

CLINICAL RESEARCH ARTICLE



The first proteomics analysis of tonsils in patients with periodic fever, aphthous stomatitis, pharyngitis, and adenitis syndrome (PFAPA)

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BACKGROUND: Periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) syndrome is a recurrent fever syndrome. The exact etiopathogenesis of PFAPA syndrome remains unknown. Biological fluids or tissues may provide disease-specific biomarkers that may help clinicians to find new pathogenic pathways.

METHODS: Tonsil tissues of seven patients with PFAPA were collected during the tonsillectomy. Seven patients who underwent tonsillectomy for reasons other than chronic tonsillitis were enrolled as a control group. The nHPLC LC-MS/MS system was used for protein identification and label-free quantification. Bioinformatics analysis was carried out using the UniProt accession numbers of the identified proteins.

RESULTS: Proteomics analysis revealed to identity of proteins of which at least 23 were up and 57 were downregulated. Bioinformatics analysis of differentially regulated proteins by STRING indicated that protein folding and clearance machinery were interrupted in PFAPA patients compared to the controls. The affected pathways underlined the importance of the mitochondrial electron transport chain and ATP biosynthesis process.

CONCLUSION: Although it is not clear that changes in tonsil protein expression whether directly related to pathogenesis or simply result of chronic inflammation, the identification of tonsil biomarkers for PFAPA may provide clinicians an opportunity to understand disease pathogenesis or develop new molecular targets for treatments.

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IMPACT:

- Proteomics analyses of tonsils revealed the identity of 80 proteins of which at least 23 were up and 57 were downregulated.
- Bioinformatics analysis underlined the importance of mitochondrial ETC and regulation of ATP biosynthetic process.
- This is the first study evaluating the proteomics of the tonsils of PFAPA patients.
- The identification of tonsil biomarkers for PFAPA may provide clinicians an opportunity to understand disease pathogenesis or develop new molecular targets for treatments.

INTRODUCTION

Periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) syndrome is a recurrent fever syndrome of unknown etiology characterized by regular episodes of fever, pharyngitis, oral aphthous, and cervical lymphadenopathy.¹ Despite being considered the most common periodic fever syndrome, the exact etiopathogenesis of PFAPA syndrome remains elusive. While studies suggest that family history is a risk factor for disease development, the pathogenesis of PFAPA is complex and likely arises from a combination of genetic disposition and environmental factors.^{2–4} Furthermore, in a previous study, genetic similarities were observed among recurrent aphthous stomatitis,

PFAPA, and Behçet's disease, positioning these disorders on a spectrum. Recurrent aphthous stomatitis was found at the mild end, Behçet's disease at the severe end, and PFAPA in between.⁵

As evidenced by the resolution of symptoms post-tonsillectomy, the involvement of tonsils in the pathophysiology of the illness is apparent. Although prior research has extensively examined histopathologic changes in tonsils, the etiopathogenesis of the disease is still unclear.^{6–11} The objective of this study was to examine alterations in histopathologic and proteomics profiles of the tonsils of PFAPA patients. Our objective was to deepen the insight into the underlying pathophysiology, to provide a valuable foundation for future protein research.

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MATERIAL-METHODS

This prospective observational study was performed between March 2022 and March 2023. The study protocol was approved by the Ethics Committee and strictly adhered to the principles of the Declaration of Helsinki. Before informed consent was obtained from all participants.

Patients

The study group consisted of 7 patients diagnosed with PFAPA and the control group consisted of 7 patients who underwent tonsillectomy for reasons other than chronic tonsillitis. The inclusion criteria for the PFAPA patients, aged 0–18 years, followed the classification outlined by Vanoni et al.¹² These individuals had been selected for tonsillectomy as a therapeutic intervention. Patients with any additional systemic disease were excluded. Furthermore, individuals who underwent tonsillectomy due to reasons other than chronic tonsillitis were included in the control group. All patients in the control group underwent tonsillectomy due to obstructive sleep apnea.

Tonsil collection and preservation

The tonsillectomy procedure was performed by cold dissection method under general anesthesia. Following tonsillectomy, the tonsil tissues were finely diced into small pieces and thoroughly washed with an ice-cold buffer consisting of 50 mM Tris.Cl, pH 7.4 supplemented 250 mM sucrose. The tissue pieces were stored at -80°C until use.

Histological examination of the tonsil

From archived paraffin-embedded, formalin-fixed tonsil blocks, five-micrometer sections were prepared. Histology examination was performed using Hematoxylin-eosin (H&E) staining. A senior pathologist (BYB) evaluated H&E slides from both children with PFAPA and the controls. The first measurement was taken at one randomly selected germinal center. Next, measurements were taken at the germinal center that was nearest to the original germinal center. This process was repeated, selecting the germinal center closest to the last one measured, until five germinal centers were eventually chosen. For each selected germinal center, the area of the germinal center and the width of the widest part of the mantle (a group of tiny lymphocytes surrounding the germinal center) were measured. This procedure was then repeated in a different area of the tonsil, starting with a new randomly selected germinal center, until a total of ten germinal centers were measured for each subject.

Five crypts and five squamous epithelium regions were chosen at random, and their respective widths were measured. The selection of ten measurements was based on the abundance of crypt and squamous epithelium areas in most tonsil pieces, ensuring a representative sampling from at least five distinct parts of each characteristic. Subsequently, the average germinal center area, mantle width, interfollicular distance, crypt width, and squamous epithelium width for each subject's tonsil were calculated based on the collected measurements.

Immunohistochemical examination of the tonsils

Immunohistochemical staining was performed using CD3 (clone F7.2.38, 1:75; Dako, Glostrup, Denmark), CD4 (clone 4B12, 1:100; Novocastra, Newcastle upon Tyne, UK), CD8 (clone C8/144B, 1:50; Dako), CD15, (clone MMA, 1:20; Thermo Scientific, Waltham, MA), CD20 (clone L26, 1:1000; Dako), CD45 (LCA) (clone 2B11 + PD7/26, 1:500; Dako), CD57 (clone NK-1, 1:25; Novocastra, Nussloch, Germany), CD61 (clone 2F2, 1:100; CellMarque, Rocklin, CA) and CD56 (clone MRQ-42, 1:100; CellMarque, Rocklin, CA) antibodies on 5 μm thick sections from paraffin blocks containing resection materials. Staining was done on a Ventana automatic device (RocheVentana), using Ventana Optiview and Ultraview DAB kits. Five different randomized germinal centers were selected for

each case. The number of positive cells according to CD3, CD4, CD8, CD15, CD61, CD56, and immunohistochemistry were recorded and averaged in a field of view (at 40x) in the germinal centers of the palatine tonsils. The germinal centers were densely populated with CD20. Virtually all lymphocytes were CD45+ throughout the entire tonsil.

Protein extraction and proteomic analysis

The tissue samples were finely diced into small pieces, thoroughly washed with an ice-cold wash buffer (50 mM Tris.Cl, pH 7.4 supplemented with 250 mM sucrose), and then subjected to centrifugation at $10,000 \times g$ to remove traces of contaminating blood. The washed tissue pieces were then treated with a lysis buffer composed of 10 mM Tris-HCl pH 8.0, 7 M urea, 5 mM magnesium acetate, 4% CHAPS, and 2 M thiourea at pH 8.0. Homogenization was achieved using a Scilogex homogenizer for 10 s at $20,000 \times g$ while maintaining a low temperature on ice. To further facilitate homogenization, a bead-beater (Bullet Blender, Next Advance, Troy, NY) equipped with 1.4 mm stainless steel beads was used. The tissue lysate was then centrifuged at $10,000 \times g$ for 15 min to obtain a cell-free supernatant, which was subsequently subjected to a second centrifugation step at $15,000 \times g$ for 45 min to ensure clarification. Protein concentration was measured using the Bradford assay (Bio-Rad) with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The protein concentrations of the pooled protein samples were measured by Qubit Protein Assay (Thermo Fisher Scientific).

Nano-liquid chromatography-mass spectrometry (nLC-MS/MS)

For analysis of peptides Ultimate 3000 RSLC nano system (Dionex, Thermo Scientific, CA) coupled to a Q-Exactive mass spectrometer (Thermo Scientific) was used. The entire system was controlled by Xcalibur 4.0 software (Thermo Fisher Scientific, CA). High-performance liquid chromatography (HPLC) separation was performed using mobile phases of A (0.1% Formic Acid) and B (80% Acetonitrile + 0.1% Formic Acid). Digested peptides were pre-concentrated and desalted on a trap column. The peptides were then transferred to an Acclaim PepMap RSLC C18 analytical column ($75 \mu\text{m} \times 15 \text{ cm} \times 2 \mu\text{m}$, 100 Å diameter, Thermo Scientific, CA). The gradient for separation was 6% for 8 min, 6–10% B for 12 min, 10–30% B for 160 min, 30–50% B for 25 min, 50–90% for 10 min, 90% for 15 min, 90–6% B for 5 min and 6% B for 5 min with the flow rate of 300 nL/min in a 240-minute total run time. Full scan MS1 spectra were acquired with the following parameters: resolution 70,000, scan range 400–2000 m/z, target automatic gain control (AGC) 3×10^6 , maximum injection time 60 ms, spray voltage 2.3 kV. MS/MS analysis was performed by data-dependent acquisition selecting the top ten precursor ions. The MS2 analysis is composed of collision-induced dissociation (higher-energy collisional dissociation (HCD)) with the following parameters: resolution 17,500, AGC 1×10^6 ; maximum injection time 100 ms, isolation window 2.0 m/z normalized and collision energy (NCE) 27. The instrument was calibrated using a standard positive calibrant (LTQ Velos ESI Positive Ion Calibration Solution 88323, Pierce) before each analysis.

nHPLC LC-MS/MS data analysis

Analysis of the raw data was performed with Proteome Discoverer 2.2 software (Thermo Scientific) for protein identification and the following parameters were used; peptide mass tolerance 10 ppm, MS/MS mass tolerance 0.2 Da, mass accuracy 2 ppm, tolerant miscarriage 1, minimum peptide length 6 amino acids, fixed changes cysteine carbamidomethylation, unstable changes methionine oxidation and asparagine deamination. The minimum number of peptides identified for each protein was 1 and obtained data were searched against Uniprot/Swissprot database.

Bioinformatics analysis

The differentially regulated proteins were subjected to STRING analysis to elucidate protein-protein interactions and perform functional enrichment. The STRING analysis was carried out using the UniProt accession numbers of the identified proteins. The organism was specified as *Homo sapiens* and functional classifications were viewed as bar charts. Selected ontologies included molecular function, biological process, cellular function, cellular component, protein class, and pathway. For each ontology, manual analysis was performed by listing the selected proteins and cross-checking their given properties with UniProt entries.

Statistical analysis

All statistical analyses were performed using IBM SPSS 20.0 (SPSS, Chicago, IL). Descriptive analyses are presented using proportions, medians, and range values as appropriate. The χ^2 /Fischer and Mann-Whitney U tests were used to compare non-normally distributed variables between the independent two groups. A *P*-value of 0.05 was considered a statistically significant result.

RESULTS

Baseline characteristics of the patients

No significant differences in age and sex were observed between the patient and control groups ($p > 0.05$). The median ages of patients and control group were 5 (4–7) and 5.5 (4.5–7) years, respectively. The median time between PFAPA diagnosis and tonsillectomy was 2 (1–3) years. Tonsillectomy was performed at least one month after the last flare, and none of the patients had taken steroids within the previous three months.

Histological findings of tonsils

The comparison of the number of positive cells by immunohistochemistry in one field of vision (at 40x) in germinal centers of palatine tonsils was depicted in Table 1. However, no significant differences were observed between the patient and control groups in terms of their immunohistochemistry features (Fig. 1).

Qualitative and quantitative analysis of pooled samples

The label-free proteome analysis revealed that 23 proteins were upregulated while 57 were downregulated when the two-fold change, a standard fold of change criteria were applied between the two groups (in log₂ scale) (Supplementary table). STRING analysis highlighted alterations in the mitochondrial electron transport chain (ETC) suggesting a regulatory effect on the ATP biosynthesis process (Fig. 2).

Western Blot analysis was used for verification of the LC-MS/MS data. An anti-OXPHOS antibody cocktail clearly showed that complex III and IV were substantially and complex II was moderately downregulated in the PFAPA group (Fig. 3). For complex V, it was not possible to infer its differential regulation due to the intensity of the saturated band. Overall, however, the complexes in the ETC were differently regulated in PFAPA patients.

After observing the downregulation of ETC complexes, we hypothesized that cells should also be regulated in the Krebs cycle to prevent wastage of catabolic activity. Several key regulation points are used to regulate the Krebs cycle. One of the key regulation points is where pyruvate is converted to acetyl-CoA. This reaction is catalyzed by an enzyme complex, the Pyruvate dehydrogenase complex (PDHC). Therefore, we analyzed the changes at the PDHC level. The PDHC consists of three enzymes: pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3). The analysis of PDHC by Western Blot analysis showed downregulation in all PDHC subunits (E1 α , E1 β , E2, and E3bp) (Fig. 4). This observation indicated a blockage or malfunction in the Krebs cycle. We also observed a clear downregulation in Complex V of ETC confirming once again that all complexes of ETC were downregulated.

DISCUSSION

In the present study, proteomics analyses of tonsils revealed the identity of 80 proteins of which at least 23 were up and 57 were downregulated. Bioinformatics analysis underlined the importance of mitochondrial ETC and regulation of ATP biosynthetic process.

To identify the etiopathogenesis of PFAPA, studies focused on histopathological changes in tonsils. Conflicted results have been reported. Initially, Peridis et al.⁹ reported complete remission in 8 (88.9%) of 9 patients with PFAPA after tonsillectomy while they found that histologic and immunohistochemical features revealed like chronic tonsillar inflammation. In contrast to this result, Dytrych et al.⁶ showed higher percentages of CD8+ and naïve CD4+ and CD8+ T cells in PFAPA tonsils compared to tonsils of obstructive sleep apnea patients. They suggested that T cells play a central role in the pathogenesis. On the contrary, Førsvoll et al.⁸ reported that tonsils from children with PFAPA showed a lower number of CD8+ cells in germinal centers compared to tonsillar hypertrophy. Subsequently, Manthiram et al.⁷ found enlargement of germinal centers and squamous epithelia in the tonsils of patients with PFAPA compared to obstructive sleep apnea. Furthermore, Gazi et al.¹¹ showed higher TLR-1 and TLR-2 on the lymphoid interior and lower on the surface epithelium of

Table 1. Number of positive cells by immunohistochemistry in one field of vision (at 40x) in germinal centers of palatine tonsils from PFAPA (n = 7) and control children with obstructive sleep apnea (n = 7).

	PFAPA median (min-max)	Controls median (min-max)	P-value
Average mantle size (μ m)	180 (100–250)	170 (150–200)	0.847
Average interfollicular distance (μ m)	170 (150–250)	220 (150–250)	0.516
Average crypt width (μ m)	900 (500–1100)	1100 (1000–1200)	0.100
Average squamous epithelium width (μ m)	11000 (7000–16,000)	8000 (6000–14,000)	0.198
CD3	230 (140–280)	250 (150–260)	0.403
CD4	210 (126–250)	200 (136–250)	0.949
CD8	13 (8–22)	18 (12–24)	0.305
CD15	1 (0–2)	0 (0–2)	0.177
CD20	800 (500–900)	820 (600–870)	0.303
CD45	970 (640–1160)	1020 (810–1080)	0.405
CD56	0 (0–5)	0 (0–0)	0.142
CD61	0 (0–0)	0 (0–0)	1.000

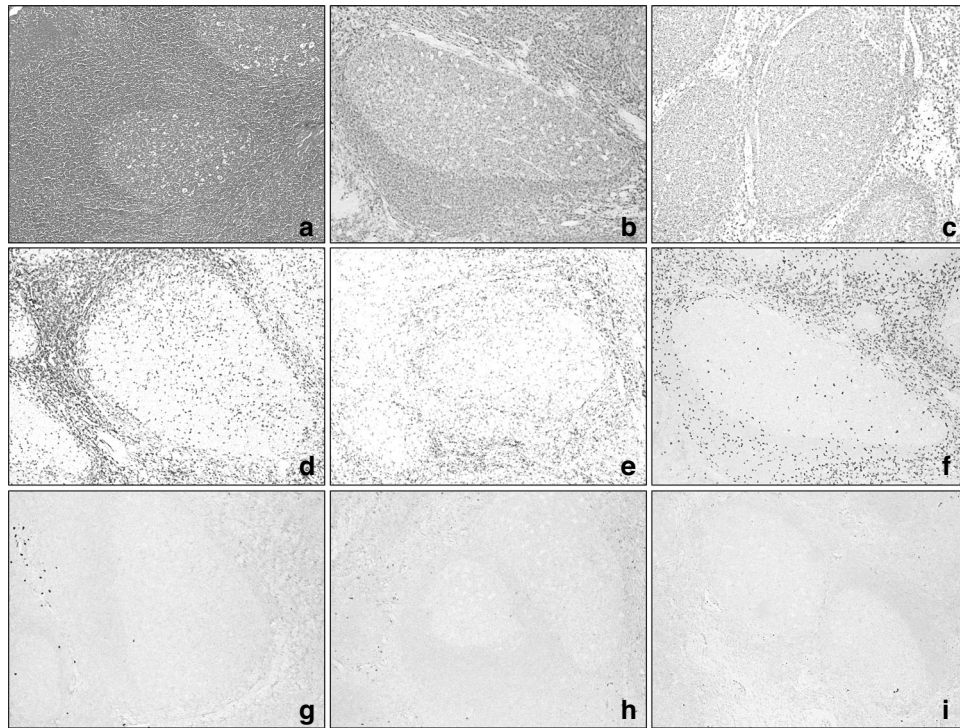
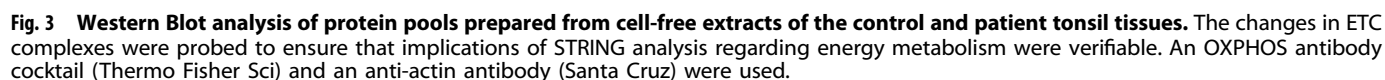
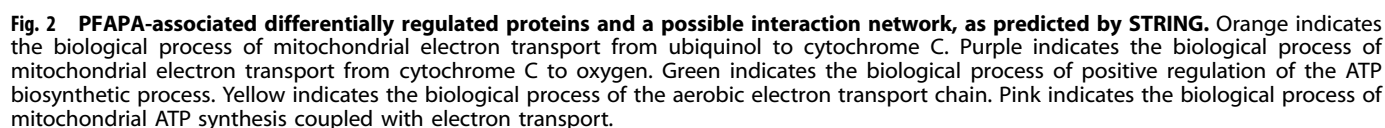


Fig. 1 Histopathological examination of tonsil tissue samples. **a** Representative H&E-stained histologic tonsil sections from PFAPA patients, showing the overall architecture of the tonsil (x100). **b** Immunohistochemical staining for LCA in palatine tonsil germinal centers, highlighting leukocyte common antigen distribution (x100). **c** CD20, indicating B-cell localization within the germinal centers (x100). **d** CD3, showing T-cell distribution (x100). **e** CD4, highlighting helper T-cell presence (x100). **f** CD8, indicating cytotoxic T-cell localization (x100). **g** CD15, showing granulocyte presence (x100). **h** CD56, indicating natural killer cell distribution (x100). **i** CD61, highlighting platelet and megakaryocyte presence (x100).

PFAPA tonsils compared to tonsils of group A beta-hemolytic streptococcal recurrent tonsillitis. Finally, Turkucar et al.¹⁰ demonstrated an increased number of CD4+ and CD8 + T cell counts on tonsils of PFAPA compared to tonsils of obstructive upper airway disorder. However, we could not demonstrate any differences between the patient and control groups in terms of immunohistochemistry features.

Although there was no histopathological difference, the proteomics analysis highlighted changes in mitochondrial electron transport pathways. The ETC is an essential component of cellular respiration, a complex metabolic process that enables organisms to derive energy from nutrients. Spanning the inner mitochondrial membrane of eukaryotic cells, the ETC comprises a sequence of multi-protein complexes and electron carriers, working in harmony to facilitate the transfer of high-energy electrons.¹³ Recent research highlighted a dysregulated tricarboxylic acid (TCA) cycle during the peak of inflammation. During the inflammatory process, activated immune cells undergo metabolic reprogramming to support activities such as cell proliferation, cytokine production, differentiation, and immune responses. Inflammatory cells prefer glycolysis while producing energy during the inflammatory phase. Subsequently, oxidative phosphorylation (OXPHOS) assumes the primary role of generating energy while inflammation resolves.¹⁴ Furthermore, inhibiting mitochondrial ATP production or mTOR activation had a detrimental effect on neutrophil chemotaxis, as indicated by previous studies.^{15,16} This implies that both glycolysis and mitochondrial respiration might play a role in mediating neutrophil transmigration. Immune cells clearly adapt their metabolism according to inflammation phase. But it remains unclear whether this metabolic switch is a result or an underlying factor in the pathogenesis. In the present study, we confirmed our findings by using Western Blot analysis. It clearly showed that

complex III and IV were substantial, complex II and V were moderately downregulated in the PFAPA group. These findings support the metabolic switch in inflammation. Downregulation of ETC complexes should have prevented oxidation of mitochondrial NADPH and FADH₂, causing halting of tricarboxylic acid (TCA) cycle. Metabolic reprogramming involves an increased rate of glycolysis and a decreased rate of mitochondrial respiration during the inflammation. The PDHC works collectively to convert pyruvate into acetyl-CoA, NADH, and CO₂ via oxidative carboxylation. Acetyl-CoA engages in a catalytic reaction with oxaloacetate, serving as the instigator of the citric acid cycle, and thereby enhancing energy production through glycolysis. Reduction in PDH activity results in influx of pyruvate levels and reduced acetyl-CoA production. Inhibiting mitochondrial pyruvate oxidation has proinflammatory consequences, as it shifts mitochondrial respiration toward glycolysis, intensifying Nod-like receptor family pyrin domain containing 3 (NLRP3) activation and interleukin-1 β (IL-1 β) secretion.¹⁷ Furthermore, pyruvate oxidation plays a role in T cell development, where different T cell subsets exhibit distinct metabolic patterns. Studies have demonstrated that steering metabolism towards glycolysis is essential for the differentiation of proinflammatory T cells, while metabolic reprogramming towards pyruvate oxidation is necessary for the development of anti-inflammatory regulatory T cells (Tregs).¹⁷ Upon activation, macrophages generate nitric oxide (NO), exerting pleiotropic effects within the cell. This leads to the inhibition of the electron transport chain (OXPHOS), as well as the E2 and E3 subunits of the pyruvate dehydrogenase (PDH), along with select enzymes involved in the TCA cycle (ACO2, IDH, OGDH).¹⁸ As we discussed above, in the zenith of inflammatory processes, immune cells exhibit a predilection for prioritizing glycolytic pathways as their primary energy source. Conversely, as the resolution phase ensues, these cells predominantly shift towards relying on



Apoptosis is considered a non-inflammatory, programmed process of cellular death. It is triggered by specific signals that prompt the formation of the apoptosome, a cytosolic protein

complex. The apoptosome utilizes Apaf-1 as a sensor to detect cytochrome c released from stressed or damaged mitochondria.²⁰ Once assembled, Apaf-1 oligomerizes and recruits caspase-9, which, in turn, activates caspase-3, ultimately leading to apoptosis.²¹ The inflammasome is another cytosolic protein complex that also activates caspases. NLRP3 is one of the inflammasomes which recruits the adapter protein Asc and caspase 1, resulting in caspase activation. The activated caspase cleaves pro-IL-1 β and pro-IL-18, leading to the release of mature cytokines. In certain

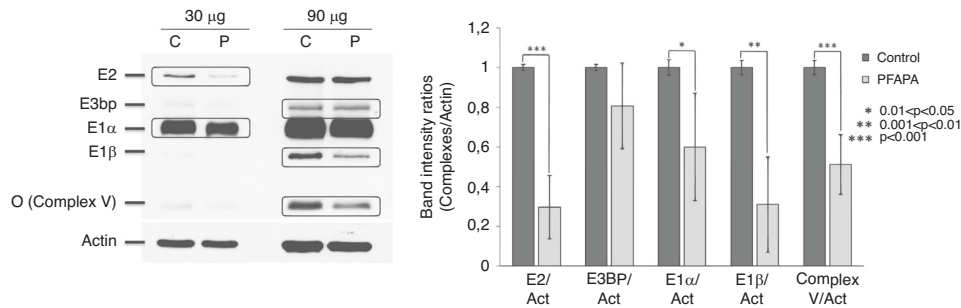


Fig. 4 The changes in PDHC and Complex V of ETC were probed by Western Blot analysis to ensure that implications of STRING analysis regarding Krebs cycle were verifiable. A PDH antibody cocktail (Thermo Fisher Sci) and an anti-actin antibody (Santa Cruz) were used.

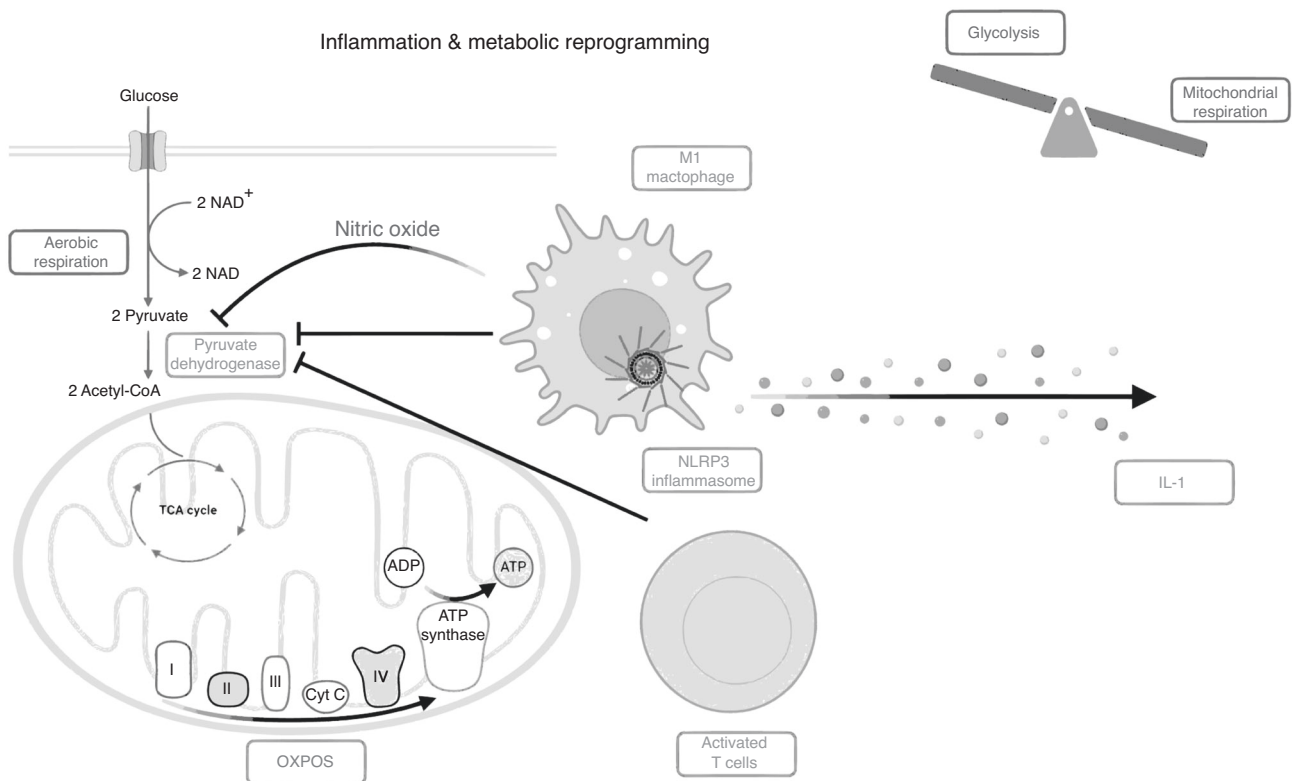


Fig. 5 A schematic representation of the metabolic programming during the inflammation.

cases, this process can trigger pyroptosis, an inflammatory form of cell death.²² Recent research highlighted an interaction between cytochrome c and NLRP3 inflammasome.²³ During intrinsic apoptosis, cytochrome c oxidizes cardiolipin, resulting in the release of cytochrome c into the cytosol, which aids in triggering apoptosome formation. The release of cytochrome c was found to inhibit the activation of the NLRP3 inflammasome, and this effect is likely achieved by inhibiting the association between NLRP3 and both cardiolipin and NEK7.²³ The aforementioned study also showed that suppressing cytochrome c levels through gene silencing resulted in an augmentation of IL-1 β production following NLRP3 inflammasome activation.²³ Since patients with PFAPA express high IL-1 β levels, changes in the mitochondrial pathways other than energy metabolism may also contribute to inflammation.

Our study is limited by its small sample size. Furthermore, PFAPA is characterized by episodic occurrences of tonsillitis, while patients are totally healthy during the attack intervals. As tonsillectomy is typically conducted during these asymptomatic

periods, they might not encompass the entirety of the underlying pathophysiological alterations. However, a notable strength of this study lies in its pioneering nature of evaluating the proteomics of the tonsils of PFAPA patients. Furthermore, we were unable to detect complex I in the Western Blots, which could be due to several factors: 1) Inefficient transfer of the complex I component NDUFB8 (NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8), 2) Precipitation of the antibody recognizing NDUFB8 in our antibody cocktail, leading to the failure of complex I detection, or 3) The need for optimization of Western Blot conditions for detecting complex I.

In conclusion, the discovery that immunometabolism has the potential to serve as a central regulator of inflammation opens new possibilities for addressing inflammation-related disorders by modulating the metabolism of vascular and immune cells. Consequently, gaining a deeper insight into the metabolic pathways within inflammatory cells could be harnessed as a therapeutic approach to mitigate overactive inflammatory reactions.

DATA AVAILABILITY

Data available on request.

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AUTHOR CONTRIBUTIONS

Conceptualization: F.M., M.K., B.Y.Y., M.S., N.Ş., A.Ö., G.A., Y.E.B., H.E.S.; Methodology: F.M., M.K., B.Y.Y., M.S., N.Ş., A.Ö., G.A., Y.E.B., H.E.S.; Formal analysis and investigation: F.M., M.K., B.Y.Y., M.S., N.Ş., A.Ö., G.A., Y.E.B., H.E.S.; Writing—original draft preparation: F.M., M.K., B.Y.Y., M.S., N.Ş., A.Ö., G.A., Y.E.B., H.E.S.; Writing—review and editing: F.M., M.K., B.Y.Y., M.S., N.Ş., A.Ö., G.A., Y.E.B., H.E.S.; Funding acquisition: F.M., M.K., B.Y.Y., M.S., N.Ş., A.Ö., G.A., Y.E.B., H.E.S.; Supervision: F.M., M.K., B.Y.Y., M.S., N.Ş., A.Ö., G.A., Y.E.B., H.E.S. All authors reviewed and revised the manuscript and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

The protocol of the study was approved by the Ethical Commission for Research from Kocaeli University School of Medicine.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41390-024-03741-z>.

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