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Research article

Increased mitochondrial proton leak and glycolysis in peripheral blood mononuclear cells in type-1-diabetes

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ABSTRACT

Changes in cellular bioenergetics such as mitochondrial respiration and glycolysis may play a role in the pathogenesis of various diseases including type 1 diabetes (T1D). We used Seahorse extracellular flux technology to analyse the efficiency of glycolysis and mitochondrial oxidative phosphorylation in peripheral blood mononuclear cells (PBMCs) obtained from fresh blood samples from fifteen long-term T1D individuals with albuminuria (five females) with an average (±SD) age of 58 (±14) years and 15 age and sex-matched healthy non-diabetic controls. In T1D PBMCs, mitochondrial proton leak was higher (T1D: 21,3 ± 1,46 pmol/min; controls: 17,3 ± 1,24 pmol/min; $p = 0{,}049$) and glucose (5 mM) suppressed mitochondrial proton leak more than in healthy controls. Further, PBMCs from T1D individuals had higher glycolysis compared with healthy controls (T1D: 9,68 ± 0,94 mpH/min; controls: 7,07 ± 0,64 mpH/min; $p = 0{,}032$). Correlation analysis of circulating inflammatory factors identified Leukaemia Inhibitor factor 1 (LIF) being negatively correlated with PBMC glycolysis. Our results suggest that mitochondrial and glycolytic pathways of PBMCs from long-term T1D individuals with albuminuria might be dysfunctional, possibly due to increased cellular metabolic load and/or oxidative stress in which inflammatory factors could play a role.

1. Introduction

Type 1 diabetes (T1D) is characterized by autoimmune destruction of the pancreatic β-cells leading to insulin deficiency and impaired blood glucose homeostasis. Despite insulin replacement therapy most patients develop long-term complications that cause reduced life expectancy and lower quality of life (Saberzadeh-Ardestani et al., 2018; Pathak et al., 2019).

Compelling evidence suggests that dysfunction of bioenergetic processes such as mitochondrial respiration, have pathogenetic roles in various diseases (Tomas et al., 2017; Smith et al., 2018; Sidarala et al., 2020). In T1D, only scarce information about mitochondrial dysfunction exists. However, in skeletal muscle, lower mitochondrial oxidative phosphorylation capacity and increased mitochondrial H_2O_2 production have been reported in young adults with T1D compared to healthy controls (Monaco et al., 2018). In addition, T cells from T1D subjects display hyperpolarization of the mitochondrial inner membrane potential correlating with increased cytokine production, increased generation of reactive oxygen species (ROS) and lower ATP content (Chen et al., 2017). Whether these mitochondrial abnormalities in T1D have pathogenetic effects or are merely a consequence of dysregulated glucose metabolism are unclear.

In relation to diabetes-associated complications, peripheral blood mononuclear cells (PBMCs) from patients with diabetic nephropathy were reported to have impaired mitochondrial respiration compared to PBMCs from patients with diabetes without nephropathy (Czajka et al., 2015). This suggests that changes in the bioenergetics of PBMCs correlate with the dysfunction of other organs in diabetes. A better understanding of PBMC bioenergetics may therefore provide both molecular insight into disease mechanisms and inform about disease status in a broader context.

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Importantly, however, the extent to which PBMC bioenergetics is dysregulated in patients with T1D compared to healthy individuals has not yet been established.

Based on the hypothesis that PBMC bioenergetics are impaired in T1D, we aimed to use the Seahorse extracellular flux technology to compare various parameters of mitochondrial and glycolytic bioenergetics in PBMCs from individuals with long-term T1D and albuminuria with healthy controls.

2. Results

2.1. PBMCs mainly represent purified lymphocytes and monocytes

This study was a cross-sectional sub-study investigating mitochondrial and glycolytic function from a randomised controlled trial, which investigated the acute effect of dapagliflozin on kidney oxygenation in 15 individuals with T1D and albuminuria and 15 age- and sex-matched non-diabetic controls (for baseline comparison). Trial registration details are provided in the Material and Methods section. The baseline characteristics of the study participants have been published in (Laursen et al., 2022) and are presented in Table 1. We used density gradient centrifugation of whole blood for isolation of fresh PBMCs followed by assessment of sample purity. As expected, lymphocytes and monocytes constituted the majority of the PBMC fraction with platelets and neutrophils almost being absent compared to whole blood (Table 2).

2.2. T1D PBMCs have increased mitochondrial proton leak

We evaluated bioenergetics of the PBMCs using Seahorse extracellular flux technology which allows tracking of cellular metabolic activity in real-time. First, we determined basal and glucose-induced mitochondrial respiration by measurements of oxygen consumption rate (OCR). During the real-time OCR measurements, a combination of inhibitors specific for different complexes in the electron transport chain in the presence or absence of 5 mM glucose were injected to the cells' assay medium allowing for evaluating the energetic flux in detail (see Materials and Methods section for detailed information about inhibitors).

In the absence of glucose, basal respiration, ATP production, maximal respiration, spare respiration, and non-mitochondrial respiration did not differ between PBMCs from T1D and healthy non-diabetic individuals (Figure 1A-C). However, we observed a significantly higher mitochondrial proton leak in PBMCs from individuals with T1D compared with healthy controls (Figure 1A and 1C). In PBMCs from both healthy controls and T1D individuals, glucose suppressed mitochondrial proton leak, but the effect was significantly more pronounced in T1D PBMCs (controls: -3,11 pmol/min; T1D: -6,06 pmol/min; p = 0,012) compared with healthy controls (Figure 1A and C).

2.3. T1D PBMCs have increased glycolysis

The glycolytic function of PBMCs was measured by extracellular acidification rate (ECAR). Glycolysis in response to glucose was significantly higher (controls: 7,07 mpH/min; T1D: 9,68 mpH/min; p = 0,032) in PBMCs from T1D individuals compared with healthy control PBMCs (Figure 2A-C). The glycolytic capacity and glycolytic reserve were not different between groups.

Table 1. Baseline characteristics of study participants. Values are mean ± SD. P-values were calculated from a two-sample Wilcoxon test for each parameter.

	H (n = 15)	T1D (n = 15)	P-value
Age (years)	56 ± 15	58 ± 14	0,82
Sex (women)	5 (33%)	5 (33%)	1
Weight (kg)	78,8 ± 21,1	81,5 ± 15,2	0,576
Body Mass Index (kg/m2)	25,9 ± 7,1	26,8 ± 3,1	0,64
Disease duration	-	39 ± 16	-
Hba1c (mmol/mol)	34,5 ± 2,6	60,7 ± 7,1	<0,0001
Plasma Glucose	5,9 ± 0,4	9,6 ± 1,0	<0,0001
LDL-Cholesterol	2,52 ± 0,3	2,09 ± 1,1	<0,01
Lipid-lowering medication	1 (7%)	11 (73%)	<0,001

Table 2. Complete cell count in blood samples and isolated PBMCs Values are mean ± SD of 10^9 cells/L for all cell types (except platelets where values are 10^{12} cells/L) for all participants.

	Red Blood cells	Platelets	Neutrophils	Monocytes	Lymphocytes
Blood	4,71 ± 0,810	258 ± 68,314	5,1 ± 3,135	0,637 ± 0,398	2,22 ± 0,759
PBMCs	0,007 ± 0,0046	6,18 ± 2,758	0,034 ± 0,037	0,613 ± 0,395	2,76 ± 0,759

2.4. Correlation between plasma LIF and PBMC glycolysis in T1D

Evidence suggests a relationship between increased glycolysis and release of inflammatory cytokines from PBMCs of individuals with cognitive decline (Wolfe et al., 2018). We therefore applied an inflammation biomarker panel to measure inflammatory biomarkers in the plasma of the study participants and performed correlation analysis between glycolysis and inflammatory markers. We observed 39 inflammatory markers upregulated in the T1D participants compared to healthy controls (Supplementary table 1). Correlation analysis showed that Leukaemia Inhibitor factor 1 (LIF) negatively correlated with glycolysis in T1D PBMCs (Supplementary table 2).

2.5. Lipid-lowering medication is a confounder of PBMC mitochondrial function

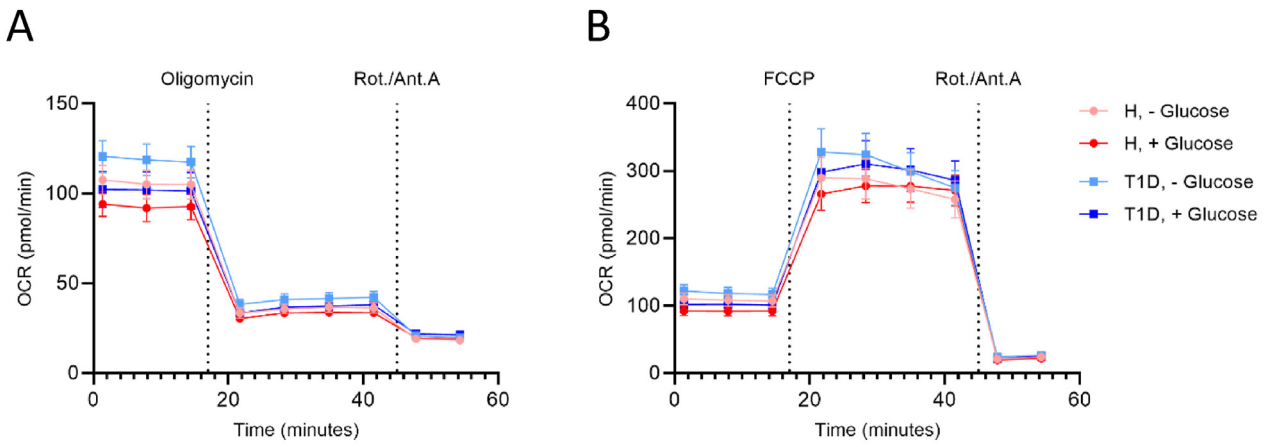
We questioned if some of the baseline characteristics of the study participants could be possible confounders of the changes in PBMC bioenergetics observed in T1D. To deal with this, we performed correlation analysis and linear regression on the mitochondrial and glycolytic parameters presented above in relation to plasma glucose, LDL-cholesterol and body weight but found no significant interactions with these (Supplementary figure 1, 2 and 3).

Although study participants should abstain from medication on the trial visit days (except for insulin for T1D participants), possible interference of medication on the results cannot be excluded. In fact, 73% of T1D participants were on lipid-lowering medication, which arguably could decrease substrate availability for PBMC mitochondrial respiration. Indeed, correlation analysis with mitochondrial respiration parameters revealed a significant interaction of lipid-lowering medication with maximum and spare respiration (supplementary figure 3, D4 and D5). However, lipid-lowering medication was not a confounder for proton leak.

3. Discussion

Here, we compared bioenergetics of freshly isolated PBMCs from individuals with T1D with that of healthy non-diabetic controls. While most parameters measured did not differ between groups, mitochondrial proton leak and glycolytic response were significantly higher in T1D. Further, glucose-inhibited mitochondrial proton leak was significantly more pronounced in T1D compared to controls.

Proton leak has been reported to be followed by excessive cellular ROS production which may lead to cellular dysfunction and death (Ana et al., 2021). We hypothesize that individuals with a long duration of T1D, like those included in the present study, are at increased risk of cellular oxidative stress in circulating immune cells. This is in agreement with previous studies reporting increased biomarkers of oxidative stress and decreased antioxidant defence mechanisms in both T1D and T2D (Dzugkoev et al., 2012; Gawlik et al., 2016; Alghazeer et al., 2018).



C

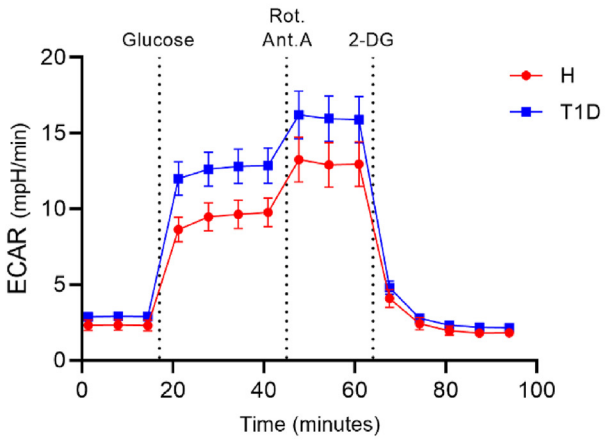
	Mitochondrial respiration (no Glucose)			Glucose-inhibited mitochondrial respiration		
	H	T1D	P-value [95%CI]	H	T1D	P-value [95%CI]
Basal Respiration	86,5±7,04	95,5±7,08	0,373 [-29,5; 11,4]	-12,9±2,9 ***	-15,2±3,94 **	0,642 [-7,7; 12,3]
ATP Production	69,3±5,55	76,7±5,9	0,371 [-24; 9,2]	-9,31±3,15 *	-11,2±3,68 **	0,704 [-8,1; 11,8]
Proton Leak	17,3±1,24	21,3±1,46	**0,049** [-7,9; -0,01]	-3,11±0,73 ***	-6,06±0,82 ***	**0,012** [0,7; 5,2]
Maximal Respiration	257±27,6	283±27,9	0,513 [-106,5; 54,4]	-2,91±15,8	-4,69±12,9	0,775 [-36,7; 28,1]
Spare Respiration	170±21,6	190±22,5	0,541 [-83; 44,5]	10,5±14,9	8,54±9,99	1 [-29,6; 20,7]
Non-Mitochondrial Resp.	19,5±1,8	21,7±1,92	0,419 [-7,6; 3,2]	-0,57±0,96	-0,72±0,78	0,903 [-2,4; 2,7]

Figure 1. T1D PBMCs have increased mitochondrial proton leak. A and B: PBMC OCR traces with addition of Oligomycin or FCCP, incubated with or without glucose for one hour. N = 15 for all four groups, values are mean ± SE of OCR (pmol/min). C: Mitochondrial respiration parameters calculated from Seahorse analysis without glucose or in response to glucose. Values are OCR mean ± SE. P-values were calculated from a two-sample unpaired t-test or Wilcoxon test for each parameter. Difference within each group in relation to the presence of glucose presented as * = $p < 0,05$; ** = $p < 0,01$ and *** = $p < 0,001$; calculated from a paired t-test or paired Wilcoxon.

From our results we cannot infer whether the changes in T1D PBMC bioenergetics are a consequence of the dysfunction of other organs, like the pancreas. We can solely establish that PBMC bioenergetic dysfunction is present in T1D. Our cell population of interest is lymphocytes and monocytes, which have been reported to exhibit similar mitochondrial oxidative phosphorylation function (Chacko et al., 2013). Potentially, our PBMC isolation method could have resulted in contamination of our cell population of interest with platelets, which are highly metabolically active cells (Kramer et al., 2014). However, we used a Sysmex Xn550 haematology analyser to obtain complete blood cell analyses and assert the purity of the cell population and concluded that our method successfully isolated the cells of interest to a high purity with very low levels of platelets. We therefore believe our obtained results on the PBMC cell fraction reflect the bioenergetics of lymphocytes and monocytes.

The basal glycolysis rate was not different between T1D and healthy controls, possibly because T1D participants did not stop taking insulin during the study. However, we found that the glycolytic response to glucose in PBMCs from T1D individuals was significantly higher compared to healthy individuals. This result suggests that PBMCs from T1D individuals are more adapted to using glycolysis for energy as opposed to PBMCs from healthy individuals which seemingly utilize mitochondrial oxidative phosphorylation for ATP production to a higher degree. Overall, this fits well with the fact that individuals with T1D have more fluctuating and on average higher blood glucose levels than non-diabetic subjects. Hence, the PBMCs as well as other tissues adapt to this and utilize glucose as the main fuel thereby possibly explaining the higher glycolysis rate observed. Interestingly, a recent study reported that increased glycolysis correlates with augmented production of inflammatory cytokines in PBMCs obtained from individuals with cognitive decline (Wolfe et al., 2018). As T1D is associated with increased levels of circulating pro-inflammatory cytokines (Fatima et al., 2016) which may play important roles in both T1D pathogenesis and development of complications, it is intriguing to speculate that the increased level of glucose-induced glycolysis in PBMCs in T1D is associated with increased

A



B

	Glycolysis		
	H (n=14)	T1D (n=15)	P-value [95%CI]
Glycolysis	7,07±0,64	9,68±0,94	**0,032** [-4,98; -0,2]
Glycolytic Capacity	10,7±1,16	13,1±1,33	0,189 [-6; 1,2]
Glycolytic Reserve	3,66±0,69	3,44±0,54	1 [-1,4; 1,4]
Non-Glycolytic Acid.	0,57±0,13	0,79±0,11	0,197 [-0,6; 0,1]

C

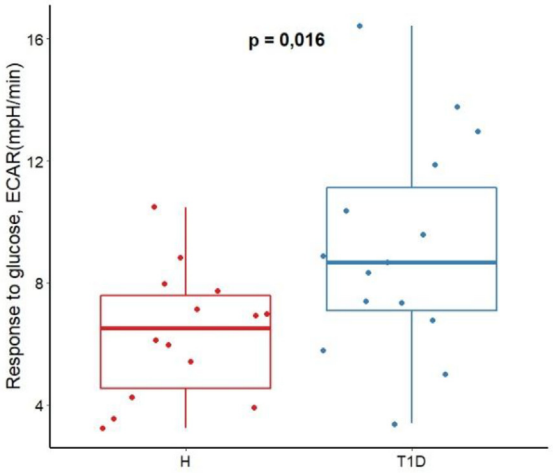


Figure 2. T1D PBMCs have increased glycolysis. A: ECAR traces of PBMCs, values are mean ± SE of ECAR (mpH/min). B: Glycolysis parameters calculated from PBMC Seahorse analyses. P-values were calculated from a two-sample unpaired t-test or Wilcoxon test for each parameter. Values are mean ± SE of ECAR. C: Response to glucose injection, values are mean ± SD of ECAR. N = 14 for H and N = 15 for T1D.

cytokine production. In this study, a plasma inflammatory biomarker panel including 91 inflammation-related human markers was utilized on the participant's blood samples (Laursen et al., 2022). We observed a significant upregulation of 39 inflammatory markers in T1D patients in comparison to healthy controls. These included IL-6, IL-8 and TNF, which is in agreement with earlier observations in T1D (Gomes, 2017; Linhartova et al., 2018; Overgaard et al., 2020). EN-RAGE (extracellular newly identified receptor for advanced glycation end products binding protein) was also higher in T1D and increased EN-RAGE levels have been observed previously in T2D patients with diabetic retinopathy (Dong et al., 2015) and under hyperglycaemic conditions (Mossel et al., 2020). We also found LIF to be negatively correlated with glycolysis in T1D patients. LIF is a pleiotropic cytokine member of the IL-6 family and has been shown to have anti-inflammatory roles in neural immunity (Knight, 2001). Recently, a paper showed that cells exposed to slow infection by Enterovirus isolates from cases of T1D showed decreased expression of LIF among other cytokines (Poma et al., 2020). Interestingly, LIF also protects against high glucose-induced oxidative stress and apoptosis in podocytes (Xu et al., 2012). These observations lead us to speculate that LIF may be a regulatory factor of mitochondrial function in T1D. This should be further investigated in future studies.

We speculated if a T2D population with albuminuria would present a similar phenotype to what we observed. Recent studies have also linked mitochondrial dysfunction and inflammation to T2D progression and associated complications (Lee et al., 2013; Rovira-Llopis et al., 2018; Chang et al., 2020). As an example, mitochondrial dysfunction in PBMCs has been reported to precede end-stage renal disease in T2D subjects (Altintas et al., 2021). Hence, we hypothesize that chronic inflammation present in T2D could equally impair mitochondrial function, which could be involved in causing renal complications. We speculate that if we performed a similar study in a population of T2D with albuminuria we would likely observe a similar phenotype of PBMC mitochondrial dysfunction as seen in our present T1D study.

Participants with T1D did, obviously, not stop insulin medication during the study. It is difficult to know to what exact extent insulin-regulated substrate availability, i.e., mainly glucose, differed in T1D participants versus healthy participants. It would have been interesting to take into consideration the insulin doses used by the study participants, but unfortunately insulin dosage data was not collected. It is therefore not possible to verify if there is a significant interaction between daily insulin dose and mitochondrial and/or glycolytic function.

Our study participants had long-duration T1D. Hence, we hypothesize that metabolic impairment of PBMCs might not be so visible at the stage of disease where we performed this study. T1D is a disease that undergoes several changes in the initial pathogenetic phases and especially the stage around time of diagnosis. Immune attack of the beta cells

leading to a decrease in insulin availability and an increase in glucose in the circulation, could lead to PBMCs shifting to a more glycolytic state. Long-term disease may not lead to many systemic changes if the individual is able to maintain good blood glucose regulation. Yet, it is known that even T1D individuals that overall have well-regulated blood glucose levels still experience frequent fluctuations (Carlson et al., 2019). In addition, high glucose has been shown to upregulate cytokines (Hu et al., 2018). Hence, complications may still arise with disease progression and PBMCs could still be exposed to signals that may lead to metabolic function changes. Others have equally suggested that PBMCs inform about and may be involved in progression of diabetic nephropathy (Czajka et al., 2015) and cardiovascular complications (Shenouda et al., 2011; Hartman et al., 2014) pointing towards mitochondrial function being altered. Yet, precaution should be taken when extrapolating our findings as our study participants with T1D all had albuminuria i.e., kidney dysfunction, and therefore our findings may not apply to T1D patients without albuminuria. Future studies addressing this are warranted.

Baseline characteristics of the study participants could be the cause for the observed differences in PBMC bioenergetics. We considered that glucose in the blood, as well as lipids could have a direct effect on PBMC metabolism. However, we found no associations of this when analysing for it. Medication taken by the participants could also be a confounding factor, especially if it interferes with metabolic substrates or metabolic pathways. Eleven T1D participants (and one healthy control) were treated with lipid-lowering medication that arguably could lead to decreased fuel substrate availability that would impact mitochondrial respiration. We observed that lipid-lowering medication was a strong confounder of maximum and spare respiration, yet these parameters did not differ between the groups. In contrast, lipid-lowering medication was not a confounder of proton leak, inferring that the observed increased proton leak in T1D PBMCs most likely reflects a true disease-associated change in mitochondrial phenotype. We do believe, however, it is an important consideration when designing future studies where metabolic measurements will be performed, to examine for medication use as confounding factors with impact on cell metabolism.

Except for the analysis of inflammatory biomarkers, we did not correct for multiple testing in our statistical analyses. The reason for this is that we consider our study of primarily exploratory nature with a relatively small sample size. We are well aware that our findings should be confirmed in other studies preferably with larger sample sizes in order to substantiate our observations and allowing for more solid conclusions. However, we still believe that our findings provide novel aspects of changes in immune cellular respiration in T1D and merits further studies.

To our knowledge, this is the first study to report changes in PBMC bioenergetic function in T1D compared to healthy controls. Our results suggest that PBMC mitochondrial and glycolytic pathways are possibly dysfunctional in T1D. This may be a consequence of increased cellular metabolic load and/or oxidative stress potentially driven by inflammatory factors. However, further investigations are required, and especially supportive data from other studies with a bigger and more heterogeneous T1D population to confirm our observations are wanted.

4. Material and Methods

4.1. Participants and trial design

This study was a cross-sectional sub-study from a randomised controlled trial investigating the acute effect of dapagliflozin on kidney oxygenation in 15 persons with type 1 diabetes and albuminuria (urine albumin creatinine ratio $\geq$30 mg/g in two out of three consecutive samples) and also included 15 age- and sex-matched non-diabetic controls for comparison at baseline (Laursen et al., 2021). The study took place at Steno Diabetes Center Copenhagen between February 2020 and September 2020. The study protocol was approved by the Regional Ethical Committee and the Danish Medicines Agency. All participants provided written consent. The study was registered in the EU Clinical Trials Register (EudraCT 2019-004557-92, registration date: 18/11/2019) and on ClinicalTrials.gov (NCT04193566, registration date: 10/12/2019). The study complied with the Declaration of Helsinki and Good Clinical Practice Guidelines.

Blood samples for the present study were taken after participants had fasted from food and liquid from midnight the evening before the study visit and abstained from medication (except insulin), alcohol, tobacco use and strenuous exercise for 24 h. All samples were taken prior to dapagliflozin or placebo treatment.

4.2. PBMCs isolation

Twenty mL of fasting blood samples for PBMCs bioenergetic analysis were collected from each participant. PBMCs were isolated from fresh peripheral blood through density gradient centrifugation, within 1h after collection. Briefly, blood was diluted 1:1 in DPBS without Ca^{2+} and Mg^{2-} (Gibco) and after a gentle mix it was layered on top of Lymphoprep (Stemcell technologies) in equal volumes on Sepmate tubes (Stemcell technologies). After 10 min centrifugation at 1200 g, plasma and PBMCs layer was collected onto a new 50 mL tube and centrifuged for 5 min at 300 g. PBMCs pellet was washed in DPBS without Ca^{2+} and Mg^{2-} and centrifuged one last time at 170 g for 8 min to remove platelets. PBMCs were resuspended in a volume of DPBS without Ca^{2+} and Mg^{2-} equal to the initial blood volume for cell counts.

4.3. Complete blood cell count for isolation quality assessment (Sysmex)

Using the manual mode in a Sysmex Xn550, a minimum of 100 μL of blood or PBMCs were analysed for complete blood cell count (CBC) for each participant visit. The number of red blood cells (RBC), white blood cells (WBC) and platelets (PLT) were obtained. We also recorded the number of each white blood cell, namely, neutrophils (NEU), lymphocytes (LYMPH) and monocytes (MONO).

4.4. Seahorse analysis

Seahorse XFe24 Analyzer (Agilent Technologies, Santa Clara, CA, USA) was used to measure in realtime parameters of glycolysis and mitochondrial oxidative phosphorylation of PBMCs.

For mitochondrial oxidative phosphorylation analysis, 5×10^5 PBMCs were seeded per well in a Seahorse XFe24 plate coated with poly-D-lysine (Millipore A-003-E) in XF base medium without phenol red (Agilent 100335) supplemented with 5mM HEPES, 2mM L-glutamine, 1mM sodium pyruvate (Gibco 11360070) and with or without 5mM glucose (Sigma G8644). Cells were incubated for 1h at 37 °C in a CO2-free incubator prior to the start of the experiment. Three basal OCR measurements were performed, followed by an injection of 0,5 μM oligomycin (Sigma O4876) to inhibit ATP synthase (and determine the ATP production efficiency of the cells) or an injection of 3 μM FCCP (Sigma C2920) which uncoupled mitochondrial respiration (to determine the maximal substrate oxidation capacity). Both oligomycin and FCCP effects were recorded during four OCR measurements and followed by a combined injection of 2 μM rotenone (Sigma R8875) and 2 μM antimycin A (Sigma A8674) and two OCR measurements to inhibited mitochondrial respiration (correcting the data for non-mitochondrial OCR).

For glycolysis analysis 5×10^5 PBMCs were seeded per well in a Seahorse XFe24 plate coated with poly-D-lysine (Millipore A-003-E) in XF base medium without phenol red (Agilent 100335) supplemented with 5mM HEPES (Gibco 15630056) and 2mM L-glutamine (Gibco 25030149). Cells were incubated for 1 h at 37 °C in a CO_2-free incubator, prior to the start of the experiment. Three basal ECAR measurements followed by a 5mM glucose (Sigma G8644) injection were done to determine glycolytic rate. After four ECAR measurements, a combined injection of 2 μM rotenone (Sigma R8875) and 2 μM antimycin A (Sigma A8674) was performed to inhibit? mitochondrial respiration. To finish,

after three ECAR measurements, 2-DG (Sigma D6134), an analogue of glucose, was injected and 5 ECAR measurements were obtained, to shut down glycolysis and allow data correction for non-glycolytic medium acidification.

Mitochondrial oxidative phosphorylation parameters were calculated after non-mitochondrial respiration correction (last measurement after rotenone/antimycin A injection). Basal respiration corresponds to last OCR measurement before first injection, subtracting non-mitochondrial respiration. Proton leak corresponds to average of all four OCR measurements after oligomycin injection, subtracting non-mitochondrial respiration. ATP production was calculated as the difference in OCR between basal respiration and proton leak. Maximum respiration corresponds to the average of all four OCR measurements after FCCP injection, subtracting non-mitochondrial respiration. Spare respiration corresponds to the difference in OCR between maximum respiration and basal respiration. Glucose treatment response for each parameter was calculated as the difference between incubation with and without glucose, for each participant.

Glycolytic parameters were calculated after non-glycolytic medium acidification correction (last ECAR measurement after 2-DG injection). Glycolysis corresponds to the average of all four ECAR measurements after glucose injection, subtracting basal ECAR (last ECAR measurement before glucose injection). Glycolytic capacity was calculated as average of all three ECAR measurements after rotenone/antimycin A injection, subtracting basal ECAR. Glycolytic reserve is the difference between glycolytic capacity and glycolysis. Glycolytic response to glucose was calculated as the difference between the first measurement after glucose injection and the last measurement before injection.

4.5. Inflammatory biomarkers on peripheral blood samples

Inflammatory biomarkers were measured on peripheral blood samples with the Olink® Target 96 Inflammation Panel (Olink, Uppsala, Sweden) and has been published in Laursen et al. (2022). Briefly, it included 91 inflammation related human protein biomarkers. Biomarker levels were obtained as the Normalized Protein Expression (NPX), a relatively arbitrary unit on a log2 scale. Although NPX directly correlates with initial protein concentrations, no comparisons of absolute protein levels can be done.

4.6. Statistical analysis

The main endpoint was to test if mitochondrial respiration and glycolysis differ between PBMCs from T1D individuals and healthy subjects. All variables are presented as mean and standard deviation (SD) or standard error (SE), as indicated in each figure.

Seahorse data was exported from Wave software and OCR and ECAR trace graphs were done using GrapPad Prism 9. Data was treated using R (3.6.2) and all other graphs and tables were prepared using the packages dplyr, ggplot and ggpubr).

Statistical analysis was done in R (3.6.2) by employing a two-sample unpaired t-test or Wilcoxon test when data was not normally distributed from the package stats. For analysis within groups paired t-test or paired Wilcoxon from the package stats was used.

Linear regression analysis to check for possible confounders were calculated from base R.

Declarations

Author contribution statement

Joana Mendes Lopes de Melo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jens Christian Laursen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Niels Søndergaard-Heinrich; Ida Kirstine Bull Rasmussen; Christian Stevns Hansen: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Marie Frimodt-Møller: Analyzed and interpreted the data.

Peter Rossing: Conceived and designed the experiments; Analyzed and interpreted the data.

Joachim Størling: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no competing interests.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e12304.

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