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Intracellular pyruvate levels positively correlate with cytokine production capacity in tolerant monocytes from patients with pneumonia

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ABSTRACT

Background: Community-acquired pneumonia (CAP) is responsible for a high morbidity and mortality worldwide. Monocytes are essential for pathogen recognition and the initiation of an innate immune response. Immune cells induce intracellular glycolysis upon activation to support several functions.
Objective: To obtain insight in the metabolic profile of blood monocytes during CAP, with a focus on glycolysis and branching metabolic pathways, and to determine a possible association between intracellular metabolite levels and monocyte function.
Methods: Monocytes were isolated from blood of patients with CAP within 24 h of hospital admission and from control subjects matched for age, sex and chronic comorbidities. Changes in glycolysis, oxidative phosphorylation (OXPHOS), tricarboxylic acid (TCA) cycle and the pentose phosphate pathway were investigated through RNA sequencing and metabolomics measurements. Monocytes were stimulated *ex vivo* with lipopolysaccharide (LPS) to determine their capacity to produce tumor necrosis factor (TNF), interleukin (IL)-1β and IL-10.
Results: 50 patients with CAP and 25 non-infectious control subjects were studied. When compared with control monocytes, monocytes from patients showed upregulation of many genes involved in glycolysis, including *PKM*, the gene encoding pyruvate kinase, the rate limiting enzyme for pyruvate production. Gene set enrichment analysis of OXPHOS, the TCA cycle and the pentose phosphate pathway did not reveal differences between monocytes from patients and controls. Patients' monocytes had elevated intracellular levels of pyruvate and the TCA cycle intermediate α-ketoglutarate. Monocytes from patients were less capable of producing cytokines upon LPS stimulation. Intracellular pyruvate (but not α-ketoglutarate) concentrations positively correlated with IL-1β and IL-10 levels released by patients' (but not control) monocytes upon exposure to LPS.
Conclusion: These results suggest that elevated intracellular pyruvate levels may partially maintain cytokine production capacity of hyporesponsive monocytes from patients with CAP.

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1. Introduction

Community-acquired pneumonia (CAP) is the most prevalent infectious disease in the world and the fourth common cause of death worldwide [1]. Furthermore, pneumonia is the main cause of sepsis [2], a life-threatening syndrome characterized by a systemic dysregulation of the host response during infection, which is associated with a high morbidity and mortality [3]. Development of disease after infection of the airways largely depends on local immune responses mediated by respiratory epithelial cells together with resident and recruited leukocytes [4]. The clinical severity of CAP is highly variable, ranging from mild disease to fulminant sepsis and shock [5,6]. Importantly, even in the absence of sepsis, CAP is associated with marked systemic inflammatory responses [7]. Recently, our group reported that hospitalized CAP patients without sepsis show clear signs of systemic activation of inflammatory, vascular and procoagulant pathways, coinciding with a reduced responsiveness of blood leukocytes upon exposure to bacterial agonists *ex vivo* [8]. These findings resembled the well-documented concurrent hyperinflammation and immune suppression reported in patients with sepsis [9,10]. We subsequently demonstrated that blood monocytes from CAP patients are less capable of releasing cytokines upon stimulation with lipopolysaccharide (LPS) [11], thereby reproducing the phenomenon also known as "LPS tolerance" [10,12].

In recent years it has become evident that many immune cells induce intracellular glycolysis upon activation to support several functions [13,14]. Glycolysis is the breakdown of glucose to pyruvate, generating energy in the form of ATP and biosynthetic intermediates that support protein synthesis and cell growth. Normally, the majority of pyruvate enters the mitochondria to be metabolized further in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) [13]. This process is slow, but yields considerably more ATP compared to glycolysis. Pyruvate can also be reduced to lactate and transported out of the cell, which enables an increased glycolytic flux to obtain energy and biosynthetic intermediates very quickly. Activated immune cells utilize glycolysis for specific purposes. For instance, the biosynthetic intermediates obtained by glycolysis enable T-cells to proliferate [15,16] and macrophages to produce reactive oxygen species and/or nitric oxide to kill phagocytosed pathogens [17–19]. Interestingly, peripheral blood mononuclear cells (PBMCs) from sepsis patients with a severe tolerant phenotype – as shown by nearly abolished cytokine production upon LPS stimulation *ex vivo* – displayed broad metabolic defects as indicated by a reduction of ATP and NAD+ content, lactate production and oxygen consumption [20].

Monocytes are key components of the innate immune system [21]. Recently, our group reported a multi-omics integrative analysis of the transcriptome and DNA methylome of blood monocytes from CAP patients and non-infectious controls [11]. An unbiased analysis of the monocyte transcriptome exposed >6000 genes that were differentially expressed between CAP patients and control subjects. Pathway analysis of the genes that were upregulated in patients revealed associations with various canonical signaling pathways, among which glycolysis [11]. In this study we elaborated on this finding and examined the metabolic profile of monocytes from CAP patients, with an emphasis on glycolysis and branching pathways, by integrating targeted gene expression data with the metabolome in these monocytes. Furthermore, seeking to obtain insight into the functional consequences of metabolic changes, we investigated the association between the intracellular abundance of pyruvate and α-ketoglutarate, metabolites found to be elevated in monocytes of patients with CAP, and the cytokine production capacity of monocytes upon LPS exposure *ex vivo*.

2. Materials and methods

2.1. Study population and sample collection

This study was part of the ELDER-BIOME project (clinicaltrials.gov identifier NCT02928367) approved by the medical ethical committees of the Amsterdam UMC – location AMC and BovenIJ Hospital in Amsterdam [8,11]. Consecutive patients older than 18 years admitted between April 2017 and May 2018 to the Amsterdam UMC – location AMC or BovenIJ Hospital were screened by trained research physicians. Patients were included if they were admitted with an acute infection of the respiratory tract, defined as at least one respiratory symptom (new cough or sputum production, chest pain, dyspnea, tachypnea, abnormal lung examination, or respiratory failure) and one systemic symptom (documented fever or hypothermia, leukocytosis or leukopenia) and had an evident new or progressive infiltrate, consolidation, cavitation, or pleural effusion on chest X ray or computed tomography scan. Patients were excluded if there was a clinical suspicion of aspiration pneumonia or hospital-associated pneumonia, or if patients were previously diagnosed with malignant hematological disease or exposed to chemotherapy, systemic corticosteroids and/or other immunosuppressive drugs, or if patients were exposed to oral and/or intravenous antibiotics within 48 h prior to hospital admission. Written informed consent was obtained from all eligible participants, or their legal representatives. Heparin anticoagulated blood was obtained within 24 h of hospital admission and approximately 1 month after admission. Age and sex-matched subjects without acute infection served as controls.

2.2. Monocyte isolation

Heparinized blood was diluted (1:1) in PBS. Isolation of PBMCs was performed by density-gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare, Chicago, IL). CD14+ monocytes were subsequently purified using MACS CD14 microbeads for positive selection, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte purity was analyzed by flow cytometry and was consistently higher than 90 %. After isolation, the monocytes were split into three fractions for three different analyses: aliquots of 5×10^5 cells were stored for gene expression analysis and metabolomics and the third fraction was used to determine the cytokine production capacity. The monocytes used for gene expression analysis were immediately stored in RNAprotect cell reagent (Qiagen) at −80 °C and the monocytes designated for metabolomics were immediately snap frozen in liquid nitrogen and stored at −80 °C until further processing.

2.3. RNA isolation and transcription analysis

Total RNA was isolated using the AllPrep DNA/RNA mini kit according to the manufacturer's instructions (Qiagen). RNA quality was assessed by bioanalysis (Agilent), with all samples having RNA integrity numbers >9. Total RNA and genomic DNA concentrations were determined by Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). RNA-sequencing libraries were prepared from 200 ng total RNA using KAPA RNA Hyperprep with RiboErase (Roche) library kits. Libraries were sequenced using the Illumina HiSeq4000 instrument (Illumina) to generate single reads (50 bp). The sequencing depth was approximately 40 million reads per sample. Sequence libraries are publicly available through the National Center for Biotechnology Information (NCBI) gene expression omnibus (GEO) under accession number GSE160331.

2.4. Bioinformatics analysis of RNA sequencing data

The sequence read quality was assessed using FastQC methods (version 0.11.5; Babraham Institute, Babraham, Cambridgeshire, UK). Trimmomatic version 0.32 [22] was used to trim the Illumina adapters and filter low quality reads and ambiguous nucleotide-containing sequences. Low quality leading (3 nucleotides) and trailing (3 nucleotides) bases were removed from each read. A sliding window trimming using a window of 4 and a phred score threshold of 15 nucleotides was used to access the quality of the reads. After pre-processing, the remaining high-

quality reads were aligned against the Genome Reference Consortium Human Genome Build 38 patch release 7 (GRCh38.p7) using Bowtie2 version 2.3.4.3 [23] with default parameters. Count data were generated by means of the FeatureCounts method [24], and differential expression analyzed using the DESeq2 method [25] in the R statistical computing environment (R Core Team 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). Statistically significant differences were defined by Benjamini & Hochberg adjusted probabilities <0.05. Pathway analysis was based on the Reactome database (https://reactome.org/) [26]. The Gene Set Enrichment Analysis approach was applied to determine the level and direction of enrichment for targeted metabolic pathways [27]. For each pathway Gene Set Enrichment Analysis calculates a Normalized Enrichment Score, which reflects the degree to which a gene set is overrepresented by up- or down-regulated genes. Transcriptional and metabolomics data were visually integrated using Escher, a web application for pathway visualization [28].

2.5. Metabolomics

One part of chloroform was added to one part of methanol/water (1/1) to the dry cell-pellet and centrifuged for 10 min at 14000 rpm. The polar phase was then dried using a vacuum concentrator at 60 °C and reconstituted in 100 μl methanol/water (6/4; v/v). Metabolites were analyzed using a Thermo Scientific ultra-high pressure liquid chromatography system (Waltman, MA, USA) coupled to Thermo Q Exactive (Plus) Orbitrap mass spectrometer (Waltman). Chromatographic separation was performed using a Merck Millipore SeQuant ZIC-cHILIC column (PEEK 100 × 2.1 mm, 3 μm particle size). Mobile phase consisted of (1) 1:9 acetonitrile:water and (2) 9:1 acetonitrile:water, both containing 5 mM ammonium acetate. Using a flow rate of 0.25 ml/min, the LC gradient consisted of 100 % B for 0–2 min, ramp to 0 % B at 28 min, 0 % B for 28–30 min, ramp to 100 % B at 31 min, 100 % B for 31–35 min. MS data were acquired using negative and positive ionization in full scan mode over the range of *m/z* 50–1200. Data were analyzed using Xcalibur software (version 3.0, Thermo scientific). Metabolites abundance were normalized to internal standards which were added prior to the extraction. For metabolite identification, a combination of accurate mass, (relative) retention times and fragmentation spectra, compared to the analysis of a library of standards were used.

2.6. Monocyte stimulation

To assess the cytokine production capacity of the monocytes, they were plated in 48-wells plates with cell-repellent surface to maintain non-adherent monocytes (5×10^5 cells/well; Greiner Bio-one, Kremsmünster, Austria). Monocytes were cultured in RPMI 1640 medium (no glutamine; 31,870,074; Gibco; Thermo Fisher, Waltham, MA) supplemented with 10 μg/ml gentamicin (Gibco), 1 mM sodium pyruvate (Gibco), 2 mM glutamax (Gibco), 20 mM HEPES (Gibco) and 10 % fetal bovine serum (FBS; HyClone; GE Healthcare) and stimulated for 24 h with 100 ng/ml ultrapure LPS (from *Escherichia coli* 0111:B4; InvivoGen, Toulouse, France).

2.7. Cytokine measurements

TNF-α, IL-1β and IL-10 levels were measured using a Luminex multiplex assay (R&D Systems Inc., Minneapolis, MN) and BioPlex 200 (BioRad, Hercules, CA).

2.8. Statistical analysis

To compare metabolite levels in monocytes from CAP patients and non-infectious controls, the values were Box-Cox transformed to obtain a normal distribution for each metabolite. Data is shown as *Z*-scores (the number of standard deviations by which an individual value is above or below the mean of all values measured). Metabolite levels were subsequently tested with Student's *t*-tests and corrected for multiple testing by the False Discovery Rate (FDR) method of Benjamini and Hochberg; the significance threshold was set at a FDR of 5 %. Correlations were performed with the original non-transformed metabolite levels using two-tailed Spearman correlations for nonparametric parameters. Analysis were performed using GraphPad Prism version 8 (Graphpad Software, San Diego, CA) and R version 4.1.2 (Vienna, Austria).

3. Results

3.1. Patients and outcome

Fifty patients admitted for CAP and 25 age- and sex-matched controls without infection were included in the study (Table 1). To asses monocytes after CAP recovery, we re-evaluated 31 CAP approximately one month (mean 31.8 ± SD 6.8 days) after inclusion. Patients and controls were similar in terms of demographics and chronic comorbidities. *Streptococcus pneumoniae* was the most frequently identified causative pathogen (n = 7; 14 %). Of CAP patients, four (8 %) had a SOFA score of two or higher. The median length of hospital stay was four days (IQR 3–6.75). 28-day mortality was 8 % (n = 4), 8 % (n = 4) of the patients were readmitted to the hospital within 28 days and six patients (12 %) were lost to follow-up.

3.2. RNA sequencing of blood monocytes obtained from CAP patients shows enhanced expression of genes encoding proteins involved in glycolysis

In order to obtain insight into energy metabolism pathways utilized by monocytes during CAP, we performed RNA sequencing on monocytes purified from blood of CAP patients on hospital admission (acute samples) and non-infectious matched controls. We performed targeted gene

Table 1
Baseline characteristics of patients with community-acquired pneumonia and control subjects.

	CAP Patients	Non-infectious Controls	P-value*
Patients, n	50	25	
Demographics			
Sex (male (%))	27 (54)	13 (52)	>0.999
Age, years (mean (SD))	69.64 (14.29)	68.08 (9.70)	0.625
BMI (mean (SD))	25.91 (6.34)	27.49 (5.71)	0.297
Chronic comorbidity			
Malignancy in medical history (%)**	12 (24)	5 (20)	0.777
COPD (%)	16 (32)	3 (12)	0.091
Hypertension (medicated) (%)	22 (44)	14 (56)	0.341
Diabetes (%)	14 (28)	3 (12)	0.151
Disease severity			
Pneumonia severity index (mean (SD))	3.46 (1.13)		
CURB-65 score (mean (SD))	1.67 (1.14)		
qSOFA score (mean (SD))	0.73 (0.62)		
Causative pathogen			
Unknown (%)	30 (60)		
Streptococcus Pneumoniae (%)	7 (14)		
Influenza A virus (%)	2 (4)		
Influenza B virus (%)	3 (6)		
Haemophilus influenzae (%)	2 (4)		
Other (%)	6 (12)		

CAP = community-acquired pneumonia; COPD = chronic obstructive pulmonary disease; SD = standard deviation of the mean; CURB-65 = mortality prediction score in CAP; qSOFA = quick Sequential Organ Failure Assessment.

* Fisher's exact test for dichotomous parameters, Students t-test for continuous parameters.

** No active malignancies or currently under treatment.

set enrichment analysis of the glycolysis pathway, the pentose phosphate pathway, the TCA cycle and OXPHOS (Fig. 1A). This analysis showed that the majority of genes involved in glycolysis was upregulated in monocytes from CAP patients, resulting in positive normalized enrichment scores of 1.46 (adjusted P = 0.0213). Key genes implicated in glycolysis upregulated in monocytes from CAP patients included *HK1*, *HK3*, *PFKP*, *ALDOA*, *GAPDH*, *PGK1*, *ENO1* and *PKM* (Fig. 1B). Of note, the gene expression of two out of three isoforms of *PFK* were downregulated (*PFKM* and *PFKL*; Figs. 1B and 2). However, *PFKP*, known for its role in immune cell induced glycolysis [29], was upregulated (Fig. 1B). Gene set enrichment analysis of the pentose phosphate pathway, the TCA cycle and OXPHOS did not reveal differences between monocytes from patients and controls (Fig. 1A). RNA sequencing and gene set enrichment analysis of monocytes harvested from the same patients one month after their hospital admission, showed that the induction of genes involved in glycolysis was largely normalized and did not significantly differ from controls (Supplementary Fig. 1; other energy metabolism pathways also shown). These results indicate a transient activation of gene transcription relating to glycolysis during the acute stage of CAP, normalizing after CAP recovery.

3.3. Integration of metabolomics and gene expression data of glycolysis and branching pathways

We next measured polar metabolites with a specific focus on metabolites that are involved in central metabolic pathways such as glycolysis and branching pathways. We integrated these metabolomics data with gene expression data using Escher, a web application for

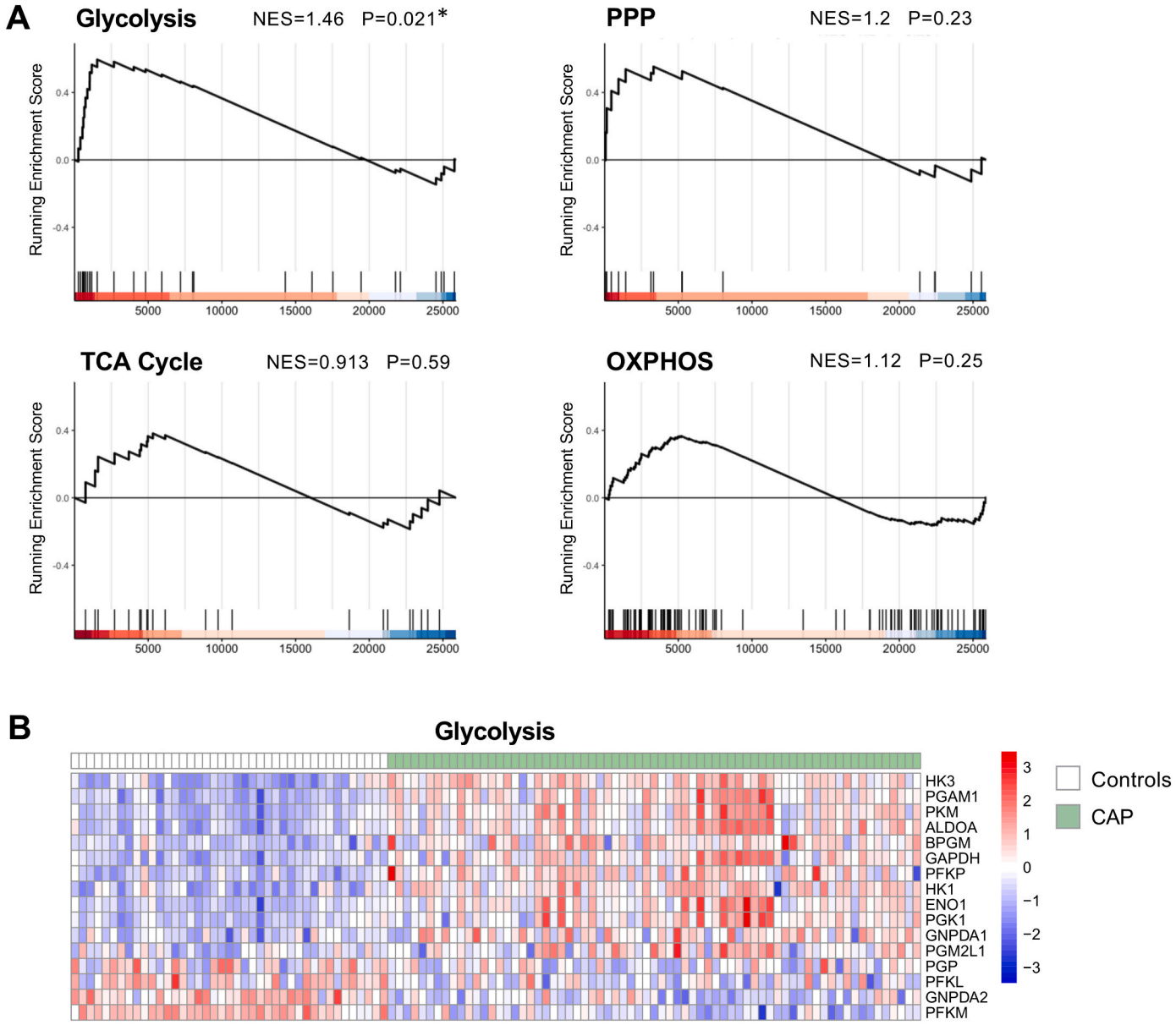

Fig. 1. Glycolysis is transcriptionally upregulated in monocytes from CAP patients compared to non-infectious controls
Gene Set Enrichment Analysis (GSEA) [27] plots for the glycolysis pathway, the pentose phosphate pathway, the TCA cycle and oxidative phosphorylation (OXPHOS) of monocytes from CAP patients compared to age- and sex-matched non-infectious controls including NES scores (Normalized Enrichment Scores; reflects the degree to which a gene set is overrepresented by up- or down-regulated genes) and P-values (A). Pathways are based on the Reactome Database [26]: *Glycolysis* (R-HSA-70171); *Pentose Phosphate Pathway* (R-HSA-71336); *TCA cycle* (R-HSA-71403); *OXPHOS* (R-HSA-611105). Heatmap comparing the transcription of the glycolysis pathway in monocytes from CAP patients with those of non-infectious controls (B), only showing significant genes (BH adjusted P < 0.05). Expression is scaled per gene (*Z*-score).

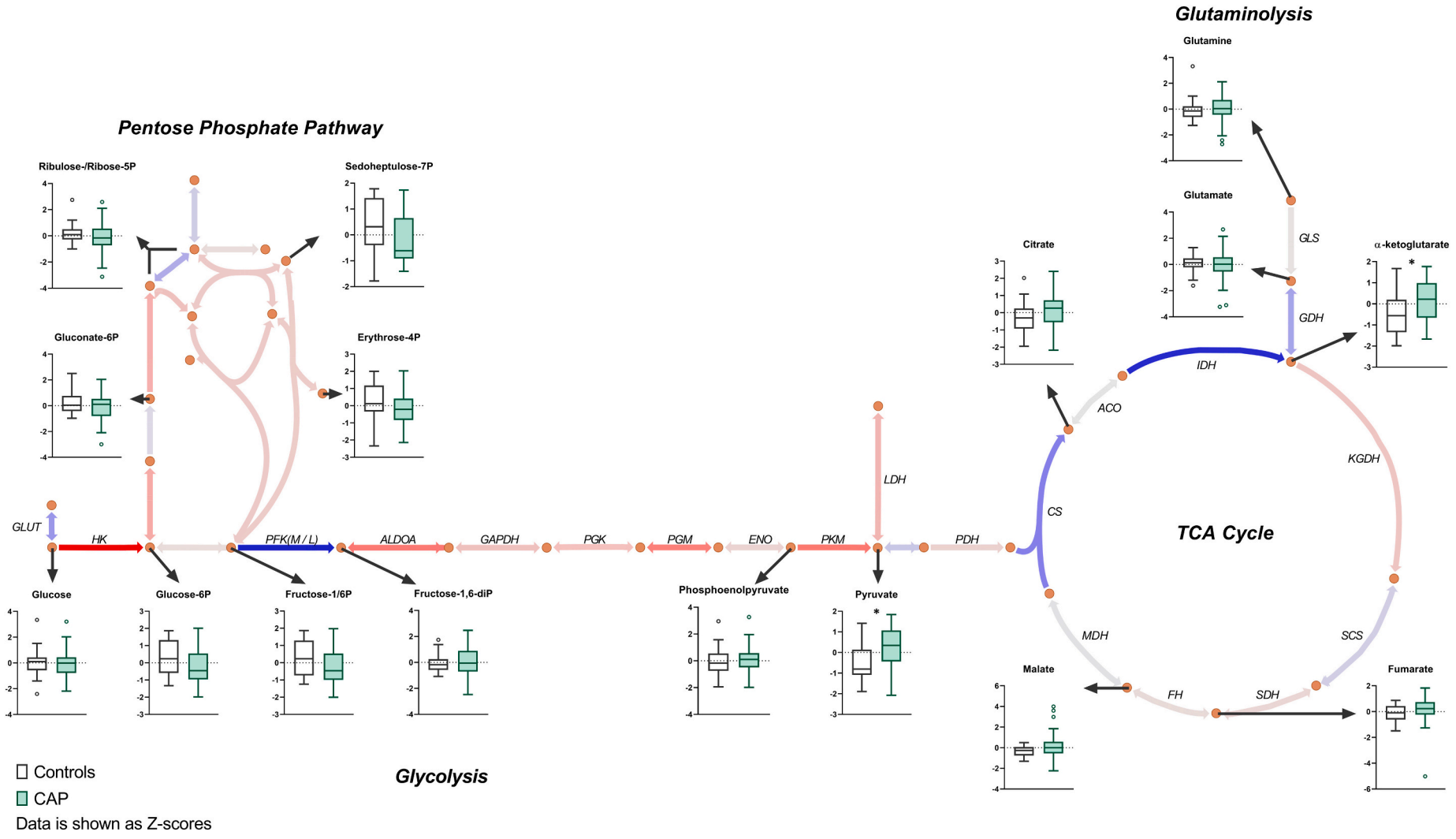

Fig. 2. Intracellular pyruvate and α-ketoglutarate levels are higher in monocytes from CAP patients compared to non-infectious controls

Escher plot showing gene expression of the glycolysis pathway and branching pathways and the relative levels of intracellular metabolites (red depicts upregulated genes, blue depicts downregulated genes, and gray depicts unchanged genes). Metabolite levels in monocytes from CAP patients (green) and non-infectious controls (white) were normalized using Box-Cox transformation. Data is shown as Z-scores (the number of standard deviations an individual value is above or below the mean). Metabolite levels were compared using Student *t*-tests and corrected for multiple testing by the False Discovery Rate (FDR) method of Benjamini and Hochberg: the significance threshold was set at a FDR of 5 %. Unrepresented metabolites (*e.g.* glyceraldehyde-3-phosphate, lactate and succinate) were not (reliably) measurable. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pathway visualization [28]. After correcting for multiple testing, only pyruvate and α-ketoglutarate levels were significantly higher in monocytes from CAP patients compared to levels in monocytes from non-infectious controls (Fig. 2). Integration with gene expression data demonstrated that elevated pyruvate levels coincided with increased expression of *PKM* (pyruvate kinase) and decreased expression of *CS* (citrate synthase), suggesting increased production of pyruvate and decreased efflux of pyruvate towards the TCA cycle (Fig. 2). Indeed, citrate levels were not enhanced in monocytes from CAP patients compared to non-infectious controls. Unfortunately, we do not have data on intracellular lactate levels to assess the efflux of pyruvate to lactate. However, it should be noted that lactate is readily exported by diffusing through the cell membrane *via* MCT (monocarboxylate transporter) pores [30]. MCTs therefore prevent intracellular accumulation of lactate by removing excess lactate produced. We did analyze the expression of *LDH* genes (overall difference indicated in Fig. 2) and found that isoform *LDHA* was significantly upregulated in CAP monocytes (BH adj P = 1.3*10–14; log2 FC = 0.82) and isoform *LDHB* was significantly downregulated (BH adj P = 2.4*10–7; log2 FC = −0.48). LDHA and LDHB form homo- or heterotetramers that interconvert pyruvate and lactate. The LDHA subunit has a higher affinity for pyruvate and results in an increase in pyruvate to lactate conversion, while the LDHB subunit has a higher affinity for lactate resulting in more lactate to pyruvate conversion [31,32]. Our data therefore suggests an increase in pyruvate to lactate conversion. Nevertheless, this did not result in normalization of the pyruvate levels as they were still higher in monocytes from CAP patients.

Elevated intracellular α-ketoglutarate levels in patients' monocytes could not be readily explained by concurrently measured expression of genes encoding possibly involved enzymes: expression of *IDH* (isocitrate dehydrogenase), the gene encoding the enzyme responsible for the conversion of isocitrate to α-ketoglutarate, was strongly reduced. Alternatively, α-ketoglutarate could be derived from glutaminolysis; however, *GLS* and *GDH*, the primary genes responsible for the conversion from glutamine to glutamate to α-ketoglutarate, were not significantly altered in patients' monocytes. Moreover, intracellular glutamine and glutamate levels were not different between patients and controls. Gene set enrichment analysis of the glutaminolysis pathway demonstrated both upregulated and downregulated genes in approximately equal distribution (Supplementary Fig. 2), further supporting the absence of glutaminolysis alteration in monocytes from CAP patients. Elevated pyruvate and α-ketoglutarate levels could also be caused by reduced activity of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH) respectively. Gene expression of the specific subunits of these complexes were not altered; however both are regulated by lipoic acid, which acts as a cofactor in the PDH and KGDH complexes and is essential for their function [33,34]. Both complexes also require NAD for their function. Reduced activity of these complexes could therefore result in elevated pyruvate, α-ketoglutarate and NAD levels. Although NAD levels were not increased in monocytes from CAP patients compared to those of non-infectious controls, we did find that pyruvate and α-ketoglutarate concentrations positively correlated with intracellular NAD levels in monocytes from CAP patients (Supplementary Fig. 3A). If both PDH and KGDH complexes are regulated by a similar mechanism, pyruvate and α-ketoglutarate levels would also correlate with each other. This was indeed the case in monocytes from CAP patients (Supplementary Fig. 3B), supporting the hypothesis of co-regulation in these cells. Monocyte pyruvate and α-ketoglutarate levels of monocytes from recovered patients did not return to normal levels after one month, but were intermediate between levels in CAP patients in the acute phase and non-infectious controls (Supplementary Fig. 4).

3.4. Pyruvate levels correlate with the cytokine production capacity in monocytes from CAP patients but not in monocytes from non-infectious controls

We recently reported that monocytes from CAP patients have a reduced capacity to produce cytokines upon stimulation with LPS *ex vivo* when compared with non-infectious controls [11]. We confirmed this finding for IL-1β and IL-10 in the subgroup of patients studied here (Fig. 3). Elevated pyruvate levels generated through glycolysis has been shown to support a proinflammatory cellular phenotype [13,19], which led us to hypothesize that these might also support cytokine production in tolerant monocytes. On the other hand, α-ketoglutarate has been identified as an anti-inflammatory metabolite that promotes LPS tolerance in macrophages by a mechanism that does not rely on its conversion into succinate [35]. Therefore, we investigated possible associations between intracellular pyruvate and α-ketoglutarate concentrations and the responsiveness of monocytes to LPS. Intracellular pyruvate levels positively correlated with IL-1β and IL-10 levels released by LPS-stimulated monocytes from CAP patients (Fig. 4A), but not from controls (Fig. 4B). There was no correlation between intracellular pyruvate levels and TNF production capacity by LPS stimulated monocytes from CAP patients. In monocytes harvested from CAP patients one month after admission pyruvate levels still correlated with LPS-induced IL-1β levels, but not with TNF and IL-10 concentrations (Supplementary Fig. 5). Intracellular α-ketoglutarate levels did not correlate with cytokine levels released by LPS-stimulated monocytes from CAP patients upon admission (Fig. 4C) or non-infectious controls (Fig. 4D). Together these data suggest a possible role for pyruvate (but not α-ketoglutarate) levels in partially maintaining cytokine production by monocytes from CAP patients in response to LPS.

3.5. Pyruvate levels correlate with parameters of cellular energy availability in monocytes from CAP patients but this does not correlate with the cytokine production capacity

We found intracellular pyruvate levels to correlate with the production capacity of IL-1β and IL-10 by monocytes from CAP patients. Elevated pyruvate levels could be indicative of enhanced glycolysis which is mainly known as an energy generating pathway [13]. To determine whether energy availability could explain the correlation with the cytokine production capacity, we assessed the intracellular ATP:AMP ratio. ATP is a high energy molecule that is converted to ADP and phosphate during almost all cellular processes requiring energy. Because adenylate kinase maintains the reaction 2ADP ↔ ATP + AMP close to equilibrium in all eukaryotic cells, the AMP:ATP ratio tends to vary as the square of the ADP:ATP ratio [36], so that the former is a more sensitive indicator of energy deprivation [37] than the latter. To make interpretation of the results easier, we chose to assess the cellular energy status as the ATP:AMP ratio where higher levels indicate more energy availability. First we compared the ATP:AMP ratio of monocytes from CAP patients to those of the non-infectious controls and found no differences herein (Fig. 5A). However, pyruvate levels positively correlated with the ATP:AMP ratio in monocytes from CAP patients on admission and in “recovery” monocytes, but not in monocytes from non-infectious controls (Fig. 5B). These results suggest that elevated pyruvate levels through enhanced glycolysis indeed result in enhanced energy availability. To see if this could be the mechanism by which the cytokine production capacity is supported, we also correlated the ATP:AMP ratio with the cytokine production capacity, but found no correlation (Fig. 5C). Therefore, the relationship between elevated pyruvate levels and maintained cytokine production capacity remains unclear.

4. Discussion

Glucose metabolism, and in particular glycolysis, has been shown to be important for proper activation of several immune cell types [13–15].

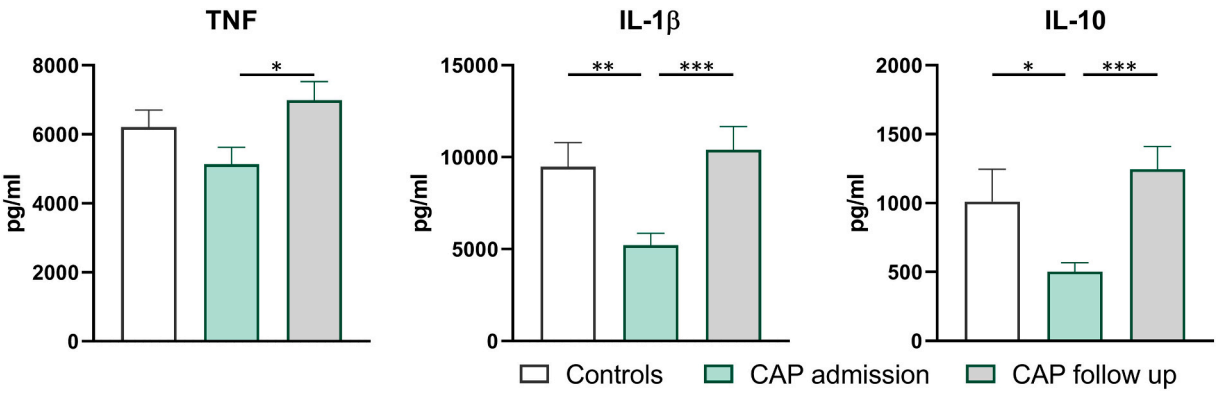

Fig. 3. Monocytes from CAP patients have a reduced capacity to produce IL-1β and IL-10
Cytokine production of monocytes from CAP patients on admission (green; n = 40–46[i]), from recovered CAP patients upon follow-up (green/gray; approximately one month after admission; n = 28–32[i]) or from age- and sex-matched non-infectious controls (white; n = 22–23[i]) stimulated *in vitro* with LPS for 24 h. [i]Values outside of the detection range were excluded. Data is shown as bar graphs with mean ± SEM. Groups were compared using ordinary one-way ANOVAs with Holm-Sidak's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In this manuscript we show that genes of the glycolysis pathway are significantly enriched in monocytes from CAP patients compared to monocytes from non-infectious controls. Concurrently, intracellular pyruvate levels, the end-product of (aerobic) glycolysis, and α-ketoglutarate levels, an important signaling metabolite in the TCA-cycle [35,38], were increased in monocytes from CAP patients, possibly due to reduced activity of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes. Monocytes from CAP patients demonstrated a reduced capacity to produce cytokines upon LPS stimulation when compared to monocytes from non-infectious control subjects, and monocyte pyruvate but not α-ketoglutarate levels positively correlated with the cytokine production capacity of IL-1β and IL-10 by monocytes from patients. Interestingly, in monocytes from non-infectious controls, which contained less pyruvate and had a higher cytokine production capacity, there was no correlation between pyruvate levels and cytokine production capacity. The finding that LPS tolerant monocytes from CAP patients with relatively high intracellular pyruvate levels had a higher capacity to produce cytokines, suggests that an increase in pyruvate levels might counteract or reduce LPS tolerance in these cells.

We show that several key glycolytic genes are upregulated in monocytes from CAP patients compared to non-infectious controls. However, the expression of two out of three isoforms of PFK, the first committed step in the glycolysis pathway, were downregulated (*PFKM* and *PFKL*), while only *PFKP* was upregulated. Interestingly, in immune cells, *PFKM* transcripts are virtually absent and while PFKL is the predominant isoform in monocyte-derived macrophages during baseline conditions, upon activation by LPS, *PFKP* transcript levels increase 10-fold [29]. Since PFKP is relatively insensitive to ATP inhibition, switching to PFKP would permit high enzymatic activity irrespective of cellular ATP concentrations, as opposed to PFKL which is more responsive to ATP inhibition [29]. Similarly, *LDH* expression was overall slightly upregulated, however isoform *LDHA* was highly upregulated while *LDHB* was downregulated. Interestingly, while the LDHB subunit is associated with lactate to pyruvate conversion, the LDHA subunit is associated with pyruvate to lactate conversion because of their increased affinity for resp. lactate and pyruvate [31,32]. Our study is limited in that we determined mRNA levels of glycolytic enzymes, not protein concentrations or activities. Previous studies have shown that upregulation of the genes encoding the glycolytic enzymes reported here is associated with enhanced lactate production by monocytes and macrophages [39–41], making it likely that our findings based on mRNA expression indicate enhanced glycolytic activity.

Our group previously reported that monocytes from CAP patients, relative to monocytes from non-infectious control subjects, demonstrated a diminished release of TNF, IL-1β and IL-10 upon stimulation with LPS *ex vivo* [11]. The current study entailed a subgroup of this cohort in which combined RNA sequencing and metabolomics data were analyzed. Of note, the difference in TNF production capacity of monocytes from CAP patients *versus* controls was just above the statistical significance threshold of 0.05. In the entire cohort, from which the current subgroup was derived, patients' monocytes did show a significantly reduced TNF production capacity as compared to monocytes from controls [11]. Likely, this difference can be explained by a lower sample size and an overall lower disease severity of the subgroup reported here. While "LPS tolerance" of monocytes has been described in many investigations involving patients with sepsis [10,12], the vast majority of CAP patients included in the present study did not fulfil the diagnostic criteria for sepsis [3], *i.e.*, only 8 % had a SOFA score of two or higher. Notably, in sepsis patients "LPS tolerant" monocytes had an unaltered or even increased production capacity of the anti-inflammatory cytokine IL-10 [12,42], which contrasts with the reduced IL-10 release by monocytes from CAP patients reported here. Possible explanations could be culture conditions used (we used cell-repellent culture plates in order to investigate non-adherent rather than – commonly studied – adherent monocytes, thereby avoiding bias introduced by adherence and monocyte-to-macrophage differentiation [39,43]), and/or differences between septic and non-septic infection.

Based on our data we formed the hypothesis that pyruvate and α-ketoglutarate levels are elevated in monocytes from CAP patients *via* reduced activity of the PDH and KGDH complexes. Both complexes are regulated by lipoic acid, also known as α-lipoic acid, which acts as a cofactor in the PDH and KGDH complexes and is essential for their function [34]. Reduced availability of lipoic acid could therefore result in both elevated pyruvate as well as α-ketoglutarate. Reduced availability of lipoic acid could result from inhibited biosynthesis or salvage pathways of lipoic acid, or less lipoic acid uptake from plasma [34], while lipoic acid could also be inactivated [33]. This can occur *via* 4-hydroxy-2-nonenal, a highly toxic product of lipid peroxidation, which interacts with the sulfur atoms of lipoic acid, thereby diminishing the activity of the PDH or KGDH complex the lipoic acid is attached to [33]. In monocytes, lipid peroxidation can occur as a result of reactive oxygen species (ROS) production during activation or ageing [44]. However, more research is needed to support our hypothesis. Although both elevated pyruvate levels and α-ketoglutarate could therefore be regulated by lipoic acid and/or lipid peroxidation in monocytes from CAP patients, only pyruvate levels correlated with a maintained capacity to produce IL-1β and IL-10.

Although little is known about the mechanism by which pyruvate

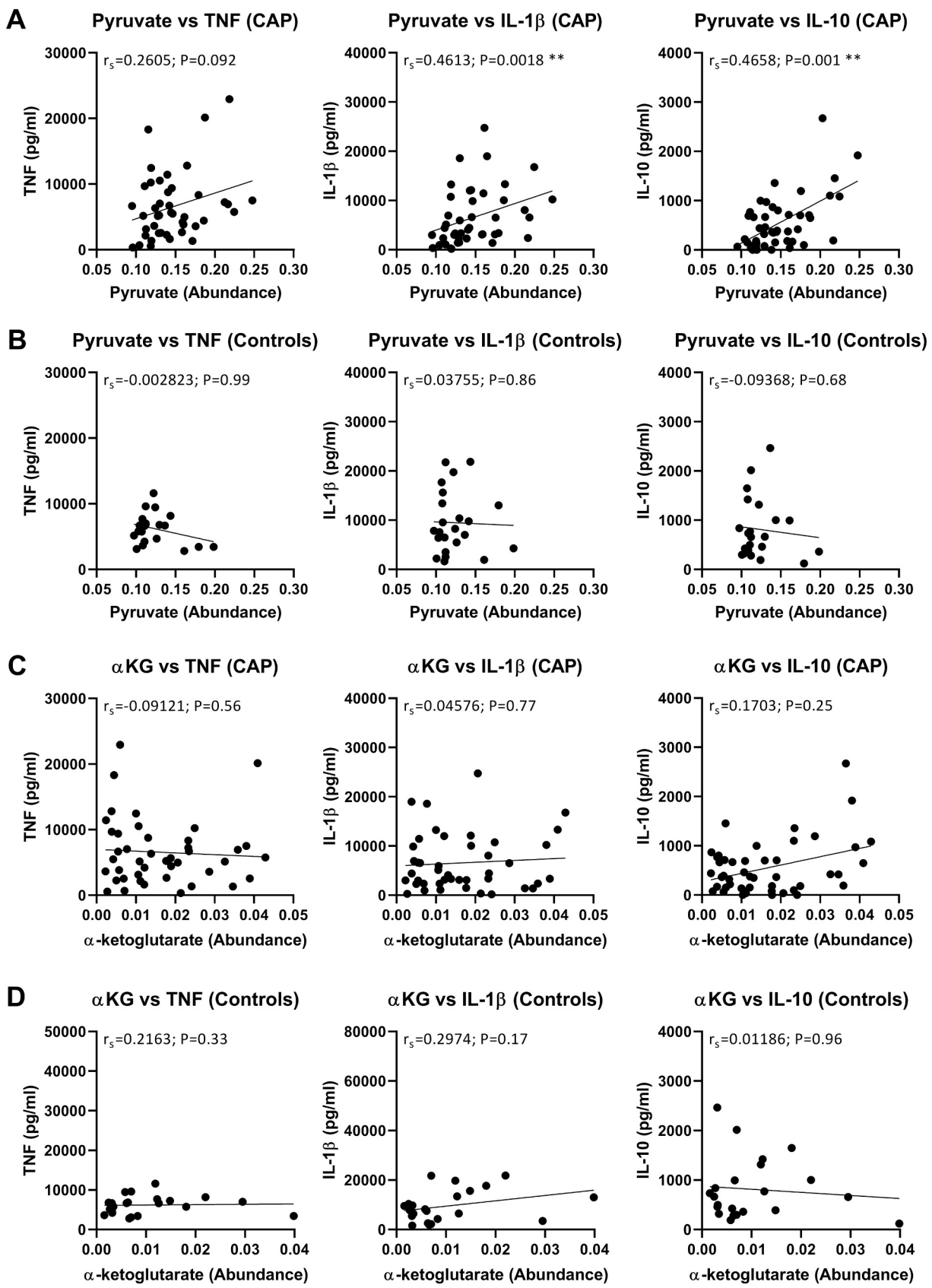

Fig. 4. Monocyte intracellular pyruvate, but not α-ketoglutarate, levels correlate with the capacity to produce IL-1β and IL-10 in patients
Scatterplots showing the correlation between intracellular pyruvate levels (of directly harvested monocytes) and the cytokine production capacity of TNF, IL-1β and IL-10 (after *in vitro* stimulation with LPS) of monocytes from CAP patients (A) or from non-infectious controls (B) including Spearman correlation coefficients (r_s) and the P-values. Scatterplots showing the correlation between intracellular α-ketoglutarate levels and the cytokine production capacity of TNF, IL-1β and IL-10 of monocytes from CAP patients (C) or from non-infectious controls (D) including Spearman correlation coefficients (r_s) and the P-values.

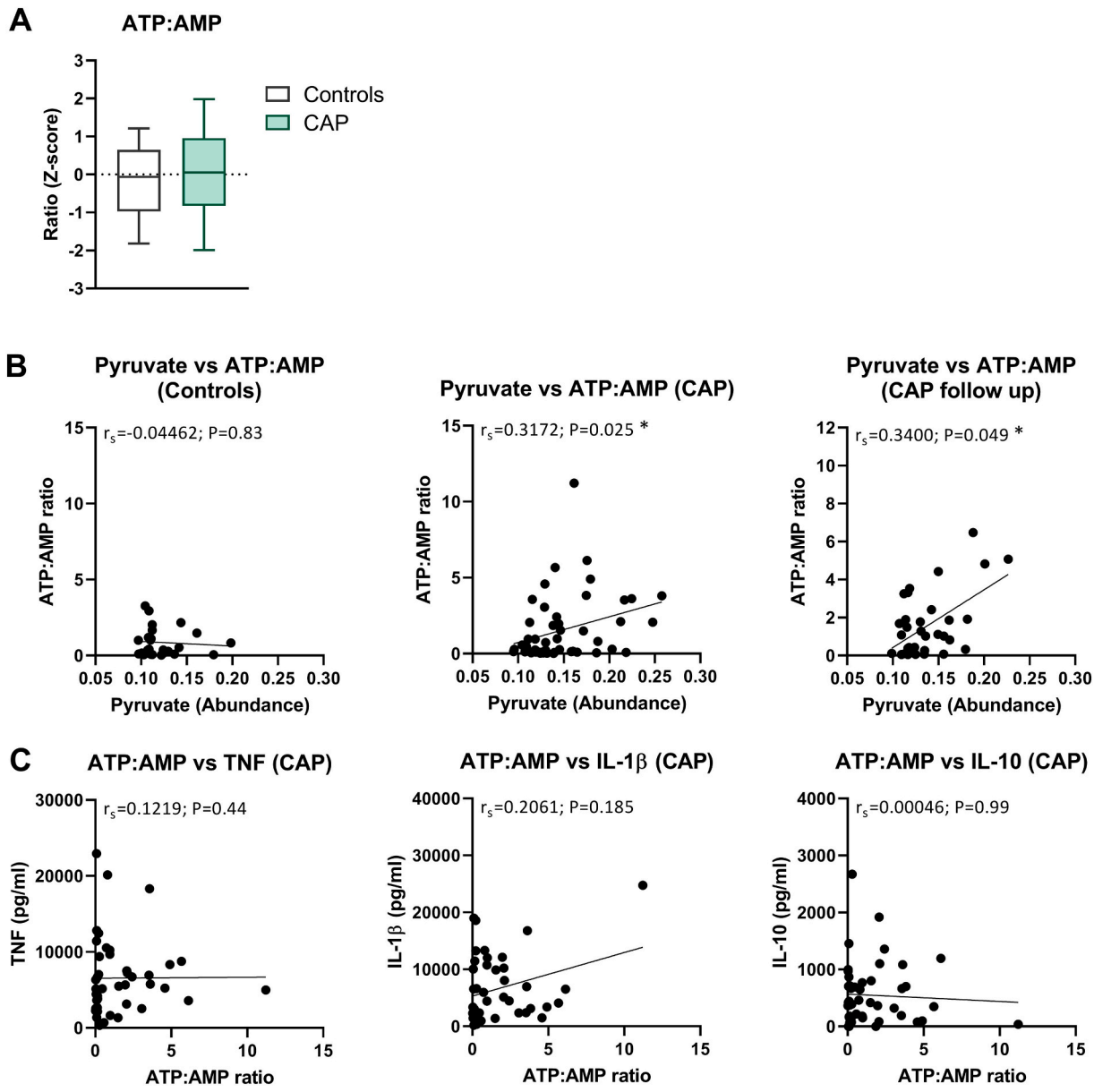

Fig. 5. Monocyte cytokine production capacity does not correlate with energy availability
Energy availability, as shown by the ratio ATP:AMP, in directly harvested monocytes from CAP patients (green; n = 50) and non-infectious controls (white; n = 25) (A). Data is shown as the *Z*-score of the ratio. Scatterplots showing the correlation between intracellular pyruvate levels and the ATP:AMP ratio in monocytes from non-infectious control, CAP patients and recovered CAP patients approximately one month after admission including Spearman correlation coefficients (r_s) and the P-values (B). Scatterplots showing the correlation between the ATP:AMP ratio and the cytokine production capacity of TNF, IL-1β and IL-10 in monocytes from CAP patients including Spearman correlation coefficients (r_s) and the P-values (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

affects the cytokine production capacity of monocytes, several reports show that addition of (extracellular) pyruvate could exerts anti-inflammatory effects *in vitro* [45,46] and *in vivo* [47,48]. For example, sodium pyruvate added to the medium of murine bone marrow-derived macrophages reduced the expression and production of TNF, IL-1β and IL-6 after stimulation with Influenza A virus [46] and co-incubation with lactate and pyruvate enhanced IL-10 production and attenuated the release of pro-inflammatory IL-1β and IL-6 by LPS-stimulated human leukocytes [48]. However, we observed increased intracellular pyruvate which was likely produced by the monocytes themselves as opposed to an extracellular signal. Furthermore, we did not only find a positive correlation between pyruvate and the pro-inflammatory cytokines TNF and IL-1β but also the anti-inflammatory cytokine IL-10. Therefore, the increase in pyruvate did not favor a pro- or anti-inflammatory cytokine response, but rather cytokine production in general.

Monocytes from CAP patients demonstrated enhanced expression of *PKM*, the gene encoding pyruvate kinase, the rate limiting enzyme in the last step of (aerobic) glycolysis, that converts phosphoenolpyruvate to pyruvate. PKM exists in two isoforms: PKM1 and PKM2, generated by alternative splicing [49]. PKM2 is expressed as enzymatically inactive monomers or dimers and enzymatically active tetramers [50]. LPS induces a switch to the dimeric form of PKM2 in macrophages, which translocates to the nucleus and mediates hypoxia-inducible factor (HIF) 1α-induced glycolytic and inflammatory gene expression [50]. PKM2 dimerization and nuclear translocation is also shown to be promoted by ROS [51]. HIF1α has been shown to be important for IL-1β and IL-10 production [17,52]. Unfortunately, HIF1α protein is unstable and rapidly degraded [53,54] and *HIF1A* gene expression is a poor indicator

of HIF1α protein levels [55]. Furthermore, although glycolytic genes were upregulated in patient monocytes, other classic HIF1α-target genes like EPO, ALDOC and VEGFA were not (data not shown). Therefore, it remains to be established whether HIF1α plays a role in partially sustaining the capacity of CAP patients' monocytes to produce IL-1β and IL-10 upon LPS stimulation.

Our study has strengths and limitations. We compared CAP patients with non-infectious control subjects who were matched for age-, sex- and comorbidities, allowing evaluation of differences related to acute pneumonia rather than pre-existing chronic conditions. We purified monocytes from patients and controls to perform an integrative analysis of transcriptomics and metabolomics, linked with cytokine production capacity. While the differences on the metabolite level seem small, we do consider them significant, since these are derived from "naïve" circulating monocytes which have not extravasated from the blood vessels to the site of infection. Additionally, circulating monocytes, like non-adherent monocytes (cultured on cell-repellent plates), might show a limited glycolytic response to LPS in comparison to adherent monocytes/ macrophages [39]. Nonetheless, while "omics" analyses provide comprehensive insight into the metabolic and immunological status of cells, interpretation is hampered by the fact that a single sample obtained shortly after admission generates static "snap-shot" information; more dynamic and metabolic flux analyses would likely offer better insight into which metabolites and energy pathways contribute to the functional phenotype of cells. We therefore believe that these results are a starting point for more targeted studies in the future to elucidate the link between metabolism and tolerance in monocytes during infectious diseases.

In conclusion, this study provides evidence for an increased glycolytic rate in blood monocytes from patients with CAP, as indicated by enhanced expression of key enzymes involved in glycolysis, including PKM, and elevated intracellular pyruvate levels. In patients, monocyte pyruvate concentrations were positively correlated with the capacity of these cells to produce IL-1β and IL-10 upon stimulation with LPS. Further research is warranted to establish a mechanistic link between monocyte metabolism, pyruvate levels and LPS tolerance in acute infections such as pneumonia.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2022.166519.

Declaration of competing interest

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Data availability

Data will be made available on request.

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