Whole blood transcriptome profiling in juvenile systemic sclerosis patients reveals active immune upregulation and enhanced fibrotic signature

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Whole blood transcriptome profiling in juvenile systemic sclerosis patients reveals active immune upregulation and enhanced fibrotic signature

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University of Pittsburgh, 2024

Juvenile Systemic Sclerosis (jSSc) is a rare chronic autoimmune disorder with understudied pathogenesis, characterized by skin thickness and multisystem organ involvement, associated with high morbidity. The University of Pittsburgh Medical Centers (UPMC) Children’s Hospital of Pittsburgh hosts a Pediatric Scleroderma Center (Torok Director) allowing the collection of blood for the biorepository paired with clinical phenotypic data to start to identify the factors associated with disease in jSSc.

RNA sequencing was performed on peripheral blood samples of jSSc (n=22) with age/sex matched healthy controls (n=17) using Illumina NovaSeq6000. Data was analyzed in Partek software and DESeq2. Differentially expressed genes (DEGs) were defined based on with a significance cut off (false discovery rate ≤0.05) and fold change of ≤1.5 or ≥ 1.5. Principal component and t-distributed stochastic neighbor embedding analyses were used to cluster samples based on gene expression profiles. Further analysis was conducted to determine pathway enrichment and dysregulated pathways

DEseq2 analysis identified 263 DEGs comparing jSSc with HC. Upregulated genes included several protein coding genes which play roles in collagen, extracellular matrix (ECM) formation, and adaptive immune responses. All findings have important roles in connective tissue disorder. Gene enrichment analysis showed activation in inflammatory, adaptive immune response pathways in jSSc.
As a result, jSSc transcriptional profile provides valuable insights into the gene expressions patterns in immune cells. It demonstrated upregulation of genes highlighting their roles in pathogenesis of jSSc involving fibrosis formation, vasculopathy, and dysregulation of immune cells
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Preface

Special thanks to my thesis advisor Dr. Kathryn S. Torok for giving me the opportunity to work on this project. It was a privilege to work with such a kind, knowledgeable and supportive person. Thanks to Anwesha and Theresa and all Torok lab members who helped and guided me in this research project.

I am proud of the challenging work during my master program journey. It was a challenge for me as a wife and mother of two children with full time job to pursue my master’s degree and this would not have been possible without the support of my professors, classmates, program advisor, and job flexibility. Thanks for the Health Science Sequencing Core at Pittsburgh children’s hospital where I learned about Next generation sequencing technology as a Research staff and motivated me to conduct bulk RNA sequencing study for my master thesis. I would like to thank all mentors who guided me in this study, especially Dr. Urban who has been supportive and helped me in my essay writing. Thanks to my academic advisor, Dr. Roman, who guided me throughout the program. Thanks to the Health Science Library staff for offering great analysis workshops. Special thanks to Sri Chaparah for helping and always being available for consultation hours.

Special thanks to my family specially my husband and our kids for the support and engorgement during this journey. I wish my parents were alive to share with them my achievement. Their unlimited love guided me through this journey. God bless their soul.

Thank you all.
1.0 Introduction

Systemic sclerosis (SSc) is a rare complex disease with multi organ involvement characterized by vasculopathy, fibrosis, and immune system dysfunction caused by autoimmunity. The resulting vascular damage triggers excessive buildup of extracellular matrix (ECM) proteins by the fibroblasts. Also, abnormality in keratinocytes leads to the accumulation of collagen resulting in skin hardening and other organ system failures, which ultimately is the main cause of mortality in SSc.

Only a handful of whole blood transcriptome analysis studies have been performed in patients with SSc. Since immune dysregulation is an early event in SSc, discovering the gene expression pattern in circulating white blood cells will provide relevant insights into the disease pathogenesis. In this study, we performed RNA-Seq analysis on cells derived from whole blood of pediatric patients and healthy control samples to determine the differential expression of genes in the two groups. Further gene set enrichment and pathway analyses were also performed among the highly significant genes to determine the biological pathways, which may play a crucial role in jSSc pathogenesis.

1.1 Overview of Juvenile Systemic Sclerosis

Systemic sclerosis is a rare chronic autoimmune disorder of connective tissues that cause fibrosis in the skin (characteristic skin thickening) and other internal organs. It is a disorder that affects children and adults. Like the adult-onset disease, jSSc is a multisystem disease, involved
in many of the body organs such as skin, blood vessels, lungs, and gastrointestinal tract [1]. Early symptoms in jSSc disease include thickening and tightening of the skin, stiff joints, and telangiectasias (small-widened blood vessels) [2]. In addition, this disease is further characterized by 3 subtypes: diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc), and overlap SSc. Distinguishing limited and diffuse SSc is based on the distribution of skin involvement. Skin thickening travels distal (fingertips/toes) to proximal (upper arms/legs and trunk), and once crosses proximal to the antecubital fossa and/or popliteal fossa, it is considered dcSSc [3]. Detection of autoantibodies such as anticentromere, anti-topoisomerase I, and anti-RNA polymerase III are useful to complement clinical observations in the diagnosis of SSc [4]. A combination of cutaneous involvement and autoantibody status can help predict organ involvement, for example dcSSc and positive topoisomerase antibody have a strong propensity towards developing interstitial lung disease (ILD) and these patients should be monitored closely for these clinical developments [5]

The most common organ system involved in jSSc is the vascular system, with approximately 95% of patients demonstrating Raynaud phenomenon, which is often the first clinical manifestation of this disease, and two-thirds having digital tip ulcers from repeated ischemia. In scleroderma, blood vessels lose their thermoregulatory control, which is often a result of excessive vasoconstriction in response to cold exposure and become smaller with reduced oxygen and nutrient supply to the skin causing discoloration of the digits from pink to white. These vascular changes can be observed by microscopic examination of the nailfold capillaries of the fingers and toes, which demonstrate abnormal architecture and blunting [6].

The second most impacted organ system in jSSc is gastrointestinal tract (GI), which includes esophagus (most frequently affected), stomach, small and large intestine [7]. Excessive
collagen deposition and fibrosis of the gut wall causes smooth muscle and neuronal atrophy, which interferes with normal motility to propel food through the GI tract, resulting in reflux, nausea, and bloating. As a result of this dysmotility, more complications can develop such as delay in gastric emptying (gastroparesis), bacterial overgrowth in the small intestine, and failure to thrive (growth delay) in children [8].

Interstitial lung disease (ILD) results from inflammation driven fibrosis in SSc and is the lead cause of mortality in these patients causing respiratory insufficiency. ILD occurs in 40% of both adult and pediatric onset cohort studies and recent guidelines support the active screening for this process with CT chest imaging early in disease [9]. Another lung-related and vascular-related phenomenon that occurs in SSc is pulmonary arterial hypertension (PAH), in which fibrosis in the lung causes narrowing of the pulmonary arteries, raising the pressure in the lung circulation and trouble with gas exchange, especially during exercise (increased cardiac demand) [10].

1.2 Epidemiology

Systemic sclerosis is a rare autoimmune disease which affects adults and children, with only 5% of all cases being pediatric onset. The average age of onset in the adult population is 45 years old, and in the pediatric population it is 8 years old. In both age spans, it is female predominant, with a female: male ratio of 4:1 in children and 7:1 in adults. The incidence rate of jSSc is 0.27 to 1 per million children in Europe and United States populations [11]. A recent study in the United States demonstrated the prevalence rate is 3 per 1 million children, estimating 250 to 300 children with jSSc [12]. The racial group characterization of jSSc in the largest international cohort study reports 83% White predominance [13]. The morbidity of jSSc compared with adults
has a better outcome due to lower rate of major visceral organ involvement. About 60% of jSSc deaths are within 5 years of the disease course [14].

1.3 Pathogenesis

SSc is multisystem autoimmune disease resulting in accumulation of fibrosis through a common pathologic cascade across many organs. SSc develops through common pathologic cascades across multiple organs and organs in addition to organ-specific pathologies making it a systemic disease [15]. The pathogenesis of pediatric SSc is extremely understudied since it is a much rarer disease than adult SSc. Since adult and juvenile scleroderma patients are characterized by similar clinical manifestations, such as skin findings, vasculopathy manifestations, fibrosis, and autoantibodies, it is assumed that the pathogenetic mechanism of adult SSc and jSSc are probably shared. In general, autoimmune disease is characterized by abnormality of innate and adaptive immune cells. The immune system is unable to distinguish between our cells and foreign invaders and attack normal components of the body. This immune system dysregulation causes vascular injury, which triggers inflammation, and subsequently resulting in activation of myofibroblasts to produce excessive collagen and extracellular matrix deposition resulting in fibrosis in many organs (Figure 1) [4].

Vasculopathy is the first indicator of pathology in SSc, occurring early at disease onset. Structurally, both the tunica interna (the inner lining) and tunica media (the muscular area layer) of the blood vessels become thick resulting in endothelial dysfunction that leads to Raynaud phenomenon, fingertip pitting scars and digital ulcers due to ischemic injury. Production of disease specific autoantibodies is an additional risk factor for vascular involvement. There are some
antibodies associated with elevated risk of PAH, such as anti-centromere anti-Th/To, anti-U1 ribonucleoprotein (RNP), anti-U3RNP antibodies, and anti-endothelial cell antibodies. Autoantibodies are produced by loss of self-tolerance of B cells and mediated auto-inflammatory process, promoting the clinical manifestation of vasculopathy [16].

T cell and B cell hyperactivation had been linked to the production of autoantibodies, microvasculopathy, inflammatory infiltrate and fibrosis. The overexpression of the receptor CD19 in B cells and loss of the activity of CD22 which downregulates B cell activation, are indicators of B cell dysfunction. In addition, B cells function as antigen-presenting cells (APCs) for CD4 and CD8 T cells. Also, B cells induce dendritic cell maturation, which promotes the production of the profibrotic cytokines IL-4 and IL-13 [17].
1.4 Current Treatment

jSSc is a life threatening disease that is associated with poor prognosis and incomplete understanding of the disease pathogenesis. Therapy for jSSc is still a challenge due to lack of clinical trials and rarity of the disease. There is no cure for jSSc but some FDA (Food and Drug Administration) approved medications have helped manage and control the disease in adults. Therapy in juvenile one set is based on adult treatments[18]. All therapeutic trials that have been done on SSc have usually been towards immunomodulating or anti-fibrotic targets.
Several studies show evidence of involvement of B cells in SSc pathogenesis. Abnormality of B cells and infiltration in skin has been detected in SSc patients. Rituximab (RTX) has been shown to be an effective treatment for SSc in SSc-ILD in children. RTX is a B-cell depletion therapy. It is a monoclonal antibody against the CD20 antigen on B lymphocytes. This treatment improves skin fibrosis and lung involvement in SSc patients [19]. Another monoclonal antibody treatment is Tocilizumab which has an immunosuppressor function and has been used in adult SSc. This medicine has anti-inflammatory and anti-fibrotic effects by binding and inhibiting the IL-6 receptor [18].

Intracellular signaling is also involved in SSc pathogenesis inducing the production of fibrotic extra cellular matrix (ECM) in the skin and various body organs. Certain protein kinases play a role in that process. Tyrosine kinases (TKs) drive the profibrotic effect in the signaling pathway of TGF-β [20]. Tyrosine kinases inhibitors (TKI) such as Imatinib are used as anti-fibrotic therapy. Signaling of Janus Kinase/signal transducer and activation of transcription (JAK/STAT) mediates the inflammation response in the transduction of cellular signals and induces fibrosis. Inhibitors of (JAK/STAT) pathway prevent fibrosis in many organs. Tofacitinib is JAK3 inhibitor that is effective in the treatment of skin fibrosis and ILD-SSc [21].

Hematopoietic stem cell transplants (HSCT) have been used as an intervention for treating severe cases of SSc [22]. This approach is still limited in pediatric patients due to the elevated risk of the procedure [23].
1.5 Genetics and Molecular Features of SSc

SSc is a complex disease caused by genetics and environmental factors. However, twin concordance risk is relatively low (4.7% in both monozygotic and dizygotic twins), indicating a relatively small genetic risk [24]. Having a first degree relative with SSc, increases the risk of having SSc by 10-16-fold [25]. In addition to genetic concordance, anti-nuclear antibodies (ANAs) were compared in the twin study, resulting in significantly higher concordance of ANAs in monozygotic than dizygotic twins, despite the fact that the MZ twins were largely discordant for SSc. Thus, just having a genetics background or relatives with SSc is not sufficient to predict whether there will be SSc disease development in the individual, it just predisposes the individual to higher risk of autoimmunity [26].

Genome wide associations studies (GWAS) have pinpointed single nucleotide polymorphisms (SNPs) in interferon regulatory factors (IRF1, IRF5, and IRF8) which are susceptibility genes for other autoimmune diseases as well. Interferon regulatory factor 5 (IRF5) is a transcription factor that controls the expression of proinflammatory cytokines such as interleukin IL-6, IL-12, and IL-23, and tumor-necrosis factor (TNF)-α which are all produced by activated macrophages [25]. In addition, candidate genetic association studies in SSc revealed other intronic variants such as TNF-α-induced protein 3 (TNFAIP3), known as A20 protein. The reduced activity of A20 protein results in increasing collagen production [27]. Interestingly, the identified genes are shared among other autoimmune diseases, such as systemic lupus erythematosus [28].

Other gene analysis and genome-wide association (GWAS) study have been conducted to assess polymorphism (SNP) in related genes loci that may play a role in the disease pathways. A study identified association with SSc predisposition in gene loci HLA especially HLA-DPB1 and
DPB2 in SSc patients positive for anti-topoisomerase I antibodies. There are some contradictions about the association of HLA genes with SSc as it may be related to autoantibodies rather than SSc itself. Other SNP detected in this study was found to be located upstream of gene peroxisome proliferator-activated receptor gamma (PPARG), a gene that plays a role in collagen suppression as an anti-fibrotic effector [29].

Regarding pro-fibrotic genes, a screening study on the exons of gene fibrillin 1 (FBN1) was conducted in 2001 using genomic DNA in polymerase chain reaction-based fluorescence to identify SNPs in FBN1 gene. This gene encodes a major component of microfibrils in the ECM. The study consisted of SSc patients from Choctaw and Japanese population. The study detected SNPs in the exonic and intronic region of the targeted population, which were associated with susceptibility to SSc [30].

Overall, the literature suggests that the genetic variants that are related to dysregulation of immune system are linked to SSc susceptibility. However, only few genes are related to fibrotic and vascular process in SSc and there are environmental factors and epigenetic influences contribute to the development of SSc [25]

1.6 Relevance To Public Health

Juvenile-onset systemic sclerosis (jSSc) is a rare and severe disease often associated with mortality. Delay in diagnoses and treatment can lead to organ damage with high fatality. jSSc is an aggressive disease with multiple organ participation. Identifying differentially expressed genes and pathways in jSSc may identify new biomarkers and pathological processes helping improve the diagnosis and treatment of pediatric scleroderma. On a larger scale, identifying genes and
dysregulated pathways promoting fibrosis could lead to better treatments for multiple fibrotic
diseases, such as idiopathic pulmonary fibrosis, nephrogenic systemic fibrosis, and graft versus
host disease [31]
2.0 Rationale

Systemic sclerosis (SSc) is a heterogeneous and complex disease. It is characterized by inflammation, vascular injury, fibrosis, and internal organ dysfunction. Clinically, skin involvement is the main unifying characteristic of SSc. The degree of skin thickness and its distribution are used to assign a value to the cornerstone outcomes measure in SSc, the modified Rodnan Skin Score (mRSS) ranged as total score (0 to 51) [32]. Several clinical trials in SSc anchor the effectiveness of a medication on the ability to clinically improve the mRSS (lower it) [33]. An improved mRSS also correlates with a change in the inflammatory gene expression in a clinical study treating SSc with mycophenolate mofetil, with skin biopsies pre- and post-treatment [34]. In addition to change in molecular signature with treatment, the baseline skin gene expression patterns in SSc can be divided into 4 essential subgroups: fibroproliferative, limited, inflammatory, and normal like. These gene expression profiles (molecular subgroups) have been shown to provide biomarkers of disease and to be predictive of treatment response. This same group had been able to convert the skin molecular phenotype to peripheral blood signature and have applied it to clinical trials. The autologous stem cell study (SCOT) comparing cyclophosphamide to HSCT, demonstrated that SSc patients with baseline fibroproliferative peripheral blood profile responded better to HSCT than the inflammatory subtype [35]. Whitfield et al. (2020) paper focuses on using machine learning in overlaying skin transcriptome with peripheral blood. This study approach is an advantage to make peripheral blood marker easier for clinical approach [35]. A European based study found that whole blood transcriptome profiling of SSc using RNA-sequencing analysis reveals differential expression of genes that had roles in the pathogenesis of the disease [36].
In a similar fashion, we propose that RNA sequencing analysis on peripheral blood cells of juvenile onset of systemic scleroderma (jSSc) patients will provide intrinsic molecular subsets of jSSc in addition to biomarkers of interest. Single-cell sequencing (scRNA-seq) in jSSc is being conducted in Torok lab to obtain detailed profile of cell population that can be paired to the analysis of peripheral blood study.
3.0 Specific Aims

Juvenile systemic sclerosis (jSSc) is a complex multiorgan disease with limited understanding of its pathogenesis and deficiency of reliable biomarkers of disease severity, which hinders the development of effective treatments. Transcriptome profiling of peripheral blood cells offers a valuable approach, providing critical insights into gene expressions patterns in immune cells and their contribution to the inflammatory and fibrotic processes observed in JSSc.

**Aim 1:**

**Objective:** To identify the differentially expressed genes (DEGs) between jSSc patients and age-matched healthy controls using bulk RNA sequencing (RNA-seq) of peripheral blood.

**Hypothesis:** We hypothesize that genes associated with inflammation, such as interferon (IFN)-related genes, and fibrosis, such as collagen (COL)-related genes, will be significantly upregulated in jSSc. Conversely, genes involved in homeostasis and tissue repair will be downregulated.

**Aim 2:**

**Objective:** To elucidate gene sets enrichment and key cellular pathways involved in jSSc by performing Gene Set Enrichment Analysis (GSEA), and Ingenuity Pathway Analysis (IPA) on the identified DEGs.

**Hypothesis:** We hypothesize that pathways related to immune response and inflammation, such as cytokine signaling and TLR pathways, will be upregulated, while pathways associated with cellular homeostasis and repair, such as those involving extracellular matrix organization and cellular adhesion, will be downregulated.
4.0 Methodology

4.1 Study Cohort and Demographics

Blood samples were collected in Tempus and Paxgene blood RNA tubes from juvenile systemic scleroderma pediatric patients and healthy age-matched controls through the National Registry for Childhood Onset Scleroderma (NRCOS). The NRCOS is a single-center, prospective cohort study conducted at the University of Pittsburgh Medical Center (UPMC) Children’s Hospital. jSSc patient inclusion criteria were as follows: 1) age < 18 years at onset; 2) SSc diagnosis using the American College of Rheumatology (ACR)/European League of Rheumatology (EULAR) classification criteria; 3) enrollment in the NRCOS cohort before age 21. One physician (K.T.) collected data prospectively at the clinical visits that are paired with the blood sample. Informed consent was obtained for all participants through the University of Pittsburgh IRB# STUDY19080297. The data collection process utilized by the NRCOS cohort includes demographic information, physical examination, clinical testing variables, and patient reported outcomes. Clinical variables collected include a comprehensive extra-cutaneous manifestation screening, nailfold capillary assessment, and skin scoring utilizing the mRSS.

4.2 RNA Extraction and Sequencing

Total RNA was extracted from whole blood samples using Qiagen PAXgene tubes and Tempus tubes (PreAnalytix by QIAGEN). RNA samples concentrations were quantified using
Qubit FLEX fluorometer and the quality was determined using fragment analyzer 5300. RNA samples were of high quality with an RNA integrity number range from 7-9.

Extracted RNA was prepared for sequencing using Illumina Stranded Total Library Prep with Ribo-Zero Plus kit (Illumina: 20040529). The library generation steps shown in (Figure 2), including:

- DNA probe hybridization to deplete abundant rRNA and globin transcripts.
- Purification of rRNA-depleted RNA
- RNA fragmentation and priming for cDNA synthesis
- Adenylate 3’ ends
- Adapter ligation
- PCR amplification and indexing

For sequencing, libraries size and quality were checked using Fragment Analyzer (Agilent) and quantification was performed by qPCR (Kapa qPCR quantification kit, (Roche)) on the LightCycler 480 (Roche). The libraries were normalized and pooled, and then sequenced using NovaSeq6000 platform (Illumina) to an average of 40 million 100 base-pair (paired-end reads).
Figure 2. Illumina Stranded Total RNA Prep with Ribo-Zero Plus.

https://www.illumina.com
4.3 RNA Analysis Pipeline

Bulk RNA sequencing analyses were performed using Partek flow software as shown in (Figure 3). First, fastq files were uploaded to Partek server and ran through reads quality check to check the average of base quality score (>20). Reads that passed quality check were aligned to reference genome using STAR. We ran post-alignment QA/QC to make sure more than 95% of the reads were aligned. We quantified aligned reads to annotation model (Partek E/M) to get gene and transcript counts. Next, we applied a filter to eliminate genes with low read counts (<25) to increase the sensitivity of differential gene expression analysis.

![Diagram of Partek Pipeline](image)

**Figure 3. Partek Pipeline.**

Created with BioRender.
5.0 Statistical Analysis

Variables of the clinical measurements are summarized using the mean of the dataset. Sequencing analysis included DESq2 in Partek software for DEG analyses. DE gene list was determined based on cut off using False Discovery Rate (FDR= < 0.05) and fold change of ≤1.5 or ≥ 1.5. This cut off has been used in many RNA-seq studies, in particular studying the transcripts expression of SSc skin [37]. Principal component Analysis (PCA) and t-distributed Stochastic Neighbor Embedding (t-SNE) are used to visualize clustering differences in gene expression. Gene set enrichment analysis (GSEA) in partek was used to determine enriched gene sets. Further analysis was conducted to determine the activity of enriched pathways (activation vs inhibition) using Ingenuity Pathway Analysis (IPA).
6.0 Results

6.1 Study Cohort and Demographics

Patient demographics and clinical variables with prominent clinical manifestations of jSSc are summarized in Table 1. The majority of jSSc patients were female and White. The predominant subtype of jSSc was diffuse cutaneous and the average modified Rodnan skin score (mRSS) was 8, with one-third having a score >10. The cohort was representative of findings seen in the largest pediatric international cohort with 32% having interstitial lung disease and 60% with digital ulcers.
### Table 1. Study Cohort Demographic.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy controls (n=17)</th>
<th>JSSc patients (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, female, n (%)</td>
<td>13 (76)</td>
<td>20 (91)</td>
</tr>
<tr>
<td>Age at sample collection, years</td>
<td>12 (5-21)</td>
<td>9 (2-15)</td>
</tr>
<tr>
<td><strong>Ethnicity</strong>, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>13 (76)</td>
<td>17 (77)</td>
</tr>
<tr>
<td>African American</td>
<td>4 (23)</td>
<td>5 (23)</td>
</tr>
<tr>
<td><strong>Disease subtype, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>-</td>
<td>15 (68)</td>
</tr>
<tr>
<td>Limited</td>
<td>-</td>
<td>4 (18)</td>
</tr>
<tr>
<td>Unclassified</td>
<td>-</td>
<td>3 (13)</td>
</tr>
<tr>
<td><strong>Clinical manifestations, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ILD Ever</td>
<td>-</td>
<td>7 (32)</td>
</tr>
<tr>
<td>mRSS 10+ samples</td>
<td>-</td>
<td>7 (32)</td>
</tr>
<tr>
<td>mRSS (average)</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>Digital Ulcers</td>
<td>-</td>
<td>13 (59)</td>
</tr>
<tr>
<td>Ulceration and/or Necrosis</td>
<td>-</td>
<td>7 (32)</td>
</tr>
<tr>
<td>GI hypomotility</td>
<td>-</td>
<td>7 (32)</td>
</tr>
<tr>
<td><strong>Autoantibodies, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scl-70</td>
<td>-</td>
<td>12 (54)</td>
</tr>
<tr>
<td>ANA only</td>
<td>-</td>
<td>5 (23)</td>
</tr>
<tr>
<td>Centromere</td>
<td>-</td>
<td>3 (14)</td>
</tr>
</tbody>
</table>
6.2 Identification of DEGs in Juvenile SSc Compared to Healthy Controls

Differential expression gene analysis using DESeq2 was performed on peripheral blood samples of juvenile SSc and compared to healthy controls. Low expression gene counts were excluded, and 20,229 genes were used for further analysis. DE gene list was determined based on fold change cutoff of $\leq 1.5$ or $\geq 1.5$ and false discovery rate (FDR $< 0.05$). We identified 263 significant genes. Two hundred DEGs were up-regulated, and 63 DEGs were down-regulated. The list of top up-regulated and down-regulated DEGs is listed in Table 2.

Further analysis of DEGs using heat map clustering showed distinct gene expression differences in juvenile SSc compared to healthy controls. Heat map analysis demonstrates there is a subset of 6 jSSc patients with a more robust gene signature (Figure 4A).

Following volcano plot in jSSc compared to healthy controls. (Figure 4B). Principal component analysis on normalized data, t-distributed stochastic neighbor embedding (t-SNE) was performed to determine clusters based on gene expression similarity in each of the groups (Figure 4C). In addition, principal component analysis reveals gene expression segregations between jSSc and healthy control samples especially in the subsets of 6 patients (Figure 4D).
Figure 4. Transcriptome Analysis of the Whole Blood of Children with jSSc Compared to Healthy Controls.
Figure 4. Transcriptome Analysis of the Whole Blood of Children with jSSc Compared to Healthy Controls (cont.).

(A) Heat map clustering between all DEGs comparing jSSc to healthy samples. (B) Volcano blot between two groups. (C) tSNE analysis of genetic expression between two group. (D) PCA analysis of jSSc compared to healthy control.
6.3 Upregulated DEGs of jSSc Compared to Healthy Controls

RNA sequencing with deferential expressions analysis reveals gene signatures of 22 blood samples of jSSc compared to healthy controls. Our findings highlight upregulated/downregulated genes which contribute to disease pathogenesis and are associated with disease activity. 264 genes were found to be significant among jSSc verses healthy, of which 219 were identified to be protein coding genes. Up-regulated DE genes included membrane genes (AQP1, CLEC4E) and secreted protease genes (ADAMTS2, MMP9) (Figure 5). Aquaporin 1 (AQP1) gene encodes a membrane protein that functions as a water channel, facilitating the movement water molecules across cell membranes. Upregulation of AQP1 expression in peripheral blood cells could have effects in vascular function, fluid balance, and fibrosis in SSc [38]. The highest expression of AQP1 is in four patients with digital ulcers within the 6 jSSc patient subset. The CLEC4E gene codes for a plasma membrane protein receptor (C-type lectin domain family 4 member E) expressed as a part of the immune response in monocytes, macrophages, and neutrophils. It activates the cytokine production pathway. CLEC4E activation led to the production of pro-inflammatory cytokines and chemokines contributing to immune activation, inflammation, and fibrosis process. ADAMTS2 gene has the highest gene expression with fold change of 19 compared to healthy controls. It a encodes a key procollagen N-proteinase that is required for the maturation and fibril formation of type I, II, III and V procollagens. The expression of this gene is thus necessary for collagen accumulation as a part of fibrosis [39]. Matrix Metalloproteinases-9 (MMP9) gene is upregulated in whole blood patients. MMP9 is a matrix metalloprotease that can degrade a wide variety of ECM substrates, including several types of collagens and elastin. It is highly expressed by migratory cells and can activate pro-fibrotic signaling by releasing latent transforming growth factor beta from the ECM [40].
Upregulated inflammatory and immune responses genes are reported in my study including (TREM1, IL1R, TLRs, S100A12, LILRB5, LILRA6). These genes are highly expressed in jSSc subset group as it is presented in (Figure 6). TREM1 (Triggering Receptor Expressed on Myeloid cells 1) is a plasma membrane cell surface receptor primarily expressed on myeloid cells, such as neutrophils and monocytes [41]. It plays a key role in the regulation of innate immune responses. IL1R1 gene encodes for Interleukin 1 Receptor Type1. It is expressed in monocytes, and dendritic cells [42]. LILRB5 gene codes for the leukocyte immunoglobulin like receptor B5 inhibitory receptor, which helps in regulating immune responses [43]. Other significant upregulated genes were detected that reflect immune responses include Toll-like receptors (TLRs) genes including TLR2, TLR4, and TLR8. Peripheral blood of jSSc showed high expression of S100A12 gene compared to healthy controls. Gene count data of S100A12 expression is significantly correlated with mRSS skin score with Spearman’s Rho (rs) of 0.56002 p (2-tailed) = 0.00672. Elevation of S100A12 mRNA expression in SSc skin is associated with active disease parameters in diffuse cutaneous involvement and is positively correlated with skin findings [44].

Genes associated with macrophage activation were upregulated significantly in jSSc and include C5AR1, DYSF, and SLC11A1. Macrophage activation has been demonstrated in adult SSc and is thought to promote disease pathogenesis [45]. In adult SSc, macrophage protein1 (SLC11A1) has a function in macrophage activation pathways including interleukin-1b and MHC II expression. Altered expression of gene SLC11A1 has an impact on macrophage function and would contribute to abnormalities of antigen presentation [46].

Additionally, CD55 gene is upregulated in the whole blood of jSSc. Studies reported the expression of this gene on endothelial cells driven by intracellular adhesion molecule 2 (ICAM-2)
promoter and its expression is essential for maintaining the vascular integrity [47]. Dysregulation of CD55 lead to endothelial injury leading to vascular abnormality.
Figure 5. Upregulated Plasma Membrane and Secreted Protease Coding Genes Expressed Highly in Subsets of Patients.
Figure 6. Inflammatory and Immune Response Genes Upregulated in jSSc Compared to Healthy Controls.

Upregulated inflammatory and immune response genes expressed highly in subsets of patients.
Table 2. Top Upregulated and Downregulated DEGs.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>P-value</th>
<th>FDR step up</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS2</td>
<td>0.000005</td>
<td>0.008</td>
<td>19.500</td>
</tr>
<tr>
<td>IGF2</td>
<td>0.000000003</td>
<td>0.000</td>
<td>4.900</td>
</tr>
<tr>
<td>LILRB5</td>
<td>1 × 10^-9</td>
<td>0.000</td>
<td>3.870</td>
</tr>
<tr>
<td>MMP9</td>
<td>0.00008</td>
<td>0.022</td>
<td>2.750</td>
</tr>
<tr>
<td>CLEC4E</td>
<td>0.00002</td>
<td>0.016</td>
<td>2.480</td>
</tr>
<tr>
<td>S100A12</td>
<td>0.00029</td>
<td>0.030</td>
<td>2.470</td>
</tr>
<tr>
<td>TMEM63C</td>
<td>0.00001</td>
<td>0.016</td>
<td>2.330</td>
</tr>
<tr>
<td>SLC8A1-AS1</td>
<td>0.00005</td>
<td>0.022</td>
<td>2.320</td>
</tr>
<tr>
<td>IL1R1</td>
<td>0.00011</td>
<td>0.024</td>
<td>2.180</td>
</tr>
<tr>
<td>AQP1</td>
<td>0.00096</td>
<td>0.039</td>
<td>1.930</td>
</tr>
<tr>
<td>CLEC4D</td>
<td>0.00081</td>
<td>0.038</td>
<td>1.900</td>
</tr>
<tr>
<td>MEGF9</td>
<td>0.00004</td>
<td>0.022</td>
<td>1.880</td>
</tr>
<tr>
<td>TLR2</td>
<td>0.00073</td>
<td>0.037</td>
<td>1.870</td>
</tr>
<tr>
<td>IGF2R</td>
<td>0.00009</td>
<td>0.022</td>
<td>1.860</td>
</tr>
<tr>
<td>TREM1</td>
<td>0.00013</td>
<td>0.025</td>
<td>1.860</td>
</tr>
<tr>
<td>LILRA6</td>
<td>0.00081</td>
<td>0.038</td>
<td>1.850</td>
</tr>
<tr>
<td>SLC11A1</td>
<td>0.00005</td>
<td>0.022</td>
<td>1.810</td>
</tr>
<tr>
<td>TMEM252</td>
<td>0.00153</td>
<td>0.045</td>
<td>1.790</td>
</tr>
<tr>
<td>FOS</td>
<td>0.00048</td>
<td>0.033</td>
<td>1.741</td>
</tr>
<tr>
<td>CDA</td>
<td>0.00126</td>
<td>0.042</td>
<td>1.730</td>
</tr>
<tr>
<td>CCN3</td>
<td>0.00069</td>
<td>0.037</td>
<td>1.720</td>
</tr>
<tr>
<td>TMEM88</td>
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<td>0.030</td>
<td>1.720</td>
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<tr>
<td>TMEM30BP1</td>
<td>0.00129</td>
<td>0.042</td>
<td>1.690</td>
</tr>
<tr>
<td>TMEM164</td>
<td>0.00089</td>
<td>0.038</td>
<td>1.620</td>
</tr>
<tr>
<td>C5AR1</td>
<td>0.00091</td>
<td>0.038</td>
<td>1.610</td>
</tr>
<tr>
<td>LRRK2</td>
<td>0.00036</td>
<td>0.031</td>
<td>1.590</td>
</tr>
<tr>
<td>TLR8</td>
<td>0.00067</td>
<td>0.037</td>
<td>1.590</td>
</tr>
<tr>
<td>TLR4</td>
<td>0.00147</td>
<td>0.044</td>
<td>1.580</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>0.00068</td>
<td>0.037</td>
<td>1.530</td>
</tr>
<tr>
<td>CD55</td>
<td>0.00031</td>
<td>0.030</td>
<td>1.530</td>
</tr>
<tr>
<td>SPRY2</td>
<td>0.00093</td>
<td>0.039</td>
<td>-1.521</td>
</tr>
<tr>
<td>CCR5</td>
<td>0.00168</td>
<td>0.047</td>
<td>-1.580</td>
</tr>
<tr>
<td>CCR9</td>
<td>0.00126</td>
<td>0.042</td>
<td>-1.630</td>
</tr>
<tr>
<td>COL6A2</td>
<td>0.00000</td>
<td>0.004</td>
<td>-1.650</td>
</tr>
<tr>
<td>SLC4A10</td>
<td>0.00083</td>
<td>0.038</td>
<td>-1.890</td>
</tr>
<tr>
<td>IGHG3</td>
<td>0.00155</td>
<td>0.045</td>
<td>-1.910</td>
</tr>
<tr>
<td>S100B</td>
<td>0.00090</td>
<td>0.038</td>
<td>-2.280</td>
</tr>
<tr>
<td>IGKV1-16</td>
<td>0.00080</td>
<td>0.038</td>
<td>-2.570</td>
</tr>
<tr>
<td>IGLV7-46</td>
<td>0.00082</td>
<td>0.038</td>
<td>-2.620</td>
</tr>
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</table>
6.4 Downregulated DEGs

DEG analysis identified the significantly downregulated genes such chemokine receptors (SPRY2, CCR5, CCR9, COL6A2) (Figure 7). Downregulated SPRY2 gene is indicator of fibrosis in jSSc. Studies indicated the role of SPRY2 as a negative regulator of fibrosis [48]. Low expression of chemokines receptors (CCR5, CCR9) in peripheral blood of jSSc could be due to the migration of the CCR5-expressing cells to the affected areas that is producing chemokines ligands, which could lead to the accumulations of these cells in these tissues, contributing to chronic inflammation and depleting these cells in the blood [49].

Down-regulated genes pertaining to ECM production included a few collagen genes. COL6A2 was demonstrated in our jSSc set to be significantly lower in expression compared to healthy controls. COL6A2 has a role in modulating the fibrotic response in SSc pathogenesis via ECM regulation [50]. The downregulated expression of COL6A2 gene in peripheral blood of jSSc may have a role in imbalance ECM production and degradation and lead to fibrosis in jSSc.
6.5 Gene Set Enrichment Analysis (GSEA)

The enrichment pathways of the DEGs were determined by gene set enrichment analysis. We identified numerous statistically high enriched pathways that have roles in the pathogenies and of the disease (Figure 8). Gene set enrichment analysis identified gene sets that have overrepresentation in a filtered gene list and associated with biological context. Significantly enriched sets in a set of upregulated deferential expressed genes were determine in the analysis table (Table 3). Each pathway indicates the number of genes enriched in each pathway with statistical analysis of FDR and P-vale measurements. Collagen regulatory pathways (plasma membrane, collagen metabolic process) are highly enriched with significant P-value, which
includes genes $AQP1$, $CLEC4E$, $COL6A2$. Additional significant enriched gene sets including upregulated genes of jSSc are immune and inflammatory response pathways including (MHC class II biosynthetic process, positive regulation of cytokine production, innate and adaptive immune signaling). The above enriched pathways comprise high expression genes such as $ILR1$, $TLRs$, $TREM1$, $S100A12$. 
Figure 8. Top Over-Represented Enriched Gene Sets in jSSc Compared to Healthy Controls.
Table 3. Gene Set Enrichment Analysis (GSEA) on DEGs in jSSc Compared to Healthy Controls.

<table>
<thead>
<tr>
<th>Gene set</th>
<th>Description</th>
<th>Genes name</th>
<th>P-value</th>
<th>FDR step up</th>
<th>Genes in list</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0005886</td>
<td>plasma membrane</td>
<td><em>AQP1</em>, <em>CLEC4E</em>, <em>COL6A2</em>, <em>S100A12</em>, <em>IL1R1</em>, <em>TREM1</em>, <em>IRAK</em></td>
<td>$5 \times 10^{-11}$</td>
<td>0.000001</td>
<td>95</td>
</tr>
<tr>
<td>GO:0006954</td>
<td>inflammatory response</td>
<td><em>IL1R1</em>, <em>S100A12</em>, <em>CCR5</em>, <em>FOS</em></td>
<td>0.00003</td>
<td>0.0669</td>
<td>16</td>
</tr>
<tr>
<td>GO:0045342</td>
<td>MHC class II biosynthetic process</td>
<td><em>SLC11A1</em>, <em>TLR4</em></td>
<td>0.00010</td>
<td>0.1281</td>
<td>2</td>
</tr>
<tr>
<td>GO:0002274</td>
<td>myeloid leukocyte activation</td>
<td><em>C5AR1</em>, <em>S100A12</em>, <em>SLC11A1</em>, <em>TLR2</em>, <em>TLR4</em></td>
<td>0.00020</td>
<td>0.1664</td>
<td>7</td>
</tr>
<tr>
<td>GO:0006955</td>
<td>immune response</td>
<td><em>LILRB5</em>, <em>CD55</em>, <em>TREM1</em>, <em>CLEC4E</em>, <em>IL1R1</em>, <em>S100A12</em>, <em>TLR2</em>, <em>TLR4</em>, <em>TLR8</em></td>
<td>0.00031</td>
<td>0.2008</td>
<td>28</td>
</tr>
<tr>
<td>GO:0042116</td>
<td>macrophage activation</td>
<td><em>C5AR1</em>, <em>DYSF</em>, <em>SLC11A1</em>, <em>TLR2</em>, <em>TLR4</em></td>
<td>0.00036</td>
<td>0.2135</td>
<td>5</td>
</tr>
<tr>
<td>GO:0001819</td>
<td>positive regulation of cytokine production</td>
<td><em>CD55</em>, <em>IL1R1</em>, <em>TLR4</em></td>
<td>0.00038</td>
<td>0.2135</td>
<td>14</td>
</tr>
<tr>
<td>GO:0002682</td>
<td>regulation of immune system process</td>
<td><em>CCN3</em>, <em>CCR5</em>, <em>CCR9</em></td>
<td>0.00039</td>
<td>0.2135</td>
<td>29</td>
</tr>
<tr>
<td>GO:0002224</td>
<td>toll-like receptor signaling pathway</td>
<td><em>IRAK3</em>, <em>TLR2</em>, <em>TLR4</em>, <em>TLR8</em></td>
<td>0.00042</td>
<td>0.2179</td>
<td>47</td>
</tr>
<tr>
<td>GO:0005576</td>
<td>extracellular region</td>
<td><em>ADAMTS2</em>, <em>MMP9</em>, <em>CD55</em>, <em>COL6A2</em>, <em>S100A12</em>, <em>S100B</em>, <em>TREM1</em></td>
<td>0.00162</td>
<td>0.3976</td>
<td>33</td>
</tr>
</tbody>
</table>
6.6 Ingenuity Pathway Analysis (IPA)

Ingenuity Pathway Analysis (IPA) was performed to determine biological pathways. IPA was performed on 263 significant differentially expressed genes in pediatric jSSc compared to healthy controls to determine activated and inhibited pathways (Figure 9). Pathway analysis of the upregulated and downregulated DE genes reveals activated inflammatory response pathways including (neutrophil degranulation, S100 family, activin inhibin signaling pathway, toll-like receptors signaling, hepatic fibrosis, interleukin signaling, TREM1 signaling, and FAK signaling pathway).

The neutrophil degranulation pathway is the most overrepresented pathway. Neutrophils are essential components of the immune system and have a significant role in the immune response and autoimmune disease. Neutrophils get activated and release their granules by the elevated cytokines and chemokines in SSc [51]. Therefore, neutrophil degranulation would play a crucial role in driving inflammation, vascular damage, and fibrosis [52].

S100 family pathway signaling is the second top significant pathway (Figure 10). This pathway includes S100A12 gene, which has a key role in skin fibrosis in adult literature [44]

More enriched pathways that are related to the disease activity are inflammatory and immune response pathways. These pathways include IL-1 Signaling and Toll-like Receptor Signaling. IL1R1 a receptor, binds to proinflammatory cytokines (IL-1α) and (IL-1β) that have a role in the immune response [53]. In the IL-1pathway (Figure 11), signal transduction in the pathway of IL signaling lead to the expression of FOS gene.

TLR signaling pathways including (TLR2, TLR4, TLR8) have a role in the activation of interleukin signaling pathways. The signaling of TLRs as inflammatory response leads to the production of pro-inflammatory interleukins such as IL-1, IL-12 (Figure 12). These pro-
inflammatory cytokines activate immune responses. Dysregulation of the pro-inflammatory TLRs production contributes to the chronic inflammation and activation of fibroblast resulting in fibrosis in SSc [54].

Another potential insult early in the disease pathway could be represented by the activated focal adhesion kinase (FAK) pathway (Figure 13) in jSSc blood, contributing to vascular damage and fibrosis since it triggers downstream signaling pathways that have a key role in fibroblast activation via transforming growth factor beta signaling [55].

Furthermore, the IPA results reveal activated interleukin pathways such as (IL-1, IL-12, and IL-27). IL-1 family includes 11 cytokines that are involved in the pathogenesis of SSc by promoting pro-inflammatory and pro-fibrotic processes[56]. Activation of type 1 helper (Th1) cells in the blood of jSSc is indicated by activated IL-12 signaling [57]. While IL-10 signaling pathway indicated as inhibited pathway in IPA analysis in (Figure 14). IL-10 is an anti-inflammatory cytokine that has a key role in inhibiting immune responses [58].
Figure 9. Significantly Pathways Determined by Ingenuity Pathway Analysis of DEGs.
Figure 10. S100 Family Pathway Inhibition and TLR4 Pathway Induction in jSSc Blood.

Products of upregulated genes are shown in pink, downregulated genes in green.
Figure 11. IL-1 Signaling Pathway Alterations in Whole Blood of jSSc.

Product of upregulated genes are shown in pink.
Figure 12. Toll-Like Receptors Signaling Pathway Alterations in Whole Blood of jSSc.

Products of upregulated genes are shown in pink.
Figure 13. FAK Signaling Pathway Alterations in jSSc.

Products of upregulated genes are shown in pink.
Figure 14. IL10 Pathway Inhibition Pathway Induction in jSSc Blood.

Products of upregulated genes are shown in pink, downregulated genes in green.
7.0 Discussion

This study is the first to profile the transcriptome of juvenile systemic sclerosis (jSSc) in whole blood compared to healthy controls. By utilizing RNA sequencing of peripheral blood, we gained valuable insights into gene expressions patterns in innate and adaptive immune cells, identifying potential biomarkers for jSSc. Additionally, this study using next generation sequencing helped in identifying gene dysregulation that is crucial in understanding disease pathogenesis and progression. This non-invasive diagnostic approach can assess disease severity and progression. The study findings support our hypothesis that immune and inflammatory response pathways are upregulated whereas anti-inflammatory pathways are downregulated in jSSc.

Our findings revealed significant enrichment of genes whose products are located in the plasma membrane and the ECM, which correlate with fibrosis and inflammation in SSc pathogenesis. Notably, \textit{ADAMTS2}, a gene encoding a metalloproteinase involved in collagen synthesis [38], was highly expressed. ADAMTS2’s role in ECM formation and its anti-inflammatory properties in autoimmune diseases [59] suggest its involvement in the imbalance of collagen synthesis and accumulation in jSSc. Furthermore, deficiency of \textit{ADAMTS2} in a mice model resulted in skin disorder of fragile skin [38]. Circulating white blood cells with high expression of \textit{AMAMTS2} may infiltrate skin and other affected tissues and enhance collagen maturation and accumulation in jSSc patients.

The upregulation expression of the other plasma membrane protein \textit{AQP1} in blood of jSSc is consistent with previous studies findings, \textit{AQP1} is highly expressed in SSc lesional skin and positively correlated with tissue fibrosis [60]. \textit{TREMI} was also a significantly elevated gene
transcript of the plasma membrane gene family in the blood of jSSc and reflected significant activation in IPA analyses. The high expression of AQP1 and TREM1, both linked to tissue fibrosis and pulmonary involvement (interstitial lung disease) in diffuse cutaneous SSc patients’ serum [61] further supports the association with severe clinical manifestations. Additionally, the upregulated plasma membrane gene CLEC4E, a member of the C-type lectin receptor (CLR) family, which is activated as inflammatory response and leads to cytokines production [62] was identified in our data set. Since the expression of this gene is remarkably high in patients with severe clinical manifestations, it could also serve as blood biomarker for disease diagnosis with future validation validations.

The high expression of MMP9 and existing is in the downstream of FAK pathway as ECM degradation confirm its role as regulator in remodeling and repair tissues. Studies reported the regulatory role of MMP9 in remodeling vascular matrix in systemic sclerosis patients [63]

S100 family pathway includes molecular interaction between S100B and S100A12 genes, highlight the importances of this pathway in the disease pathogenesis [44]. Possibility of serving as therapeutic marker for jSSc.

The activation of the neutrophil degranulation pathway underscores the high inflammatory state in jSSc. Neutrophils, essential in inflammatory stage of SSc disease, produce cytokines that influence B cells differentiation [64]. Consistent with the literature, this study demonstrates activated interleukin pathways (IL-1, IL-12) in jSSc. Elevated IL-1 was demonstrated as a pro-inflammatory cytokine in adult SSc and an elevated IL-12 expressed by activated T-helper1 cells was seen in jSSc peripheral blood mononuclear cells by flow assays [56]. While downregulation, or inhibition of the anti-inflammatory IL-10 cytokine is correlated with high inflammatory and fibrosis stages of the jSSc patients [58].
In summary, transcriptome profiling of the peripheral blood in jSSc patients reveals differentially expressed genes involved in inflammation, immune response, endothelium vascular abnormalities and fibrosis. Shared dysregulated genes between jSSc blood and systemic sclerosis (SSc) skin, including pro-inflammatory cytokines and fibrotic markers, highlight common pathways. Circulating immune cells, common vascular abnormalities and shared fibrotic and inflammatory pathways are leading to the overlap of gene expression signatures. Additionally, gene set enrichment and ingenuity pathway analyses reveal immune activation and fibrosis-related pathways similar to those in SSc skin. Understanding these similarities increases the ability to identify therapeutic targets for jSSc and SSc alike.


