Transcriptomic gene profiles in an *ex vivo* model of erythropoiesis to unravel molecular pathomechanisms in sickle cell disease

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Abstract

Sickle cell disease (SCD) is an autosomal recessive, single gene disorder caused by a point mutation in the beta globin *(HBB)* gene. The single amino acid substitution of the haemoglobin molecule leads to aberrant β-chain formation, polymerizing sickled haemoglobin (HbS) molecules together during episodes of deoxygenation and therefore causing intracellular sickling. Hydroxyurea (HU) is still the most indicated drug inducing fetal hemoglobin (HbF) production for SCD treatment, especially for the SCD patients with a severe clinical profile. In this study we could identify transcriptional profiles in erythroid cells of SCD patients, which have been treated with HU in comparison to untreated SCD patients and healthy controls.

Our present transcriptomic profile analysis contained a new subset of genes identified for the first time as associated with SCD. In SCD non-treated-derived erythroid cells compared to controls, 398 differentially expressed genes (DEGs) were identified, of which 100 genes were upregulated and 298 genes were downregulated in the SCD patient cells. 65 DEGs were identified in SCD HU-treated patient-derived erythroid cells compared to controls, of which 37 genes were upregulated and 28 genes were downregulated. 212 DEGs were identified in SCD HU-treated patient-derived compared to SCD non-treated derived erythroid cells, of which 113 genes were upregulated and 99 genes were downregulated. 141 DEGs were identified in SCD derived erythroid cells compared to controls, of which 58 genes were upregulated and 83 genes were downregulated. We found biological processes such as oxidative phosphorylation pathway, Fanconi anaemia pathway, adaptive immune response or inflammatory response to be part of our significantly enriched Gene Ontology (GO) terms. Further genes encoding significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for proteasome, autophagy, immune response, platelet activation, as well as for natural killer cell (NK) cytotoxicity, oxidative phosphorylation, Fanconi anaemia pathway, ribosome biogenesis were upregulated in both SCD non treated and SCD HU-treated patient-derived erythroid cells in comparison to healthy controls. Our findings collectively suggest different as well as common molecular signatures between SCD non-treated, SCD HU-treated compared to HC. We could validate 12 of our top significant genes by qRT-PCR (calneuron 1 (CALN1), nucleoporin 85 (NUP85), tumor protein 63 (TP63), ras and rab interactor 2 (RIN2), solute carrier family 44 member 5 (SLC44A5), phenylethanolamine N-methyltransferase (PNMT), stabilin 1/ clever-1 (STAB1), SLX1 Homolog A, Structure-Specific Endonuclease Subunit (SLX1A), ATPase phospholipid transporting 9A (ATP9A), MSH5-SAPCD1, G protein subunit gamma 4 (GNG4) and glutathione S-transferase theta 2B (GSTT2B)) and compare their mRNA levels in bone marrow and spleen tissue from the Berkely SCD mouse model.

In short, our study could identify new genes and pathways that may be involved in the SCD disease pathogenesis and that may be influenced by the HU treatment. The findings encourage future characterization of their roles in SCD to establish these findings as basis for new therapeutic targets.

Introduction

Sickle cell disease (SCD) is an autosomal recessive, single gene disorder[1]. Sickled haemoglobin (HbS - $\alpha_2\beta^S{}_2$) is as described in Murayama M et al.[2] a structural variant of normal adult haemoglobin (HbA) caused by a point mutation in the *HBB* gene that leads to the single amino acid substitution of valine for glutamic acid at position 6 of the β-globin subunit (β^S) of the haemoglobin molecule[1,3]. This amino acid substitution arose from a single base change (A>T) at codon 6 (*rs334*)[4]. Any condition in which the production of HbS leads to pathophysiological consequences is referred to as sickle cell disease (SCD)[1].

An increased comprehension of the molecular mechanisms of the disease has led to a diversification of newly studied therapies, investing the HbS polymerization event to more distal pathologic processes, such as red blood cells (RBC) adhesion to the vascular endothelium[8]. Patients taking part in our study have been treated with Hydroxyurea (HU), a ribonucleoside diphosphate reductase inhibitor approved by the Food and Drug Administration (FDA) in 1998 that was first used in myeloproliferative disease[8,9]. It was originally used to reduce the need of blood transfusions and the frequency of painful crisis in adults with homozygous SCD[8]. The mechanism of HU in SCD is multifactorial, but it primarily involves increasing production of fetal haemoglobin (HbF) as described in McGann PT et al.[10]. It is shown that patients with naturally high levels of HbF have milder disease symptomatic[11]. Adult patients who had received hydroxyurea for up to 9 years of treatment in the course of a long-term analysis were associated with a significant reduction of 40% in mortality[12].

Several studies were already undertaken to analyse transcriptional signalling pathways and differentially expressed genes that may be dysregulated in SCD. The study from Raghavachari et al.[13] identified multiple platelet-specific genes involved in arginine metabolism and redox homeostasis. Top platelet-abundant genes included platelet derived growth factor receptor alpha (PDGFRA), myosin light chain kinase (MYLK), platelet factor 4 variant 1 (PF4V1), 5'-aminolevulinate synthase 2 (ALAS2), histone cluster 1, H2bg (HIST1H2BG) and solute carrier family 24 member 3 (SLC24A3)[13]. Study from Creary et al.[14] explored changes in gene expression profiles via whole blood RNA-Seq among patients with SCD hospitalized for acute chest syndrome (ACS) and acute vaso-occlusive pain crises (VOC) episodes. Top differentially expressed genes included CD177 molecule (CD177), caspase 5 (CASP5), suppressor of cytokine signalling 3 (SOCS3), free fatty acid receptor 3 (FFAR3), GRAM domain containing 1C (GRAMD1C) and annexin A3 (ANXA3), whereas top canonical pathways identified in ACS were interferon signalling, pattern recognition receptors, neuroinflammation, macrophages and in VOC were related to IL-10 signalling, iNOS signalling, IL-6 signalling and B cell signalling[14]. Desai et al[15] identified and validated the association of a circulating genome-wide gene expression profile with poor outcomes in 3 cohorts of SCD based on peripheral blood mononuclear cell (PBMC) transcriptomes. The findings highlighted the role of haemolysis and globin synthesis in SCD through gene expression upregulation in BCL2 like 1 (BCL2L1), DOCK family candidates, selenium binding protein 1 (SELENBP1), Delta-aminolevulinate synthase 2 (ALAS2), basigin (BSG), ferrochelatase (FECH), glycophorin A (GYPA), and bisphosphoglycerate mutase (BPGM), as well as upregulation of different metabolic pathways, complement and coagulation cascade, malaria signalling and downregulation of T-cell receptor signalling, immunodeficiency, and vascular endothelial factor (VEGF) pathway[15]. The Study from Hamda et al.[16] performed gene expression meta-analysis of five independent publicly available microarray datasets related to homozygous SS patients with SCD to identify a consensus SCD transcriptomic profile. Among these, heat shock protein 40 (Hsp40), DNAJ heat shock protein family (Hsp40) member C6 (DNAJC6), ring finger protein 182 (RNF182), carbonic anhydrase 1 (CA1) were the most significantly over-expressed genes, whereas wnt ligand secretion mediator (WLS), transcription factor 7 like 2 (TCF7L2), G protein-coupled receptor 171 (GPR171)

were the most under-expressed genes; highlighted pathways were related to innate immune responses, metabolism, erythrocyte development and hemostasis pathways[16].

Transgenic mouse models and erythroid cell lines as *in vitro* models have been used to understand the underlying mechanisms of pathogenesis of various diseases and as preclinical testing for new therapies. In our study we used an erythropoiesis model using a 2-phase liquid system in which we previously showed that microRNA-96 directly supress γ-globin expression by contributing to HbF regulation[17,18]. Our *in vivo* model, namely the Berkeley SCD transgenic mouse model, includes genetic disruptions of endogenous adult-type α and β-globin gene (*Hbb-a1*, *Hbb-a2*, *Hbb-b*^{maj} and *Hbb-b*^{min}) and expresses human globins via three DNA transgenes: 1.5 kb spanning the α-globin gene *HBA1*; a contiguous 39 kb genomic fragment including genes for γ-globin (*HBG2*, *HBG1*), δ-globin (*HBD*) and sickle β-globin (*HBB*^{S}); and a 6.5 kb 'mini-locus control region (LCR)' derived from an endogenous enhancer in the human β-like globin cluster that confers high-level erythroid expression[19,20]. Berkeley mice have similar pathologies as the human sickle cell disease (erythrocytic sickling, vascular ectasia, intravascular hemolysis, exuberant hematopoiesis, cardiomegaly, glomerulosclerosis, visceral congestion, hemorrhages, multiorgan infarcts, pyknotic neurons, and progressive siderosis) as well as differences (splenomegaly, splenic hematopoiesis, more severe hepatic infarcts, less severe pulmonary manifestations, moderate vascular intimal hyperplasia, and only a trend toward vascular medial hypertrophy)[21]. This SCD mouse model has been used to test lentiviral or Cas9-induced DNA repair vectors for beta-globin gene replacement or to alter the mutant SCD codon[22–27].

In this study, we aimed to identify the erythroid-specific transcriptional profiles of non-treated and HU-treated SCD patients, in comparison to healthy controls using a patient-derived erythropoiesis in vitro model. Furthermore, we could select and validate top candidate genes at mRNA levels by RT-qPCR and explored their expression in the Berkeley SCD mouse model. The identified genes and pathways could be further considered as important role-players in SCD pathogenesis and might lead to discovering new treatment targets.

Materials and methods

Study Approval

All procedures were reviewed and approved by the local ethical committee (Cantonal Ethics Committee Zurich, Switzerland). All healthy blood donors as well as SCD patients, or their legal guardians gave written informed consent prior to their enrolment in this study.

Patients

All patients were recruited at the Children's Hospital Zurich, Switzerland. Subjects with SCD had haemoglobin SS. Healthy controls were Caucasians and presented haemoglobin genotype AA confirmed by differential hemogram. In total, we recruited 17 SCD patients with median age: 9.0 ± 5.36 years; gender ratio: 8 females (F) / 9 males (M)). For comparison, 5 SCD non-treated patients (median age: 10.0 ± 6.05 years; gender ratio: 5F/0M) and 7 SCD HU-treated patients (median age: 12.0 ± 6.8 years; gender ratio: 1F/6M) were studied. In addition, we included 5 healthy pediatric controls with a median age of 8.0 ± 3.28 years (2F/ 3M). Clinical data were extracted from the medical record and included complete blood count and medical history (as described in Table 1).

Sample Collection

Erythroblast expansion and differentiation (ex-vivo erythropoiesis)

Peripheral blood mononuclear cells (PBMCs) were isolated from heparin blood using the SepMateTM PBMC isolation tubes from STEMCELL Technologies Inc. according to manufacturer's protocol. PMBCs layer was removed into a new Falcon tube, diluted 1:1 with RPMI 1640 medium (Thermo Fischer Scientific) and centrifuged at 1000g for 10 minutes. After removal of the supernatant, resuspension of the pellet and repeat centrifugation, the pellet of PBMCs was divided, one part was freezed at -80°C for further RNA extraction, the remaining part was then cultured in a two-phase liquid system as previously described by our group[28] and characterized at molecular level[29].

Transgenic and control mice

The Berkeley model (*JAX stock #003342*) sickle cell mice homozygous for the alpha-globin null allele, homozygous for the beta-globin null allele and carrying the sickle transgene (Hba$^{0/0}$ Hbb$^{0/0}$ Tg(Hu-miniLCRα1^{G}γ^{A}γδβ^{S}) was pursued from Jackson Laboratory (Strain 003342) and were kindly provided

to us by Dr. Dominik Schär and Dr. Florence Vallelian, Department of Internal Medicine, University Hospital Zürich.
The animals were dissected to expose the spleen and femur bone marrow. The tissue was then washed and processed for further RNA sequencing.

RNA-sequencing Data Analysis

Total RNA was extracted from cultured erythrocytes and PBMCs using RNeasy mini kit from Qiagen, Germany, as described in the manufacturer's manual and in a previous publication[17].
The libraries were prepared with Illumina's TruSeq Stranded mRNA kit using 300ng input total RNA. RNA sequencing was performed on an Illumina NovaSeq 6000 instrument at the Functional Genomics Center Zurich. The raw reads were cleaned by removing adapter sequences, trimming low quality ends, and filtering reads with low quality (phred quality <20) using Fastp (Version 0.20)[30]. Pseudo sequence alignment of the resulting high-quality reads to the human transcriptome (genome build GRCh38.p10, gene annotations based on GENCODE release 32) and quantification of transcript expression was carried out using Kallisto (Version 0.46.1)[31].To detect differentially expressed genes, we applied a count based negative binomial model implemented in the software package EdgeR (Rversion: 4.0.3, EdgeR version: 3.32)[32]. The differential expression was assessed using the GLM framework and applying a quasi-likelihood F-test. The resulting p-values were adjusted with the Benjamini and Hochberg method.

RT-qPCR Analysis

The extracted RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit from Qiagen as recommended by the manufacturer. RT-qPCR was performed in 96-well fast thermal cycling plates (Thermofisher Scientific, Switzerland) using PowerUp SYBR Green Master Mix (Thermofisher Scientific, Switzerland, cat. A25742) and primers with specific sequences as mentioned in Table 2. Oligonucleotides from BIO-RAD (PrimePCR SYBR Green Assay) were used for the following genes: NUP85 (UniqueAssayID: qHsaCID0018193); SLC44A5 (UniqueAssayID: qHsaCED0057217); TP63 (UniqueAssayID: qHsaCID0036332); CALN1 (UniqueAssayID: qHsaCED0047583); GNG4 (UniqueAssayID: qHsaCED003675); GSTT2B (UniqueAssayID: qHsaCID0038698), GNG4 (UniqueAssayID: qMmuCID0018380); MSH5 (UniqueAssayID: qMmuCID0022924); KIFC2 (UniqueAssayID: qMmuCID0022929) and FBF1 (UniqueAssayID: qMmuCID0021443).

Statistical Analysis

Our data was statistically analyzed by using Prism 9.5.0 (GraphPad, Software CA, USA). Data are presented as mean +/- standard deviation of the mean. Data was analyzed using t-Tests or One-way ANOVA, followed by multiple comparisons tests to compare the means between the groups. Data was considered significantly different from controls if $p < 0.05$ (*), $p < 0.0021$ (**), $p < 0.0002$ (***), $p < 0.0001$ (****).

Results

Patient demographics

Table 1 shows the patient demographics (sex, age, HU-treated) and hematologic parameters of the patients (white blood cell count, absolute neutrophil count, absolute lymphocyte count, absolute monocyte count, hemoglobin, platelet count).

Transcriptome profiling in SCD and HU-treated SCD patients versus healthy controls

We identified by RNA sequencing 1398 DEGs in SCD non-treated derived erythroid cells compared to controls, of which 519 genes were upregulated (e.g., CALN1) and 879 genes (e.g., GSTT2B, MSH5-SAPCD1 and RDH10) were downregulated (Figure 1A). 495 DEGs were identified in SCD HU-treated patient-derived erythroid cells compared to controls, of which 288 genes were upregulated (e.g., CALN1) and 207 genes (e.g., GSTT2B, FBF1 and MSH5-SAPCD1) were downregulated (Figure 1B). 786 DEGs were identified in SCD HU-treated patient-derived erythroid cells compared to SCD non-treated derived erythroid cells, of which 455 genes (e.g., GNG4 and RDH10) were upregulated and 331 genes were downregulated (Figure 1C).
We generated heatmaps to display the level of expression of each gene in relation to the mean level of expression of that gene across all samples. The expression of the 20 most significant DEGs in non-

treated SCD patients was downregulated compared to healthy controls. Overall, the HU treatment altered the SCD gene expression signatures, some being reversed to the control levels (Figure 2). Specifically, when we compared SCD non-treated to healthy controls, the HU-treated and healthy controls samples showed similar upregulation of DEGs in comparison to the downregulated DEGs of non-treated samples (Figure 2A). Whereas, in HU-treated SCD versus healthy controls, we found a clear gene downregulation expression in healthy controls, compared to upregulated DEGs of non-treated and HU-treated SCD samples for unc-51 like kinase 3 (ULK3), KIFC2, translocase of inner mitochondrial membrane 8A (TIMM8A), AC004233.2, CU638689.4, AL035461.2, crystallin beta B2 (CRYBB2), coiled-coil glutamate rich protein 2 (CCER2), SLX1A, MIR503HG, GSTT2B, PNMT, AC004832.3, solute carrier 11a1 (SLC11A1), ribonuclease A family member 1 (RNASE1) and desmin (DES). The other genes represented on the bottom half such as transmembrane 4 L six family member 1 (TM4SF1), CALN1, ADGRA2, long intergenic non-protein coding RNA 2099 (LINC02099), endoplasmic reticulum aminopeptidase 2 (ERAP2), neuron navigator 3 (NAV3), neuregulin 1 (NRG1), SLC44A5, AC083862.3, leukocyte immunoglobulin like receptor A4 (LILRA4), ras related glycolysis inhibitor and calcium channel regulator (RRAD), STEAP4 metalloreductase (STEAP4), caveolin 1 (CAV1) and phosphate regulating endopeptidase X-Linked (PHEX), showed an upregulation in healthy controls, and a downregulation in non-treated and HU-treated SCD samples (Figure 2B). Furthermore, when we compared the expression levels of 20 most significant DEGs in SCD HU-treated versus SCD non-treated samples, we found that the gene expression for AC138696.1, macrophage scavenger receptor 1 (MSR1), SMAD family member 6 (SMAD6), CAMP-dependent protein kinase inhibitor beta (PKIB), inka box actin regulator 1 (INKA1), COL23A1, glycoprotein Ib platelet subunit beta (GP1BB), heparan sulfate glucosamine 3-O-sulfotransferase 2 (HS3ST2), CD163, MAF bZIP transcription factor B (MAFB) and STAB1 was upregulated in healthy controls and SCD HU-treated samples but downregulated for non-treated SCD samples. The opposite was found for SALL4, Fc gamma receptor IIIa (FCGR3A), C2 calcium dependent domain containing 2 (C2CD2), neurocan (NCAN), desmocollin 3 (DSC3), histocompatibility minor HB-1 (HMHB1), AL359834.1, DIRAS family GTPase 2 (DIRAS2), frizzled class receptor 6 (FZD6), AC011447.7, protein tyrosine phosphatase non-receptor type 14 (PTPN14), nuclear receptor subfamily 4 group A member 2 (NR4A2), protein tyrosine phosphatase, non-receptor type 3 (PTPN3), abelson helper integration site 1 (AHI1), RDH10, TP63, GNG4, endosome-lysosome associated apoptosis and autophagy regulator 1 (KIAA1324) and ATP9A, which were downregulated in healthy controls and SCD HU-treated samples and upregulated in non-treated SCD samples (Figure 2C).

Molecular pathway analysis

Using the WebGestalt toolkit (http://www.webgestalt.org/), we analyzed the RNA sequencing datasets with two approaches: (i) by performing GSEA in which a list of all detected genes ranked by their average fold change is used to calculate enrichment scores of KEGG pathways (significantly enriched pathways are summarized in Figure 3) and (ii) by using DEGs in patient-derived erythroid cells to identify over-represented biological processes and cellular components by ORA (Supplementary Figure 2 and Supplementary Table 3). Notably, genes encoding significantly enriched KEGG pathways for Fanconi anaemia and ribosome pathways were upregulated in both SCD non-treated and SCD HU-treated patient-derived erythroid cells in comparison to healthy controls. Both autophagy and mitophagy as well as complement cascades pathways were found downregulated in SCD non treated group, whereas systemic lupus erythematosus, Toll-like receptor, NF-kappa B signalling were downregulated in SCD HU-treated patient-derived erythroid cells in comparison to healthy controls (Figure 3A-B). For HU-treated SCD patient derived erythroid cells in comparison to SCD non-treated, the significantly enriched KEGG pathways included upregulation in systemic lupus erythematosus, complement cascades, phagosome, lysosome and oxidative phosphorylation (Figure 3C). Significantly enriched downregulated pathways included Th1 and Th2 cell differentiation, antigen processing and presentation, T cell receptor signalling pathway and Th1 and Th2 cell differentiation (Figure 3C). Variants in toll-like receptor 2 (TLR2) were found to correlate with the occurrence of bacterial infection in SCD patients[33].

ORA analysis showed the significantly enriched biological processes (e.g., inflammatory response, innate immune response) that are summarized in Supplementary Table 3.

Selection and validation of top candidate genes

We further selected top significant candidates DEGs with biological functions that may contribute to the pathogenesis of SCD. CALN1, NUP85, KIFC2, MSH5-SAPCD1, GNG4, GSTT2B, RDH10 and FBF1 were identified as potential contributors (Figure 4). Interestingly, the gene expression of GNG4, MSH5, RDH10 of SCD HU-treated samples were found to be at similar levels as healthy controls suggesting that they may be influenced by the treatment. Whereas the levels of CALN1, NUP85, KIFC2 and FBF1 of SCD HU-treated and non-treated patients remained at the same levels, significantly different from the levels of healthy controls suggesting that their changed SCD expression may not be affected by the HU treatment (Figure 4).

We then technically validated by qRT-PCR 12 from 17 genes CALN1, NUP85, TP63, RIN2, SLC44A5, PNMT, STAB1, SLX1A, ATP9A, MSH5-SAPCD1, GNG4 and GSTT2B by qRT-PCR in 5 SCD non-treated patients and 5 SCD HU-treated patients, which showed the same regulation profile in line with the RNA-sequencing data, as shown in Figure 5 and Supplementary Table 1.

Moreover, due to the role of PBMCs in inflammation and in SCD disease (Desai et al 2017)[15], we investigated the mRNA levels of these top candidate genes in PBMCs isolated from these SCD, HU-treated SCD and healthy controls groups. We found downregulation of SLC44A5 and CALN1, as observed also in erythroid cells in our transcriptomic data, whereas the downregulated mRNA levels of KIFC2 in SCD patients were found the opposite than in RNA sequencing data (Supplementary Figure 4). However, GNG4, NUP85, COL23A1, STAB1, MSH5-SAPCD1, FBF1, RIN2, ATP9A, RDH10 showed no detectable mRNA values in PBMCs. This result suggests the specificity of the mRNA gene expression to erythroid cells in comparison to PBMCs in SCD disease.

Next, we investigated if the validated genes are differentially expressed in the spleen and bone marrow of the SCD Berkeley mouse model. We observed that the mRNA levels of MSH5-SAPCD, GNG4, FBF1 were upregulated in both spleen and bone marrow of the SCD mouse model compared to the wild type mouse, whereas for STAB1 the mRNA levels were significantly lower (Figure 6 and 7). These results were similar to the mRNA validated expression levels observed in SCD non-treated patients (Figure 5), suggesting that these genes could be signatures genes of SCD disease.

Discussion

A better understanding of SCD pathophysiology and its complex manifestation is needed to develop targeted new treatment modalities that may also be more widely available to patients [14]. To this end, we analysed gene expression profiles from children with SCD, treated with HU or untreated, in comparison to healthy controls in an erythropoiesis in vitro model. Overall, we observed for the first-time significant changes in gene expression and top canonical pathways for SCD, suggesting that erythroid-specific RNA sequencing is able to distinguish changes according to clinical statuses and treatment modalities. Our findings may contribute to further understanding of SCD pathogenesis, HU treatment impact on the genetic profile and development of novel therapeutic strategies.

Several transcriptomic studies performed in whole blood revealed prominent roles for proteasome, inflammatory response, innate immunity, haemostasis, hematopoiesis, haemoglobin expression, cytoskeleton, autophagy/mitophagy[15,16,34,35]. Two published meta-analyses in whole blood and PBMCs[16,35] revealed the involvement of innate immunity, response to oxidative stress, erythrocyte development and ubiquitination as most relevant biological processes to SCD. While our study could confirm the contribution some of these pathways in SCD similar to other previous reports (e.g., proteasome, autophagy, immune response, platelet activation), our transcriptomic analysis could generate also novel genes and pathways that were not previously associated with SCD disease pathogenesis (NK cytotoxicity, oxidative phosphorylation, Fanconi anaemia pathway, ribosome biogenesis) that might be specific for erythroid cells. Overall, these results might reflect the contribution of both erythroid cells and PBMCs co-culturing in our ex-vivo erythropoiesis model, since the PBMCs cannot be completely eliminated from the preparations[28].

Several studies have shown that SCD patients have a decreased T-lymphocyte count[36] and found the top significantly enriched GO terms and biological pathways to be associated with T-cell activation and differentiation[16]. T-cell lymphopenia–associated inflammatory responses have been previously linked to the inactivation of PI3Kδ and PI3Kγ[37], genes that were found to be downregulated in a previous [16] meta-analysis. Concomitant suppression of genes related to adaptive immune responses (B and T cell

signalling) was also observed during the two complications of SCD, acute chest syndrome (ACS) and acute vaso-occlusive pain crises (VOC) in the study from Creary et al.[14]. We found Th1 and Th2 cell differentiation and Th17 cell differentiation to be significantly enriched downregulated KEGG pathways for treated SCD patients in comparison to healthy controls (Figure 3A-C). This result may suggest a contribution to the altered immune response leading to an increased risk of severe bacterial infections among SCD patients. This finding may also suggest that other genes than PI3Kδ and PI3Kγ may be implicated in T-cell lymphopenia–associated inflammatory responses as they were not part of our top DEGs and we still observed similar downregulation in T-cell differentiation pathways.

Another group evaluated by RNA sequencing of whole blood analysis of patients with SCD and found most of the upregulated genes were associated with erythropoiesis, highlighting the role of globin synthesis and haemolysis (Raghavachari N et al.)[38].

DEGs from our transcriptomic profiles analysis included SLC44A5, ADGRA2, ADARB2, STAB1, RIN2, PID1, GSTT2B, PNMT, NRG1, ATP9A, RDH10, TP63, GNG4, PTPN3, NR4A2, SALL4, COL23A1, PTPN14, CALN1, STEAP4, KIFC2, RRAD, NUP85, MSH5-SAPCD1 and FBF1 (Figure 1, 2 and 4). We could validate 17 genes by qRT-PCR (CALN1, NUP85, TP63, RIN2, SLC44A5, PNMT, STAB1, SLX1A, ATP9A, KIFC2, MSH5-SAPCD1, GNG4, GSTT2B, RDH10, COL23A1, ADGRA2 and FBF1), from which SLC44A5 and CALN1 and showed the same expression pattern in PBMCs and MSH5-SAPCD, GNG4, FBF1 and STAB1 in bone marrow and spleen of SCD mouse model.

Meta-analysis and genome-wide association study by Ben Hamda C. et al.[16] showed SLC7A5 as one of the top shared upregulated DEGs in the meta-analysis that is from the same solute carrier family as solute carrier family 4 member 1 (SLC4A1) and as our top DEG SLC44A5 downregulated and overlapping in SCD non-treated over HC and SCD treated over HC. The solute carrier transporter families are known as metabolic “gatekeepers” of the cells with an important role in adaptive and innate immunity[39]. SLC4A1 also encodes Band 3 an important mebrane transporter protein in red cells, highlighting its role in membrane stability and anione transport in erythropoietic cells[15], Human SLC4A1/AE1 mutations are known to cause human spherocytosis and stomatocytosis. Therefore SLC44A5 may contribute to an abnormale membrane function and stability and could play a role in the pathophysiology of SCD.

Our study showed a downregulation of CALN1 expression for SCD treated and SCD non-treated patient-derived erythroid cells, suggesting that HU may not affect CALN1 expression, in particular calcium ion binding. Changes in CALN1 expression has not been reported in prior SCD studies. CALN1 increases calcium storage in endoplasmic reticulum (ER) to collaborate with sarcoendoplasmic reticulum transport ATPase (SERCA)[40]. Hänggi et al.[41] described significantly increased intracellular Ca^{2+} levels in SCD due to an increased number of NMDA receptors causing abnormal Ca^{2+} uptake. Hertz et al.[42] hypothesized that increased Ca^{2+} levels in RBCs may contribute to an accelerated clearance of RBCs from the blood stream and may be a common part of the molecular mechanism in anaemia. Gardos channels, μ-calpain and NOXes are known to respond to the upregulation of the intracellular Ca^{2+} levels[43]. Wang et al 2021[44] found a new calcium signalling cascade that is increased in RBCs in SCD patients through the upregulation of lysophosphatidic acid (LPA) receptor 4. Targeting Ca^{2+} entry mediating molecular pathways with Memantine for example could benefit SCD treatment[45].

GSTT2B showed an upregulated gene expression for SCD treated patient-derived erythroid cells in comparison to healthy controls and non-treated patients, suggesting a possible influence of HU on GSTT2B expression, as HU can improve antioxidant defences of SCD patients (Silva et al 2011)[46]. GSTT2B is part of the Glutathione *S*-transferase (GST) enzyme complex catalysing the conjugation of xenobiotics to glutathione, as major phase II detoxification enzymes in the cytosol[47–49]. The normal glutathione-S-transferase activity in red cells is significantly decreasing in the first weeks of life, remaining constant afterwards[50]. GSTT2B deletion is associated with high frequency deletion of the GSTT2 and GSTT1 gene, both encoding cytosolic detoxification enzyme[49]. Reduced glutathione is an important antioxidant in red cells and low reduced glutathione levels may contribute to the pathophysiology of SCD[51]. L-Glutamine, a precursor for the synthesis of glutathione (GSH), has been investigated as a

GNG4 showed an upregulated gene expression for SCD non-treated patient-derived erythrocytes in comparison to healthy controls and treated patients, suggesting that HU could modify the GNG4 expression by downregulating it. We also found an upregulated mRNA expression in the spleen and bone marrow of the SCD mouse model. GNG4 belongs to the G-protein gamma family and it is

commonly used as a marker of poor overall surviving rated in some malignancies such as bladder cancer or colorectal cancer[55,56]. GNG4 may affect tumour progression through the PI3K-AKT signalling pathway[56]. After downregulating the expression of GNG4 in the in vitro experiments, proliferation, migration and invasion of SW-620 type colon cancer cells were significantly reduced[56]. In addition, apoptosis was significantly increased and the cell cycle was blocked in the S phase[57]. Interestingly, these results show common benefit in GNG4 expression inhibition, a result that we observed in our DEG analysis as well.

FBF1 showed an upregulated gene expression for both SCD treated and non-treated patient-derived erythroid cells in comparison to HC, suggesting that HU may not affect FBF1 expression. We also found an upregulated mRNA expression in the spleen and bone marrow of the SCD mouse model. Fas and its ligand (FasL) interactions are known as major inducer of apoptosis and of inflammatory response[58]. In line with our results, high levels of sFas/sFasL ratio were previously reported in SCD, this ratio being used as a marker for vascular dysfunction and assessing renal dysfunction in SCD patients, in relation to inflammation, iron deposition and albuminuria[58].

Our results indicated that the expression of STAB1 was downregulated in both SCD HU-treated and non-treated groups compared to healthy controls. We also found a downregulated mRNA expression in the spleen and bone marrow of the SCD mouse model. STAB1 is expressed in alternatively activated macrophages, with an important role in tissue homeostasis and prevention of autoimmunity[59,60]. STAB1 is required for anti-inflammatory macrophage genotype differentiation[61]. Moreover, it was shown that blocking STAB1 in macrophages results in defective engulfment of aged red blood cells[60]. In which relation STAB1 may be involved in SCD pathogenesis in red blood cells remain to be elucidated in future studies.

HU treatment is up-to-date the only approved FDA-treatment for SCD and beta-thalassemia (Chondrou V. et al. (2017)[62]. The mechanisms of its HbF induction and its effects on the global transcriptome are not yet fully elucidated. Chondrou V. et al. (2017)[62] performed a whole transcriptome analysis in erythroid cells derived from human hematopoietic tissues of various developmental stages where they identified VEGFA gene to be associated with elevated fetal haemoglobin levels in β-type hemoglobinopathy patients. They indicated that *VEGFA* genomic variants were associated with disease severity in β-thalassemia patients and HU treatment efficacy in SCD/β-thalassemia compound heterozygous patients. Whereas Cui P. et al. (2010)[63] analysed global transcriptomic modification of mouse embryonic stem cells in response to HU-treatment. They found the global transcriptional activity to be suppressed after HU-treatment exposition. HU-treatment altered multiple key cellular pathways, including cell cycle, apoptosis, inflammatory response. DNAs- and alternative splicing mechanisms and suppressed non-coding RNA expression. Similar to the upregulation of DNA repair and replication pathways observed in our analysis, many DNA damage repair enzymes were upregulated in HU-treated embryonic stem cells, including *trex1*, *mus81*, *nthl1* and *xrcc1*. Cui P. et al. (2010)[63] suggest that the pathways of DNA damage repair may be activated when embryonic stem cells are exposed to genotoxic stress such as HU, that may lead to changes in the replication fork, base oxidation and DNA single- or double-strand breaks.

Very few data are available regarding the transcriptomic gene profile and gene regulation mechanisms in SCD transgenic mouse models in comparison to human SCD patients. Previous work in sickle transgenic mouse showed the role of NF-kB signalling in blood mononuclear cells in regulating endothelial tissue factor expression[64] or that the loss of NRF2, a master regulator of oxidative stress and heme activator, could exacerbate the pathophysiology of SCD disease[65]. We could show that some of the genes that demonstrated an upregulation of the mRNA levels in the human SCD erythroid cells were also upregulated in the bone marrow and spleen from the SCD mouse model (e.g., STAB1, GNG4, FBF1). These results suggest that SCD disease might share some common molecular mechanisms in both human cells and mouse model.

Despite the limitations of our small, hospital-based cohort, the findings stress a difference in genetic expression for HU-treated and non-treated SCD samples. Limitations of this study include small sample size, the inclusion of SCD patients recruited at a hospital only, lack of a validation by a cross a cross-over treatment design and lack of a validation by a semiquatitative protein detection methods like western blot. Our restriction to peripheral blood transcriptomes and ex-vivo erythropoiesis limited possible expression comparisons to other transcriptomic studies. Still, our analysis contains a new

subset of genes identified for the first time as associated with SCD. These findings encourage future characterization of their roles in SCD and provide more insights into relevant mechanistic pathways.
In conclusion, our study could identify new genes and pathways involved in the SCD pathophysiology that might be influenced by HU treatment. Importantly, this transcriptomic analysis could serve to identify putative biomarkers of SCD disease and could indicate treatment response parameters in HU treated patients[66,67].

Contributions

The study was conceived and coordinated by FDF and MS. The experiments were designed by MS, DS, FV and FDF. The experiments were conducted by MT, DS, FV and FDF. Bioinformatic analysis was conducted by LO, MT and FDF. Data analysis was conducted by MT and FDF. The project was supervised by FDF and MS. The manuscript was drafted and refined by MT and FDF with contributions from all authors.

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Figure 1: Transcriptomic profiles of SCD HU-treated, SCD non-treated and healthy (HC) patient-derived erythroid cells. A) Volcano plot of 1398 selected DEGs showing up- and downregulated genes in SCD non-treated over HC; B) Volcano plot of 495 selected DEGs showing up- and downregulated genes in SCD HU-treated over HC; C) Volcano plot of 786 selected DEGs showing up- and downregulated genes in SCD HU-treated over SCD non-treated.

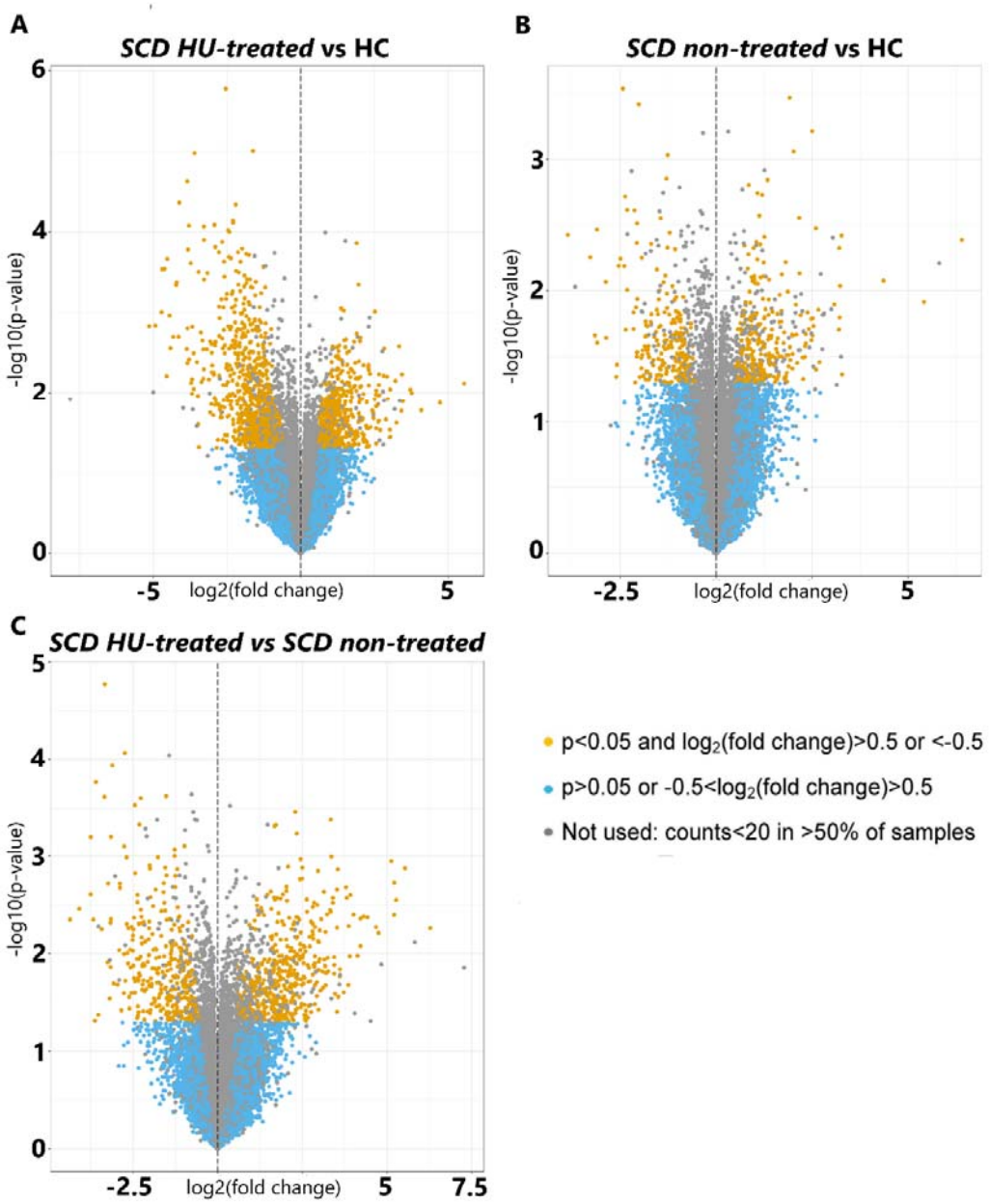
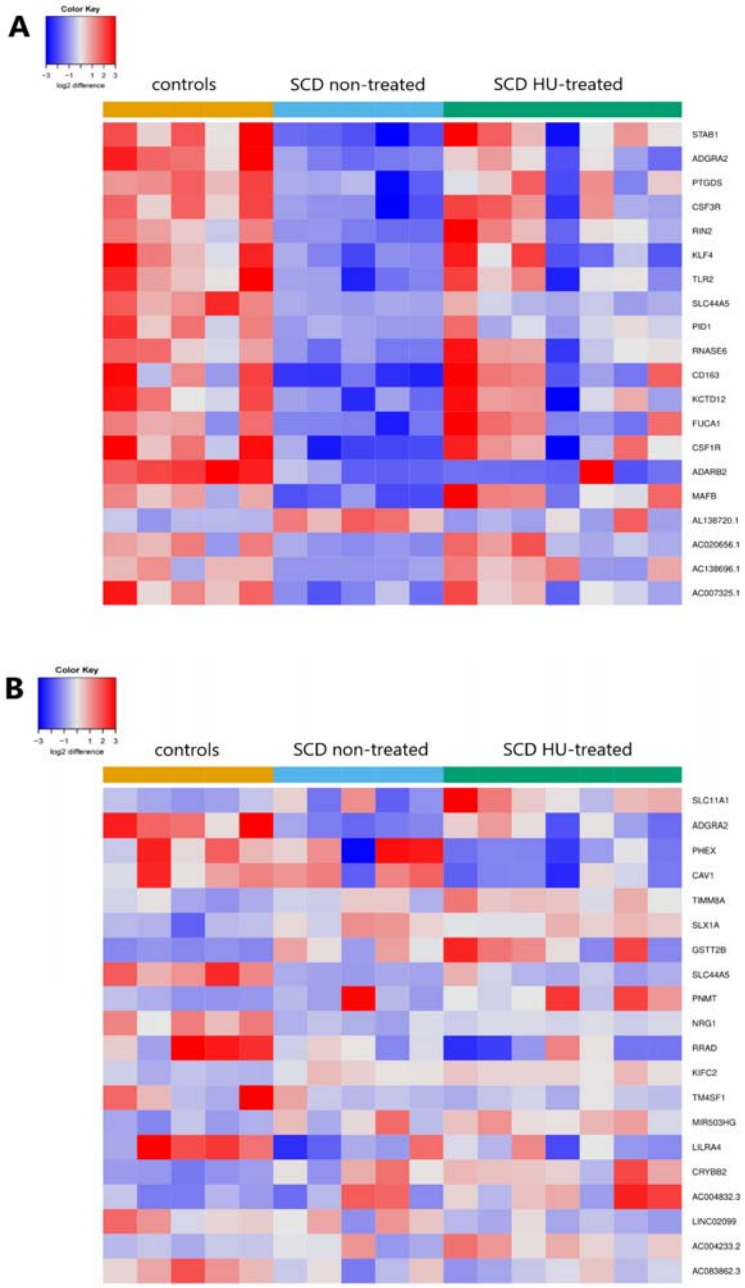


Figure 2: Heatmaps representing expression levels of top 30 most significant DEGs in A) SCD non-treated versus healthy controls B) SCD HU-treated versus healthy controls C) SCD HU-treated versus SCD non-treated. The colours keys indicate the log2 difference relative to the average expression of all samples within the comparison: in blue are shown downregulated genes and in red are shown upregulated genes. The dendrogram on the left y-axis shows clustering of the genes.



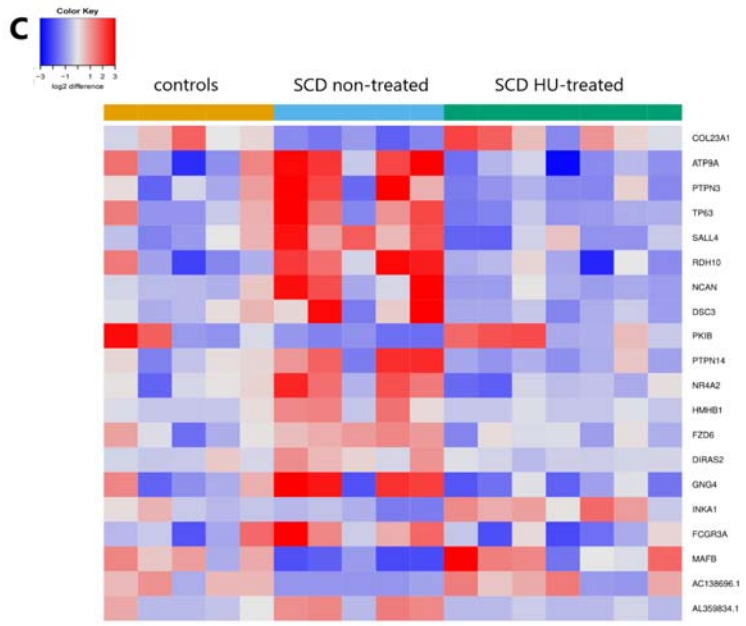
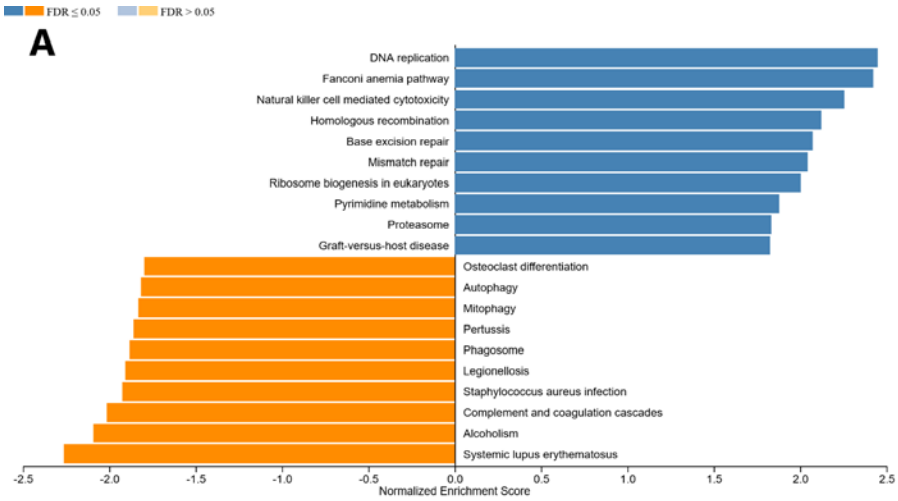
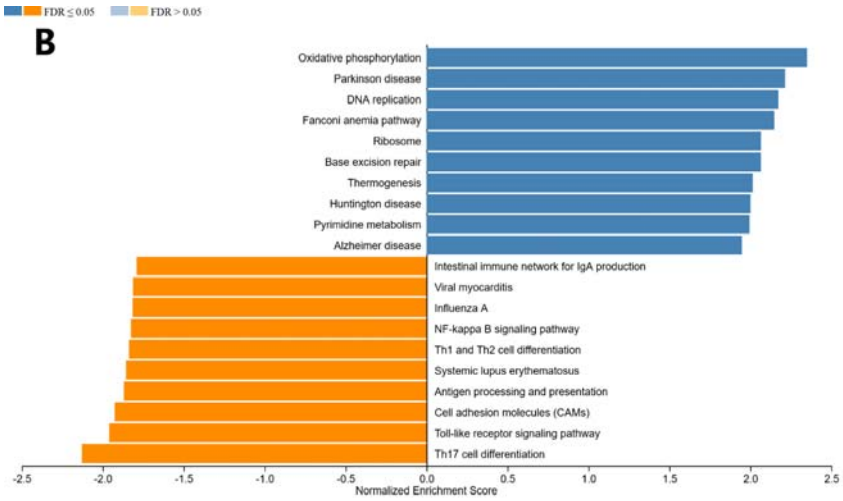


Figure 3: Bar chart depicting most significant differentially expressed pathways in our study groups. A) SCD non-treated over HC. B) SCD HU-treated over HC. C) SCD HU-treated over SCD non-treated based on FDR.




FDR ≤ 0.05
FDR > 0.05
B
Oxidative phosphorylation
Parkinson disease
DNA replication
Fanconi anemia pathway
Ribosome
Base excision repair
Thermogenesis
Huntington disease
Pyrimidine metabolism
Alzheimer disease
Intestinal immune network for IgA production
Viral myocarditis
Influenza A
NF-kappa B signaling pathway
Th1 and Th2 cell differentiation
Systemic lupus erythematosus
Antigen processing and presentation
Cell adhesion molecules (CAMs)
Toll-like receptor signaling pathway
Th17 cell differentiation
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
Normalized Enrichment Score

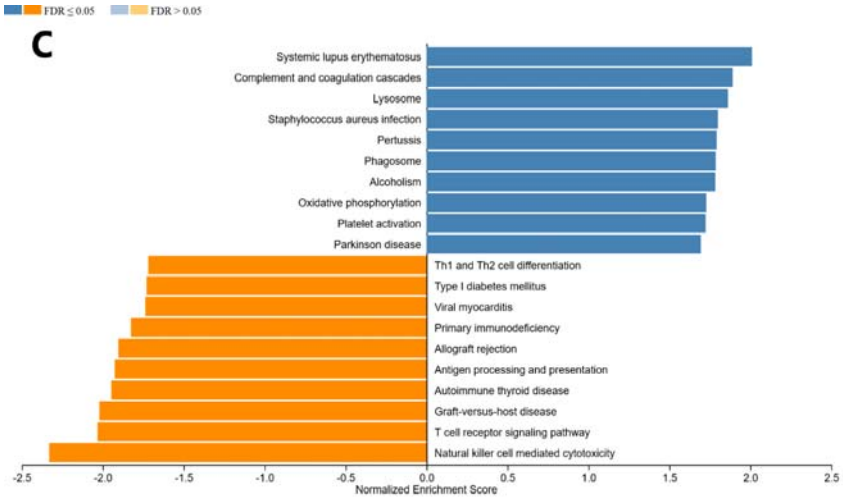

FDR ≤ 0.05
FDR > 0.05
C
Systemic lupus erythematosus
Complement and coagulation cascades
Lysosome
Staphylococcus aureus infection
Pertussis
Phagosome
Alcoholism
Oxidative phosphorylation
Platelet activation
Parkinson disease
Th1 and Th2 cell differentiation
Type I diabetes mellitus
Viral myocarditis
Primary immunodeficiency
Allograft rejection
Antigen processing and presentation
Autoimmune thyroid disease
Graft-versus-host disease
T cell receptor signaling pathway
Natural killer cell mediated cytotoxicity
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
Normalized Enrichment Score

Figure 4: Transcriptomic results for our top significant genes. Colour code: SCD non-treated in orange; SCD HU-treated in blue, HC in green. A significance threshold of 0.05 and a fold change threshold of 0.05 were used for the grouping. The y-axis shows -log10 (P-value) and the x-axis shows log2 (ratio) values.

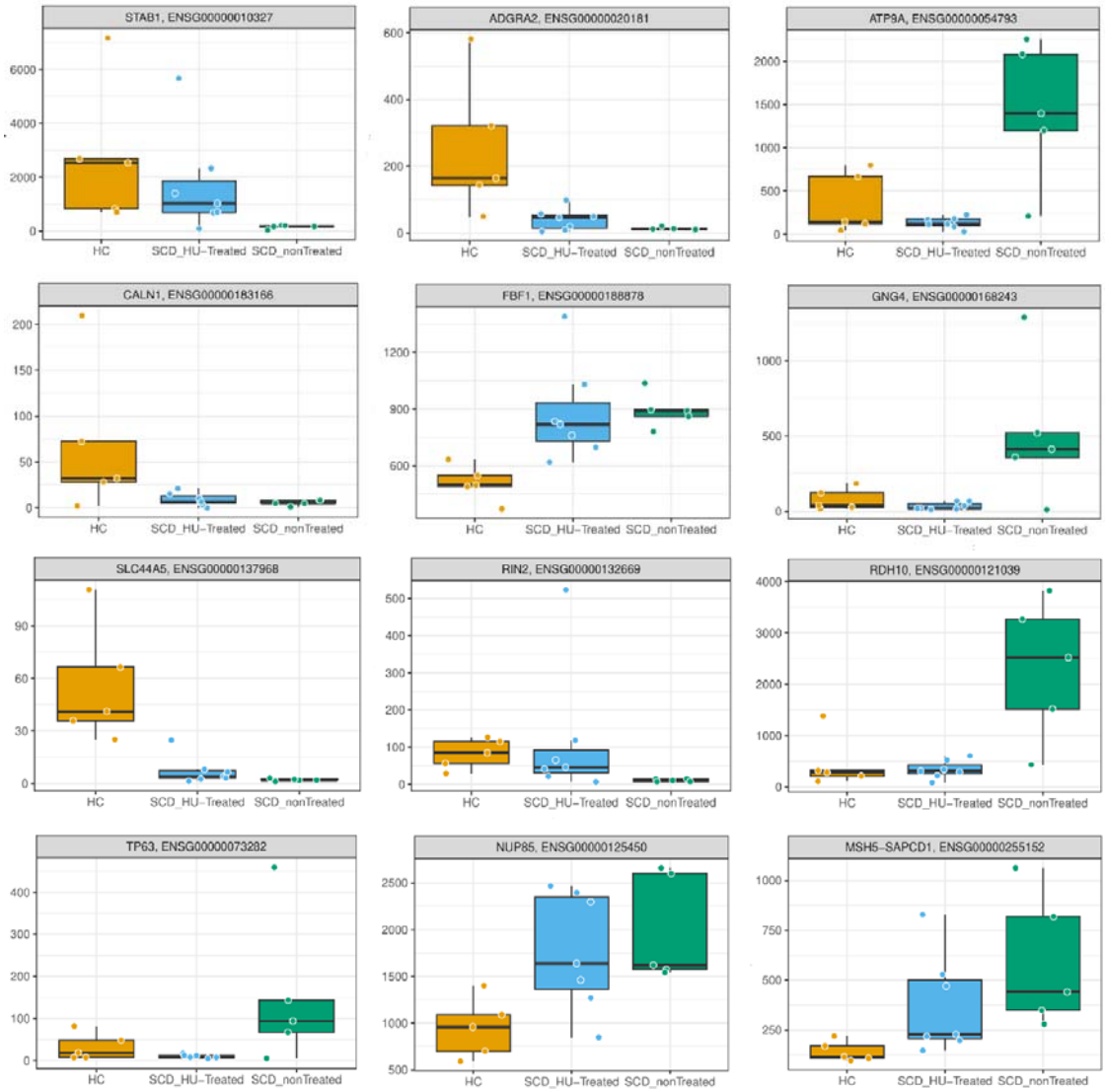


Figure 5: qRT-PCR validation for the RNA-sequencing results for our top selected genes. Gene expression was measured in four independent replicates per subject, and t-tests were performed for statistical analysis. Data are expressed as mean ± SEM.

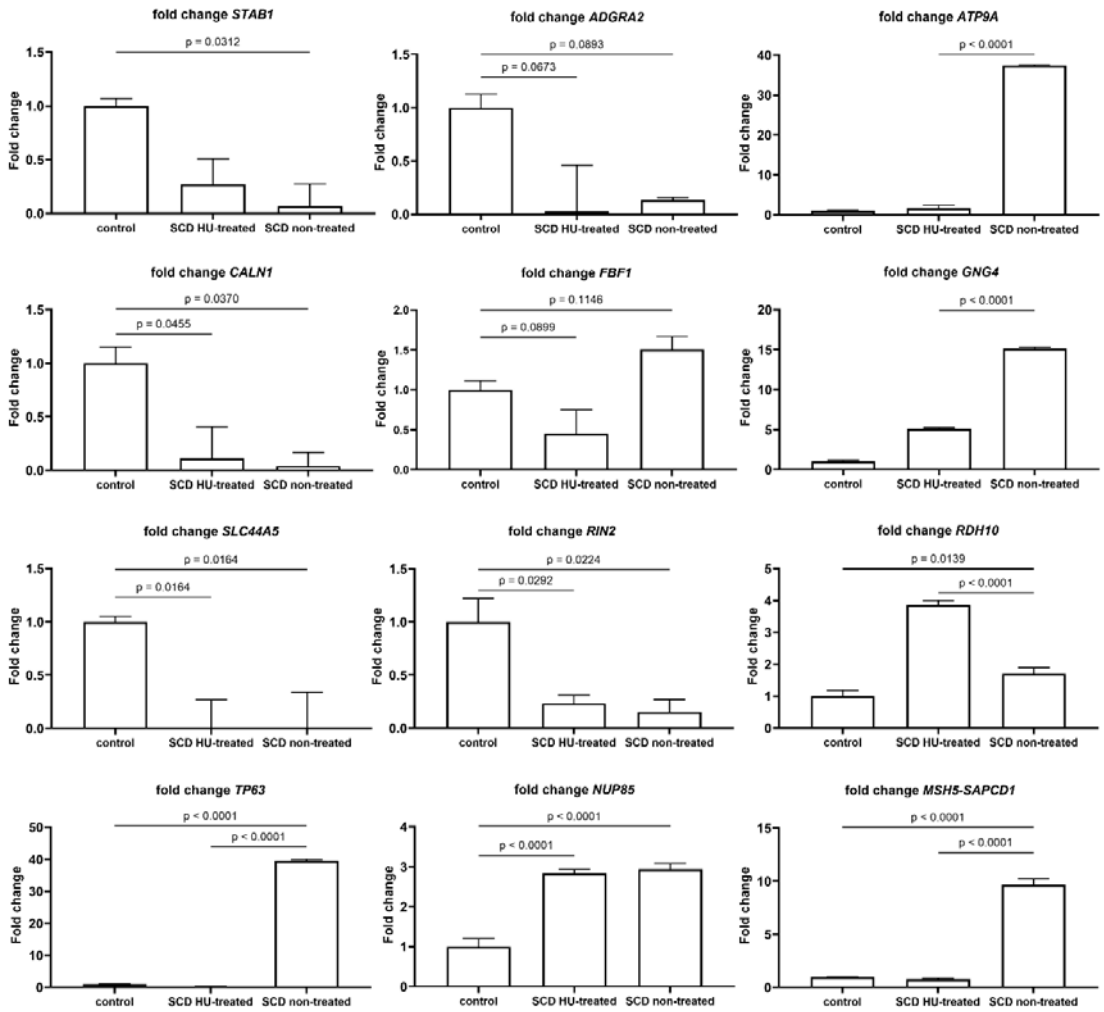


Figure 6: qRT-PCR results for our top selected genes in the bone marrow of SCD mouse model compared to wild-type mouse. Gene expression was measured in four independent replicates per subject, and t-tests were performed for statistical analysis. Data are expressed as mean ± SEM.

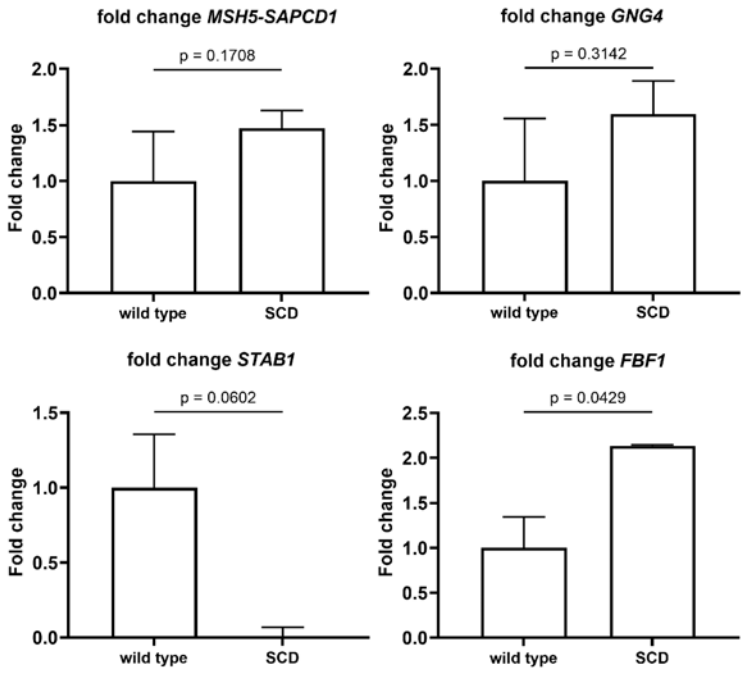


Figure 7: qRT-PCR results for our top selected genes in the spleen of SCD mouse model compared to wild-type mouse. Gene expression was measured in four independent replicates per subject, and t-tests were performed for statistical analysis. Data are expressed as mean ± SEM.

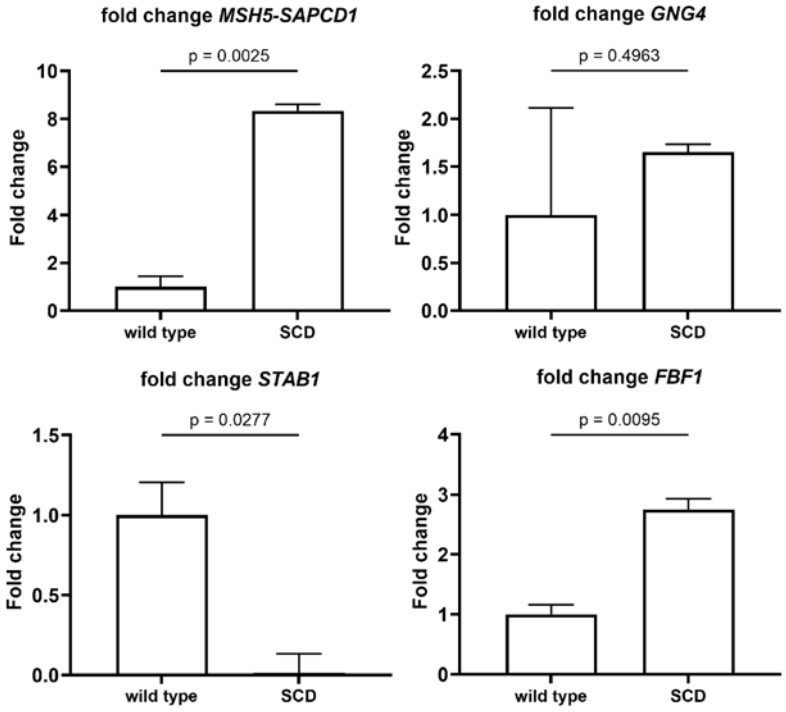


Table 1: Demographics and haematological routine parameters (means) in SCD patient groups and healthy controls

Parameter	Healthy controls (N=5)	SCD patients (N=5)	HU-treated SCD patients (N=7)
Age (years)	8.0 ± 3.28	10.0 ± 6.05	12.0 ± 6.8
Sex	2 F/ 3 M	5 F/ 0 M	1 F / 6 M
SCD Genotype (Hemoglobin SS, SC, SB+)	na	HbSS	HbSS
Hemoglobin (g/dL)	116.22	91.18	88.71
White blood cell count (x10 3/L)	8.80	8.19	10.96
Mean platelet count (x10^9/L)	305.00	317.27	333.57
Mean absolute monocyte count (%)	7.76	9.55	9.13
Mean absolute neutrophil count (%)	43.16	40.18	50.60