at 500*g*; the cellular pellet was finally resuspended in DMEM complete medium, which is constituted by XF DMEM medium pH 7.4 with 5 mM HEPES (Agilent, USA) supplemented with 10 mM XF glucose, 2 µM XF glutamine and 1 µM XF pyruvate (Agilent, USA). Assays were performed seeding cells at a concentration of $6*10^4$ cells/50 µL/well in XFp PDL miniplates (Agilent, USA). PBMCs were spun down at 1200*g* for 5 minutes at 20°C and then DMEM complete medium was added up to a final volume of 180 µL per well.

The bioenergetics of PBMCs were assessed using the Agilent Seahorse XFp analyzer, which allows the evaluation of ATP generation (ATP Rate Assay, Agilent, USA) and the cells' response to mitochondrial stress (Cell Mito Stress Test, Agilent, USA). The Agilent Seahorse XFp analyzer simultaneously measures the oxygen consumption rate (OCR), or the measure of cellular respiration, and extracellular acidification rate (ECAR), which is an index of glycolysis expressed in mpH/min. XFp sensor cartridges were hydrated overnight using XF calibrant (Agilent, USA) and, prior to the experiment, appropriate modulators were loaded in the cartridges' injection ports. In accordance with the ATP rate assay program's specifications, we loaded the ports to obtain the final concentration of 1.5 µM oligomycin (Oligo) in port A and 0.5 µM rotenone (Rot) plus 0.5 µM antimycin A (AA) in port B. The Cell Mito Stress Test set for our experiments, instead, requires the final concentrations of 1.5 µM Oligo in port A, 2 µM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) in port B and 0.5 µM Rot plus 0.5 µM AA in port C. The FCCP concentration was chosen after proper titration. Each assay was performed at 37°C in a number of independent experiments equal to the number of subjects studied. Data were analyzed using the WAVE software (Agilent) [14].

Assessing cell viability is a crucial step before undertaking any cell-based investigation. We discriminated dead and viable canine PBMC using the MacsQuant Analyzer10 Flow Cytometer and the Viobility™ Fixable Dye (Miltenyi Biotec Bergisch Gladbach, Germany). The number of viable cells in healthy dogs (identified as Control) and those affected by MMVD (Fig. 1A,B) was used for normalizing the data of cell metabolism analysis.

OCR and ECAR values were used to evaluate the ATP rate production under basal conditions. The result highlighted the activities of mitochondrial ATP production (mitoATP) or glycolysis ATP production (glycoATP) of PBMCs. Control showed a higher ATP production rate based on OXPHOS than MMVD, whereas no differences were detected on glycolysis. As a consequence, a higher rate of mitoATP of Control than MMVD PBMCs caused an increase in total ATP production (Fig. 1C). The PBMCs energy map demonstrated that Control had a more active OXPHOS pathway than MMDV, confirming aerobic energy metabolism *versus* quiescent metabolism, respectively (Fig. 1D). Finally, the ATP synthesis of PBMCs can be different. Indeed, the ratio between mitoATP Production Rate and glycoATP Production Rate (ATP Rate Index) in Control PBMCs was >1, which highlighted a prevailing oxidative phenotype, whereas in MMVD was <1 detecting a less oxidative and more glycolytic phenotype (Fig. 1E).

By considering the negative function of mitochondria in MMDV the direct measure of cell respiration showed a significant decrease in the OCR kinetic profile in MMVD compared to Control (Fig. 1F). As a result, the key bioenergetics parameters of mitochondrial function were reduced in MMVD. The non-mitochondrial respiration, evaluated as OCR in the presence of rotenone plus antimycin A (respiratory chain inhibitors), was subtracted from all the bioenergetics parameters (Fig. 1G,H,I,L,M). MMVD inhibited basal respiration detected as baseline OCR before oligomycin addition by 69%, maximal respiration evaluated as OCR after FCCP addition was inhibited by 78%, and ATP production obtained from the difference between the basal respiration and the minimal respiration (OCR in the presence of oligomycin) was decreased by 76%. Finally, spare respiratory capacity, which is the amount of additional ATP that oxidative phosphorylation can synthesize in case of an abrupt increase in energy demand [15] was calculated as the difference between the maximal and basal respiration underwent a total inhibition in MMVD. The only parameter that remained unaffected was the proton leak, which corresponds to the difference between basal respiration and

respiration in the presence of oligomycin (minimal respiration). This indicates the re-entry of H^+ in the intermembrane space of mitochondria independently of the ATP synthase activity [16].

Dysfunctions at the cardiomyocyte level contribute to determining the aetiology and pathogenesis affecting the heart in MMVD. However, being aware that an invasive approach must be used to obtain cardiomyocytes from MMVD-affected dogs, the use of PBMCs represents a valid and alternative non-invasive and low-cost method to study MMVD. By presenting an impaired bioenergetic profile of MMVD, the PBMCs represent an attractive translational approach to studying this disease. On balance, the cell metabolism analysis performed on PBMCs in the presence and absence of MMVD has highlighted mitochondrial dysfunction. CVDs are characterized by impaired mitochondrial activity and MMVD induced a metabolic rewiring of energy metabolism. In particular, the altered bioenergetics might be connected to amino acid metabolic reprogramming of canine MMVD [4,17]. Therefore, cell bioenergetics of MMVD did not fulfil the ATP demand by oxidative respiration that, in this disease, can accomplish the events to exacerbate oxidative stress and alterations in mitochondrial DNA [18], an outcome of most CVDs.

Abbreviations

AA	antimycin A
CVDs	cardiovascular diseases
ECAR	extracellular acidification rate
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
MMVD	myxomatous mitral valve disease
OCR	oxygen consumption rate
Oligo	oligomycin
PBMCs	peripheral blood mononuclear cells
Rot	rotenone

Acknowledgements

None

Funding

This study was supported by the University of Bologna, grant numbers RFO 2022 to SN and RFO 2023 to CB.

Declaration of interest

The authors declare no competing interests.

Author contributions

Silvia Granata: Formal analysis, Investigation, Writing - original draft. **Chiara Bernardini**: Conceptualization, Funding acquisition, Methodology, Writing - review & editing. **Patrycja Anna Glogowski**: Investigation, Resources, Writing - review & editing. **Giovanni Romito**: Conceptualization, Resources, Writing - review & editing. **Roberta Salaroli**: Investigation, Writing - review & editing. **Cristina Algieri**: Methodology, Writing - review & editing. **Antonia Cugliari**: Data curation, Writing - review & editing. **Micaela Fabbri**: Data curation, Writing - review & editing. **Fabiana Trombetti**: Validation, Writing - review & editing. **Augusta Zannoni**: Validation, Writing - review & editing. **Salvatore Nesci**: Conceptualization, Funding acquisition, Supervision, Visualization, Writing - original draft.

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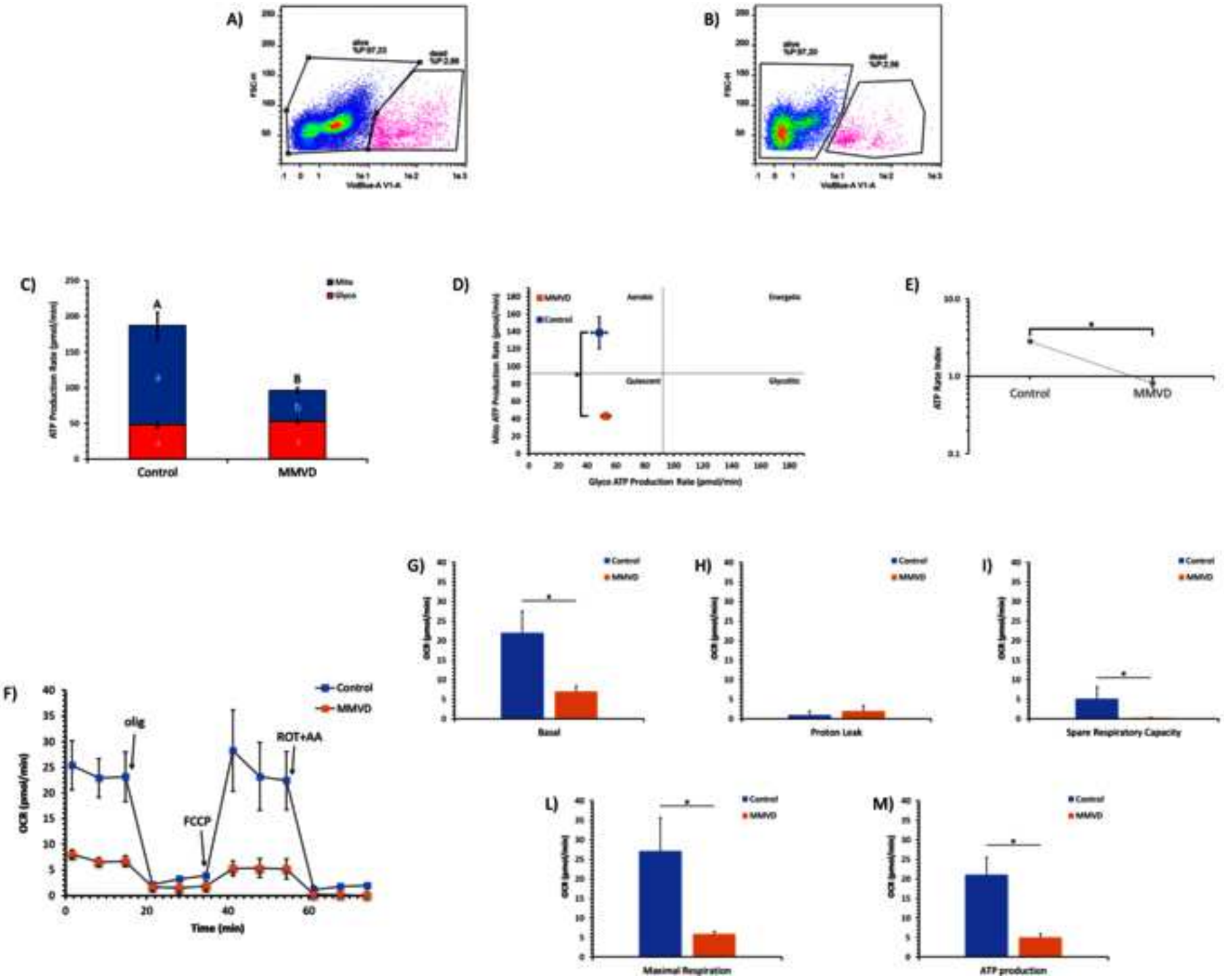
Figures

Figure 1. Evaluation of viability and bioenergetic metabolism in control and MMVD PBMCs. Representative images of vitality analysis of PBMCs in Control (A) and MMVD (B) by flow cytometry stained with Viobility 405/452 Fixable Dye. C) Quantification of ATP production by mitochondrial oxidative phosphorylation (OXPHOS) or by the glycolytic pathway. D) Energy map relative to the metabolic state of PBMCs control *vs* MMVD. E) The plot shows the ratio between the mitochondrial ATP production rate and glycolytic ATP production rate (logarithmic scale). F) The mitochondrial respiration profile in control and MMVD PBMCs was evaluated as OCR (Oxygen Consumption Rate) under basal respiration conditions. Mitochondrial parameters identified as (G) basal respiration, (H) Proton leak, (I) Spare respiratory capacity, (L) Maximal respiration, and (M) ATP production of control MMVD PBMCs are reported in the graphs. Data expressed as column charts (C,G,H,I,L,M plots) and points (D,E,F plots), represent the mean±SD (vertical or horizontal bars) from three experiments carried out on different cell preparations. Different lower-case letters indicate significantly different values ($P \leq 0.05$) among treatments (Control and MMVD) in the same metabolic pathway; different upper-case letters indicate different values ($P \leq 0.05$) among treatments in ATP production rates due to the sum of OXPHOS (Mito) plus glycolysis (Glyco). Statistically significant differences are indicated (* $P \leq 0.05$).

Figure 1

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