Network pharmacology and an in silico-based study to identify proinflammatory pathways and promising bioactive polyphenols for the treatment of sickle cell anemia

Lila Rosa Maturana Pérez  
University Cartagena

Johana Márquez Lázaro  
Corporación Universitaria Rafael Núñez

Neyder Contreras Puentes  
Corporación Universitaria Rafael Núñez

Antístio Álvis Amador  
University of Cartagena

Albeiro Marrugo-Padilla (✉ albeiro.marrugo@curnvirtual.edu.co)  
Corporación Universitaria Rafael Núñez – Torices

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Abstract

Sickle cell anemia (SCA) is a disease transmitted as an autosomal recessive Mendelian trait. It causes symptoms that worsen with age, such as acute and chronic pain, chest syndrome, pulmonary hypertension, stroke, kidney disease, and vaso-occlusive crises (VOCs), which are the leading cause of hospitalization and premature death. Although there are existing treatments to manage it, side effects related to VOCs such as patients’ inflammatory state requires the review of options for its control. In this sense, the study of polyphenols represents a potential alternative for the treatment of ACS due to their widely reported anti-inflammatory capacities. This research identified hub genes associated with inflammatory processes in SCA by extracting differentially expressed genes (DEGs) in a GEO dataset collection. Then, polyphenols with anti-inflammatory activity were selected from natural product databases, subsequently, molecular docking was performed with the polyphenols and the key protein derived from the selected hub genes. Finally, molecular dynamics were performed on the promising complexes. The comparative analysis allowed us to find 10 genes associated with proinflammatory pathways in SCA (MX1, FIT1, IFIT3, STAT1, ISG15, GBP1, OAS1, OAS2, OAS3, and RSAD); among them, STAT1 was selected as a central gene by regulating the expression of the rest. Docking and dynamics studies showed good binding energies among STAT1 and the fifteen polyphenolic extracted compounds, with quercetin, diosmetin, and fisetin showing the lowest binding energies. These flavonoids have been described in the past as compounds having anti-inflammatory and antioxidant features, as well as possible alternatives for SCA treatment.

Introduction

Sickle cell disease (SCD) is a broad term for a group of inherited conditions that include sickle cell anemia (SCA) and β thalassemia [1, 2]. These disorders are marked by changes in the gene that codes for the globin chains that make up hemoglobin (Hb), a polyfunctional molecule whose fundamental role is to transfer oxygen from the lung to tissues in a coordinated way. The Hb, found in most vertebrates, is a tetramer with two α-subunits (α1 and αx) and two β-subunits (β1 and β2) that are structurally identical and comparable in size [3, 4].

The many tetrameric configurations of Hb and the varied range of genes encoding the globin chain contribute to its potential to display a multiplicity of structural alterations throughout the embryonic, neonatal, and adult stages of development. According to Heaton et al. (2021), the HbVar database (https://globin.bx.psu.edu/globin/hbvar/) has over 1833 Hb variables, 1393 of which are Hb variations and 534 are thalassemia. Among the Hb variants, HbA is the predominant in adults (> 90%), whose structure consists of two β-globin subunits (which are encoded by the HBB gene) and two α-globin subunits (which are encoded by the HBA1 and HBA2 genes); on the other hand, HbS gives raise to the most important of the haemoglobinopathies (sickle hemoglobin), while types C, E, D and J can cause SCA when associated with HbS [5, 6].
HbS is generated by a point mutation in the gene of the β-globin chain at chromosome 11, where valine is replaced by glutamic acid at the sixth position [Glu6Val, rs334]. This mutation gives rise to the β^S allele, which codes for a Hb tetramers containing two of these abnormal sickle chains. Under hypoxic conditions, two-mutant sickle β-globin subunits could undergo polymerization, resulting in the adoption of a crescent or sickle shape by the erythrocytes, thus giving rise to the name of the illness [1, 2].

SCA is currently the most frequent genetic disorder in the SCD group, and it is handed down as an autosomal recessive Mendelian characteristic. Individuals who are heterozygous for the β^S allele have the sickle cell trait but not SCA, while those who are homozygous for the β^S allele have SCA [6].

Among the hemoglobinopathies, SCA is by far the largest global public health concern, according to the World Health Organization (WHO). Evidence suggests that approximately 43 million people worldwide are living with sickle cell trait, while 4.4 million have SCA. Despite being a global phenomenon, sub-Saharan Africa has the greatest incidence of SCA (approximately 80%), where the mortality rate for infants below the age of five varies between 50% and 80%. Inadequate access to comprehensive healthcare in the area exacerbates the disease's high prevalence in this setting [1, 7].

From 2000 to 2021, the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) examined the mortality burden and prevalence of SCA in 204 countries. National incidence rates of SCA remained stable, according to the study; however, the total number of births involving infants with the pathology rose by 13.7% to 515,000 (425,000–614,000), primarily attributable to population growth in western and central sub-Saharan Africa and the Caribbean. Additionally, the prevalence of SCA rose by 41.4% between 2000 and 2021, from 5.46 million to 7.74 million individuals. Global estimates for 2021 placed cause-specific all-age fatalities at 34,400; however, the overall mortality burden at 376,000 attributed to SCA was eleven times greater, placing it twelfth among all causes [8].

Babies born with SCA may remain asymptomatic for the first three months, as these often manifest later in life and worsen as the disease progresses. Hemolysis and vaso-occlusion result from sickling and HbS polymerization; this multi-factorial process includes activation of endothelial and blood cell components, involvement of both small and large blood vessels, hypoxia/reperfusion injury, ischemic tissue damage, and chronic inflammation [6]. According to Badawy et al. (2021) and Tebbi (2022), these processes may be exacerbated by inflammatory responses, hypercoagulability, issues with arginine metabolism, and oxidative stress [2, 9].

Patients with SCA also suffer significant acute and chronic pain, acute chest syndrome, lung hypertension, chronic anemia, strokes, renal disease, gall stones, and other long-term organ damage. These problems may occur at varying rates; some patients may have six or more episodes each year, while others may experience pain crises less often or not at all [1, 2, 6, 9]. Vaso-occlusive crises (VOC) constitute the principal reason for hospital admissions of patients with SCA and are correlated with premature death, with a median age of 43 years at which such crises occur [1, 10, 11].
In addition to reduced life expectancy, SCA is widely associated with reduced quality of life for patients, because of recurrent crises, repetitive hospitalizations, and disease-related complications (Painful VOC, acute chest syndrome, stroke, renal failure, and pulmonary hypertension). Adults with SCA frequently experience anxiety, depression, sleep disturbances, low self-efficacy, and feelings of hopelessness as psychological complications [1, 10, 11].

There are several therapy options available now to treat SCA. Since hydroxyurea (HU) has been shown to be helpful in lowering VOCs and hospitalizations, it is the drug that is advised for all patients with the most severe types of SCA. Via a poorly understood process associated with stress erythropoiesis induced by cytotoxicity, HU raises HbF and total Hb, decreasing sickle cell adherence to the endothelium. Unfortunately, owing to a lack of adherence and side effects such as headaches, nausea, and gastrointestinal discomfort, this drug is not recommended often enough or used on a regular basis [12–14]. Certain children have temporary and reversible myelosuppression, primarily neutropenia, along with dermatological changes such as hyperpigmentation of the skin or darkening of the nails. On the other side, blood transfusions have been shown to be beneficial, but, over time, they may result in iron excess and alloimmunization [15]. Similarly, although successful, hematopoietic stem cell transplantation needs a donor with the same HLA as the patient; the transplant has an 85% disease-free survival rate and a mortality rate associated with it. Its use is restricted by its 7% and 9% graft failure rates [16]. The US FDA has approved three novel agents: crizanlizumab, voxelotor, and l-glutamine; nevertheless, it is still early to determine the best way to utilize them. Lastly, research is now being done on the efficacy and safety of genetic treatments that change a patient’s own red blood cells to make them sickle cell-free [17, 18].

Together, the combination of ongoing vascular and organic damage, insufficient use of HU, and the recognized adverse effects of successful treatments contribute to the usage of healthcare resources, leading to an economic burden on medical and hospital services. In 2016, the aggregate financial cost of hospital treatment for individuals with SCD in the United States amounted to $811 million [19].

The current landscape of SCA therapies and their numerous adverse effects requires the exploration of novel alternatives and biological targets that provide greater selectivity and safety. Currently, different investigations have evaluated the role of inflammatory mediators and how oxidative stress contributes to the pathophysiology of SCA. Several mechanisms have been proposed, such as elevated auto-oxidation of HbS, repeated ischemia-reperfusion damage, excessive levels of cell-free hemoglobin, and iron overload. The precise immunological and inflammatory pathways, however, are not well understood [20–23].

Polyphenols, for instance, are one of the chemical molecules that have captured the scientific community’s attention in search for novel therapeutic agents to treat inflammatory illnesses. Secondary plant metabolites such as phenolic acids, flavonoids, catechins, tannins, lignans, stilbenes, and anthocyanidins have been shown to have key chemopreventive and protective activities for health maintenance [24–26]. Long-term consumption of polyphenol-rich diets offers protection against the development of a variety of chronic diseases, including neurodegenerative, cardiovascular, cancer,
diabetes, inflammatory disorders, infectious diseases, and anemia, according to preclinical and clinical evidence [27, 28].

Therefore, according to previously described, the goal of this study was to find novel pathways and promising molecules for the treatment of SCA using a combination of network pharmacology and in silico methods.

**Materials and methods**

**Identification of hub genes associated with inflammatory processes in SCA**

**Dataset selection and validation**

To identify possible pathways and genes associated with the inflammatory state in patients with SCA, analysis of the genomic data collections of the Gene Expression Omnibus of the National Center for Biotechnology Information was carried out (GEO, https://www.ncbi.nlm.nih.gov/gds). [29] For this, studies that evaluated the transcriptome of blood cells from patients with SCA were selected, especially those that identified proinflammatory genes and/or related pathways. The following search strategy was used: ("anemia, sickle cell" [MeSH Terms] OR sickle cell anemia [All Fields]) AND ("inflammation" [MeSH Terms] OR inflammation [All Fields]) AND "Homo sapiens" [porgn].

The dataset selection process adhered to the following inclusion criteria: i) research conducted on human subjects; ii) analysis of gene expression associated with inflammatory processes; iii) studies involving a minimum of 20 patients; iv) patients who were stable and ambulatory, devoid of any concurrent complications or comorbidities, including chronic inflammatory and autoimmune diseases; v) employ arrays as a technology for transcriptomic analysis.

Once the dataset was chosen, the intra-group data repeatability was determined per group using the Pearson correlation test and principal component analysis. Heat maps developed in the R programming language were used to show these relationships [30].

**Identification of Differentially Expressed Genes (DEGs)**

The interactive online tool GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to identify DEGs. This package compares infected SCA patients with healthy controls to detect DEGs using the GEO query and limma R tools from the Bioconductor, an open-source software project based on the R program [31]. The significant genes’ up-regulated and down-regulated were categorized based on Log2 (fold change) values, > 1.5 and < −1.5, respectively. Finally, DEGs were visualized through volcano plots and significant genes were filtered based on their adjusted P-value employing the Benjamini-Hochberg false discovery rate method [30, 32].

**Functional enrichment analysis of DEGs**
The DEGs found were subsequently analyzed using a database for annotation, visualization, and integrated discovery DAVID (https://david.ncifcrf.gov/home.jsp ; version 6.8). DAVID could perform gene ontology and functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGGG) pathway enrichment analysis. Results were considered statistically significant if $P < 0.05$ [33–35].

### Identification and validation of hub genes

A protein-protein interaction analysis was conducted on discovered DEGs in the STRING online database (https://string-db.org), to predict and trace a PPI network (PPI-Net). For it, proteins with minimum interactions of 10 and a minimum interaction score of 0.40 were considered. Subsequently, PPI-Net was visualized in Cytoscape (version 2.9). Next, using the plug-in Molecular Complex Detection tool (MCODE; version 1.5.1), we developed a topological analysis for finding the PPI-Net’s main cluster. The criteria selected for MCODE analysis were a degree of cut-off = 2, MCODE scores > 5, maximum depth = 100, node score cut-off = 0.2 and k-score = 2 [36, 37].

After identifying the key cluster genes in MCODE, we find the hub genes through the Cytoscape Cyto-Hubba application, using five calculation methods: Closeness, Degree, EcCentricity, MCC, and MNC. The intersected genes derived from these five algorithms were labeled as hub genes and may be central genes with important biological regulatory functions [38].

To validate the identified hub genes and assess the extent of gene variation across datasets, receiver operator characteristic curves (ROC) were generated for them utilizing a reference dataset. The evaluation was conducted by calculating the area under the curve (AUC) as a parameter [37].

### Identification of polyphenols exhibiting therapeutic potential for SCA

Polyphenols with potential activity for SCA treatment, owing to their ability to modulate inflammation, were obtained from different natural product databases, including Supernatural III (https://bioinf-applied.charite.de/supernatural_3/index.php), Ambinter-Greenpharma natural composite library (https://www.ambinter.com/), and AntiBase (http://sciencesolutions.wiley.com/solutions/technique/screening/wiley-identifier-of-natural-products-for-drug-discovery/) [39–41]. Once the databases were consulted, through text mining in search engines such as ScienceDirect, PubMed, and Google Scholar, the biological activity of the selected compounds was validated. [42].

### Evaluation of the promising activity of polyphenols against SCA

Following the discovery of the polyphenolic compounds, we evaluated their potential utility in the treatment of SCA. Computational molecular docking meth was used to achieve this goal, with the target protein being the most important central gene throughout the PPI-Net.
For Molecular Docking, the structures of ligands (polyphenol compounds) and selected protein (central hub gene) were obtained from Pubchem (https://pubchem.ncbi.nlm.nih.gov) and Protein Data (https://www.rcsb.org), respectively. Next, DFT method with the hybrid functional B3LYP (Gaussian 09 software) was used to improve the structure of the ligands [43–45]. The protein’s 3D crystallographic structure was optimized by adding missing amino acids using the PDBFixer Anaconda application [46]; it was subsequently improved by getting rid of water molecules and co-crystallized substructures (ligands and ions), followed by energy minimization in the UCSF Chimera program using 1000 steps of steepest descent followed by 500 steps of conjugated gradient algorithms [44]. Polar hydrogen and Kollman charges were added to the resulting structure [47, 48].

Molecular docking was performed in AutoDock Vina via shell commands. For this, the spatial dimensions of the cube (gird) were established based on the reported bibliographic information regarding the active site of selected protein using Auto dock tools. Three coupling runs were and the binding affinity for each complex was established according to the mean values of affinity for the best poses [44]. The results derived from the docking were visualized and interpreted using Discovery StudioVisualizer [49].

The reliability of the docking results was carried out by a comparative analysis (linear regression analysis) between docking made with the promising compounds and their respective ligands (inhibitors described in the literature), whose 3D structures and biological data in terms of median inhibitory concentrations (IC50 µM) were obtained from PubChem and bibliographic information sources. Docking between these standard inhibitors was carried out following the same methodology described above [44].

On the other hand, in accordance with the methodology outlined by Alviz-Amador et al. (2021), unrestrained all-atom molecular dynamics simulations were executed utilizing PMEMD from AMBER16 software. The procedure consisted of the following stages: MD minimization, equilibration, production, and analysis. The optimized structures were determined utilizing the TIP3P water model. This was achieved by minimizing conjugate gradients after 1000 steps of steepest descent, with a constant restraining force of 25 kcal/mol-2, applied to the entire solute molecule. The heating process spanned 5000 steps of MD from 100 to 300 K with a two-fs time step, utilizing a weak coupling thermostat at a constant pressure and SHAKE to constrain hydrogen bonds with a tolerance of 0.00001. An 8-inch non-bonded termination was utilized. The particle mesh Ewald (PME) was employed to account for long-range electrostatics, utilizing the default PME parameters for Amber and an automated pair list updating system. The peptides and proteins were subjected to a gradual reduction in the restraints from five to 0.5 kcal/mol-A2 over the course of five intervals following heating. After 1000 steps of steepest descent, 500 steps of conjugated gradient minimization, and a two-fs time step, each step is subsequently minimized using 50 ps of MD at 300 K, constant pressure, and temperature, with Berendsen coupling constants of 0.2 ps. Each system exhibited a total production time of 100 nanoseconds. Per system, the total production time was 100 nanoseconds. Root means square deviation (RMSD), root mean square fluctuation (RMSF), solvent accessible surface area (SASA), and radius of gyration were all evaluated utilizing simulation trajectories [50].
**Results and discussion**

**Dataset selection**

The GEO database search using the specified descriptors provided the identification of ten datasets; however, only one of them, GSE53441, satisfied the established inclusion criteria. Similarly, two data sets were also identified that examined variation in gene expression between healthy individuals and patients with SCA, but without fully emphasizing pro-inflammatory pathways (GSE35007 and GSE72999).

GSE53441 was based on individuals diagnosed with SCA with 18 years of age or older and have undergone chromatographic diagnosis. The study only included outpatients who were in good health. Subsequent evaluations were performed on patients who had experienced a VOC in the previous two weeks or an incident of acute chest syndrome in the previous four weeks. The objective of this study was to examine the role of the pro-inflammatory profile in SCA by evaluating the transcriptome of peripheral blood mononuclear cells (PBMCs) in a cohort of 24 adult SCA patients and 10 individuals without the condition (healthy controls). This was achieved by isolating RNA from PBMCs and plasma, and subsequently hybridizing them onto Affymetrix’ Human Genome U133 Plus 2.0 microarray in a single batch [51].

**Dataset validation**

The selected data collection was validated through the analysis of intra-group repeatability under the development of Pearson’s correlation analysis, which was represented by a heat map (Fig. S1). A strong positive correlation between the expression data of dataset’s samples were found. On the other hand, based on the PCA, the intra-group data repeatability was adequate; the distances among samples in the control and SCA groups were close in the dimension of the PCA1, except for five samples in the SCA group, which were close in the PCA2 (Fig. S1b).

**Identification of differentially expressed genes (DEGs)**

DEGs were identified by comparing gene expression patterns in samples from the control group (healthy subjects) with those of SCA patients through the interactive online program GEO2R. The findings are shown in Fig. 1a. Each node of the volcano plot (dot) represents a potential DEGs, which were assessed and classified as significant if they fulfilled the threshold criteria [P < 0.05, Log2 (fold change) values > 1.5 and −1.5, respectively]. The volcano plot depicted up-regulated DEGs in red, whereas down-regulated DEGs were shown in blue.

A total of 411 DEGs were identified based on log2 fold change values; 195 were down-regulated and 216 were up-regulated (P < 0.05) (supplementary table S1 – Fig. 1). The heat map in Fig. 1b illustrates the log2 fold change values from the identified DEGs. Based on the findings of the functional enrichment analysis in DAVID database, DEGs exhibited a notable enrichment in biological processes associated with the type I interferon signaling pathway and viral response; furthermore, protein tyrosine kinase activity, oxygen binding, and oxygen carrier activity were their principal molecular functions (Fig. 2).
Hub genes identification, validation, and analysis

The hub genes were identified from the selected DEGs (down- and up-regulated) by constructing a PPI-Net in the STRING online database; as a result, a network composed of 374 nodes and 809 interactions was obtained (Fig. 3). Next, the major clusters (highly linked areas) that comprised the PPI-net were discovered using Cytoscape's Molecular Complex Complement Detection Tool (MCODE; version 1.5.1). The MCODE analysis revealed nine clusters, selecting the one with the highest score (24.333) as the most important in the PPI-net, and its 25 nodes were the hub genes, which had scores greater than 10 (Fig. 3b and c) [30].

Hub genes were validated through CytoHubba (Cytoscape complement) applying the five most reported computational methods in the scientific literature: Degree (Deg), Maximum Neighborhood Component (MNC), Maximal Clique Centrality (MCC), EcCentricity (EC) and Closeness [38]. The Table 1 lists the scores of the top 10 hub genes identified by the algorithms of the evaluated methods. Next, we performed an intersection analysis of these genes on the website: http://www.ehbio.com/ImageGP/index.php/Home/Index/VennDiagram.html, in order to find the central hub genes (CHGs), which are shown in Fig. 4 and described in Table 2. These included: (i) Mx1 interferon-induced GTP-binding protein (MX1), (ii) Interferon-induced protein with tetratricopeptide repeats 1 and 3 (IFIT1, IFIT3), (iii) Signal transducer and activator of transcription 1 (STAT1), (iv) ISG15 ubiquitin-like protein (ISG15), (v) Guanylate-binding protein 1 (GBP1), (vi) 2'–5'–oligoadenylate synthase 1, 2, and 3 (OAS1, OAS2, OAS3, respectively), and (vii) S-adenosyl methionine radical domain-containing protein 2 (RSAD2).
Table 1
Hub genes identified in the cluster 1 by each Cytohubba algorithm.

<table>
<thead>
<tr>
<th>GEN</th>
<th>Algorithm*</th>
<th>MNC</th>
<th>MCC</th>
<th>EC</th>
<th>Degree</th>
<th>Closeness</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAS2</td>
<td></td>
<td>23</td>
<td>1.46x10^19</td>
<td>1</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>GBP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OAS1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OAS3</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ISG15</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>RSAD2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MX1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFIT3</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IFIT1</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

*Degree (Deg), Maximum Neighborhood Component (MNC), Maximal Clique Centrality (MCC), Eccentricity (EC) and Closeness
Table 2
Features of central hub genes identified in the cluster 1 by each Cytohubba algorithm

<table>
<thead>
<tr>
<th>Hub genes</th>
<th>Log2FC</th>
<th>Uniprot accession code</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Symbol</strong></td>
<td><strong>Name</strong></td>
<td></td>
</tr>
<tr>
<td>MX1</td>
<td>Interferon-induced GTP-binding protein Mx1</td>
<td>0.187</td>
</tr>
<tr>
<td>IFIT3</td>
<td>Interferon-induced protein with tetratricopeptide repeats 3</td>
<td>0.273</td>
</tr>
<tr>
<td>IFIT1</td>
<td>Interferon-induced protein with tetratricopeptide repeats 1</td>
<td>0.296</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal transducer and activator of transcription 1-alpha/beta</td>
<td>0.079</td>
</tr>
<tr>
<td>ISG15</td>
<td>Ubiquitin-like protein ISG15</td>
<td>0.281</td>
</tr>
<tr>
<td>OAS3</td>
<td>2’-5’-oligoadenylate synthase 3</td>
<td>0.184</td>
</tr>
<tr>
<td>OAS2</td>
<td>2’-5’-oligoadenylate synthase 2</td>
<td>0.134</td>
</tr>
<tr>
<td>OAS1</td>
<td>2’-5’-oligoadenylate synthase 1</td>
<td>0.111</td>
</tr>
<tr>
<td>GBP1</td>
<td>Guanylate-binding protein 1</td>
<td>0.126</td>
</tr>
<tr>
<td>RSAD2</td>
<td>S-adenosylmethionine-dependent nucleotide dehydratase RSAD2</td>
<td>0.347</td>
</tr>
</tbody>
</table>

All CHGs were upregulated in patients with SCA, according to the differential expression analysis performed in GEO2R (Fig. 5). Furthermore, ROC curves (acronym for Receiver Operating Characteristic) were performed to evaluate the magnitude of the variation of their expression between other datasets such as GSE35007, to validate their capability to sensitively predict the diagnosis and/or inflammatory complications in patients with SCA. The results showed a significant association between the variables evaluated (0.554 < AUC < 1; P ≤ 0.05) (Fig. 6).

A gene ontology analysis of CHGs through text mining in the PubMed search engine was carried out; results evidenced that all were closely related to the type I interferon signaling pathway (IFN-α, IFN-β), which agrees with the functional enrichment analysis performed in DAVID (Fig. 2); similarly, a high degree of co-expression of these genes was found in the ProteomeHD database (https://www.proteomehd.net/proteomehd) (Supplementary Fig. S2).

Type I interferons (IFN-I), such as IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω, are polypeptide members of innate immunity that are released in response to different autoimmune disorders and viral infections in humans [52, 53]. All IFN-I mediate their physiological effects through the interferon alpha receptor (IFNAR), which has the capacity to activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway [54], well recognized as one of the primary communication nodes involved in cell function. [55]
In summary, the IFNAR is constitutively associated with tyrosine kinase 2 (TYK2) and JAK1, proteins that are trans-phosphorylated when said receptor binds to its ligands; this in turn activates them to phosphorylate tyrosine residues conserved in the cytoplasmic tails of the IFNAR, which serve as docking sites for the recruitment of signaling molecules with the SH2 domain (Src Homology 2), such as STAT1 and STAT2, which once phosphorylated dimerize and move to the nucleus, where they associate with the factor interferon regulator (IRF9), thus forming the interferon-stimulated gene factor 3 (ISGF3), which binds to the IFN-I response elements, positively regulating the expression of genes such as ISGs (interferon-stimulated genes, ISG15); interferons themselves; interferon regulatory factors (IRFs); signal transducers and activators of transcription (STATs); oligoadenylate synthetase (OAS) enzyme genes; guanylate binding proteins (GBPs); nitric oxide synthase 2 (NOS2); interferon-induced transmembrane proteins (IFITMs), tripartite motif proteins (TRIMs); interferon-induced GTP-binding protein Mx1 (MX1), among others (Fig. 7) [52, 53, 56, 57].

All CHGs found in this research have a strong relationship with genes involved in the JAK/STAT pathway and were concordant with those obtained by Hermand-Tournamille et al. (2018), who performed a proteomic analysis by label-free mass spectrometry on polymorphonuclear neutrophils (PMNs) from four steady-state SCA patients and four healthy subjects. In this research, 50 up-expressed genes were found in patients with the pathology, including the STAT family (STAT1 and STAT2), OAS 1, 2, and 3, and many INF-I signaling genes (IFIT1, IFIT2, IFIT3, ISG15, ISG20, GBP2, IFI35, MX1, and MX2), whose concentrations in PMNs from SCA patients were on average 10 to 100 times higher than in healthy subjects [58].

Several studies have shown that individuals with SCA in steady-state have increased levels of IFN-I in their plasma as well as high expression of its inducible genes [58–60]. Liu et al. (2021) report that in the SCA is generated a significant activation of classical monocytes (CMo) linked to intravascular hemolysis processes. In fact, monocytosis is frequent in SCA and is associated with hemolysis indicators and adversely with hemoglobin levels. [61] The total plasma heme levels and circulating IFN-α in SCA patients were discovered to be positively correlated by these researchers. Additionally, they observed an elevation of IFN-I-inducible genes in genes in sort-purified SCA patients’ CMo by transcriptome analysis.

IFNs promotes chemokine synthesis, including chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein, which leads to the migration of bone marrow-derived classical monocytes into the circulation, exacerbating the inflammatory state in patients with SCA. [63–65] Furthermore, CMo from SCA patients display an activated profile, with increased expression of CD11b on their surface and increased production of proinflammatory cytokines including interleukin (IL)-1β and TNF-α as compared with healthy control monocytes [61], increasing the inflammatory status, and with it, the aggravation of the hemolytic process in the patient with SCA.

The mononuclear phagocyte system, according to Liu et al. (2023), is critical to SCA pathogenesis. They investigated the effect of hemolysis on circulating monocyte trajectories in SCA mice and discovered that
hemolysis increased CSF-1 synthesis, which was mediated in part by endothelial cells via Nrf2, enhancing CMo differentiation into blood patrolling monocytes (PMo) in SCA mice. Hemolysis, on the other hand, increased CCL-2 expression via IFN-I, promoting CMo transmigration and differentiation into tissue monocyte-derived macrophages. [66]

PMNs, on the other hand, have also been recognized as significant participants in the pathophysiology of SCA; nevertheless, the signaling mechanisms driving their activation and encouragement of a persistent inflammatory state remain unknown. In steady-state SCA patients, the absolute neutrophil count is greater than in ethnicity-matched healthy controls and positively linked with SCA severity. [61] Activated PMNs play an important role in the initiation and propagation of VOC in patients with SCA and different investigations have shown that IFN-I is able to regulate the differentiation and production of cytokines in these cells. [58]

Decker et al., (2011) demonstrated that IFN-α can increase the recruitment of PMNs and the differentiation of monocytes into dendritic cells, which present antigens from dying cells to CD4+ lymphocytes, resulting in their proliferation [67]. On the other hand, Zimmermann et al. (2016) evidenced that IFN-I prolongs neutrophil survival through the expression of CXC motif chemokine ligand 10 (CXCL10) mRNA and can potentiate IL-6 production in neutrophils stimulated with R848 (Resquimod), a TLR8 agonist [68]. In congruence with the latter results, Glennon-Alty et al. (2021) measured the effects of IFN-α on the functions of healthy neutrophils incubated in vitro in the absence and presence of proinflammatory cytokines, such as TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF), and found that IFN-α significantly increased CXCL10 expression, which was enhanced in the presence of TNF-α. These investigators identified the important role of IFN-α in the regulation of chemokine production by neutrophils. [69]

IFN-α was found to significantly decrease the expression of the chemokine genes CXCL1, CXCL2, CXCL3, CCL3, and CCL4 in response to GM-CSF and TNF-α, but enhanced the expression of CXCL10, which has important implications for neutrophil-mediated regulation of the immune response in vivo. The chemokines CXCL1, CXCL2, and CXCL3, and CCL3 and CCL4 behave as chemoattractant of neutrophils, while CXCL10 is mainly a chemoattractant of T cells, monocytes, NK cells, and dendritic cells [69].

The above-described effects exerted by IFN-I on CMo and PMN could explain the effects of this cytokine on the inflammatory status and VOC events in patients with SCA. In fact, inappropriate neutrophil activation can induce tissue damage [67], and being related to the generation of inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), where type I IFNs play an important role [67–69].

According to what was described above, IFN-I is proposed as a new target for drug design, based on the inhibition and/or reduction of its immunomodulatory effects in patients with SCA, given the effects derived from IFN-I on the pathophysiology of SCA and the large number of identified CHGs associated with it signaling pathway. At present, pharmaceutical approaches for IFN-I pathway antagonism have
relied on monoclonal antibodies such as anifrolumab, a RIFNAR1 antagonist [53], or rontalizumab, an anti-IFN- antibody utilized in lupus clinical trials [70].

By analyzing identified CHGs, the signal transducer and activator of transcription 1 (STAT1) was chosen as a biological target for molecular docking assays, due to its central role as modulator of INF-I pathways.

**Identification of polyphenols exhibiting therapeutic potential for SCA**

More than 100 bioactive phenolic compounds that inhibit inflammatory processes were found by searching the databases of natural products under examination. Text mining in search engines (PubMed and Science Direct) was used to reduce these into 32 based on their anti-inflammatory activity in experimental models (*in vitro* and *in vivo*). Supplementary Table S2 lists the classification of all chosen phenolic compounds as flavonoids, including anthocyanins, flavones, isoflavones, and flavonols. A total of 15 flavonoids were selected for molecular docking investigations out of 32 (Table 3), owing to their significant affinity towards biological targets that are implicated in chronic inflammatory processes. These targets comprise TNFα, IL1β, IL6, TLR4, CXCL8, and STAT 3 (unpublished study). To compare the binding properties of flavonoids with STAT1, a reference inhibitor (STOCK-1N-69677; ((2S,3S,4S,4S,5R)-3,4,5,6-tetrahydroxyoxane-2-carboxylic acid, or D-glucopyranuronic acid) was extracted from the literature, based on its high activity with STAT1 [71] (Table 3).

**Table 3.** Features of selected flavonoids and ligand of reference for STAT1 docking studies.
<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>2D-Structure</th>
<th>Properties</th>
</tr>
</thead>
</table>
| Acacetin   | ![Acacetin 2D-Structure](image) | **Formula:** $C_{16}H_{12}O_5$
**Molecular Weight:** 284.26 g/mol
**IUPAC:** 5,7-dihydroxy-2-(4-methoxyphenyl)chromen-4-one
**ChEMBL ID:** CHEMBL243664 |
| Cyanidin   | ![Cyanidin 2D-Structure](image) | **Formula:** $C_{15}H_{11}O_6$
**Molecular Weight:** 287.24 g/mol
**IUPAC:** 2-(3,4-dihydroxyphenyl)chromenylium-3,5,7-triol
**ChEMBL ID:** CHEMBL404515 |
| Chrysin    | ![Chrysin 2D-Structure](image) | **Formula:** $C_{15}H_{10}O_4$
**Molecular Weight:** 254.24 g/mol
**IUPAC:** 5,7-dihydroxy-2-phenylchromen-4-one
**ChEMBL ID:** CHEMBL117 |
| Diosmetin  | ![Diosmetin 2D-Structure](image) | **Formula:** $C_{16}H_{12}O_6$
**Molecular Weight:** 300.26 g/mol
**IUPAC:** 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chromen-4-one
**ChEMBL ID:** CHEMBL90568 |
| Epicatechin | ![Epicatechin 2D-Structure](image) | **Formula:** $C_{15}H_{14}O_6$
**Molecular Weight:** 290.27 g/mol
**IUPAC:** (2R,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol
**ChEMBL ID:** CHEMBL583912 |
| Fisetin     | ![Fisetin 2D-Structure](image) | **Formula:** $C_{15}H_{10}O_6$
**Molecular Weight:** 268.24 g/mol
**IUPAC:** 2-(3,4-dihydroxyphenyl)-3,7-dihydroxychromen-4-one
**ChEMBL ID:** CHEMBL31574 |
| Formononetin| ![Formononetin 2D-Structure](image) | **Formula:** $C_{16}H_{12}O_4$
**Molecular Weight:** 268.26 g/mol
**IUPAC:** 7-hydroxy-3-(4-methoxyphenyl)chromen-4-one
**ChEMBL ID:** CHEMBL242341 |
| Gallocatechin| ![Gallocatechin 2D-Structure](image) | **Formula:** $C_{15}H_{14}O_7$
**Molecular Weight:** 306.27 g/mol
**IUPAC:** (2R,3S)-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol
**ChEMBL ID:** CHEMBL125743 |
| Kaempferol  | ![Kaempferol 2D-Structure](image) | **Formula:** $C_{15}H_{10}O_6$
**Molecular Weight:** 286.24 g/mol
**IUPAC:** 3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one
**ChEMBL ID:** CHEMBL150 |
| Luteolin    | ![Luteolin 2D-Structure](image) | **Formula:** $C_{15}H_{10}O_6$
**Molecular Weight:** 286.24 g/mol
**IUPAC:** 2-(3,4-dihydroxyphenyl)-5,7-dihydroxychromen-4-one
**ChEMBL ID:** CHEMBL151 |
| Morin       | ![Morin 2D-Structure](image) | **Formula:** $C_{15}H_{10}O_7$
**Molecular Weight:** 302.23 g/mol
**IUPAC:** 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one
**ChEMBL ID:** CHEMBL9352 |
| Naringenin  | ![Naringenin 2D-Structure](image) | **Formula:** $C_{15}H_{12}O_5$
**Molecular Weight:** 272.25 g/mol
**IUPAC:** (2S)-5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one
**ChEMBL ID:** CHEMBL28626 |
| Peonidin    | ![Peonidin 2D-Structure](image) | **Formula:** $C_{16}H_{13}O_6$
**Molecular Weight:** 301.27 g/mol |
### Evaluation of the promising activity of polyphenols against SCA

Molecular docking studies were conducted to explore the possible effects of flavonoids via their interaction with the active site of known STAT1 inhibitors. Once the flavonoids 3D structures were optimized, the CHG crystal (STAT1) was selected with PDB code 1YVL, which has been frequently documented in docking studies \[71, 72\]. According to Raj et al. (2016), STAT1's active site is made up of the amino acid residues between positions 573 and 670 that made up the SH2 domain (grid dimensions: $x$: -21.914 Å, $y$: 1.074 Å, $z$: 115.493 Å; size: $30 \times 30 \times 30$) \[71\].

Docking studies were developed in triplicate with an exhaustiveness of 1000, determining nine conformations based on effectiveness value, free energy, and RMSD between conformations. In Table 4, the average binding energies are shown; these were in a range between $-6.7$ and $-4.5$ Kcal/mol for flavonoids, in contrast with the reference ligand ($-5.7$ Kcal/mol).
### Table 4
Result of molecular docking of flavonoids evaluated against STAT1.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Binding energy (Kcal/mol)*</th>
<th>Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H-bond</td>
</tr>
<tr>
<td>Acacetin</td>
<td>-7,3</td>
<td>S462</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>-7,1</td>
<td>V461, S560</td>
</tr>
<tr>
<td>Chrysin</td>
<td>-7,3</td>
<td>-</td>
</tr>
<tr>
<td>Diosmetin</td>
<td>-7,5</td>
<td>V461</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>-7,2</td>
<td>K566</td>
</tr>
<tr>
<td>Fisetin</td>
<td>-7,4</td>
<td>E524, F581</td>
</tr>
<tr>
<td>Formononetin</td>
<td>-7,1</td>
<td>S583, E524</td>
</tr>
<tr>
<td>Gallocatechin</td>
<td>-6,7</td>
<td>Q497</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>-7,3</td>
<td>-</td>
</tr>
<tr>
<td>Luteolin</td>
<td>-7,3</td>
<td>V461</td>
</tr>
<tr>
<td>Morin</td>
<td>-7,2</td>
<td>-</td>
</tr>
<tr>
<td>Naringenin</td>
<td>-7,3</td>
<td>-</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>-7,3</td>
<td>V461</td>
</tr>
<tr>
<td>Quercetin</td>
<td>-7,5</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnetin</td>
<td>-6,9</td>
<td>V461</td>
</tr>
<tr>
<td>Reference</td>
<td>-5,7</td>
<td>Q3, Q41, W43</td>
</tr>
</tbody>
</table>

* Average value of three replicates

All assessed flavonoids showed lowest binding energy in comparison to the reference ligand: STOCK-1N-69677. The flavonoids having the lowest binding energy with STAT1 were quercetin, diosmetin, and fisetin, indicating strong binding stability (Fig. 8). Hydrogen bonds with amino acids such as V461, E524,
S560, and S583 were one of the main ways that these systems interacted together. \( \pi \)-alkyl interactions, on the other hand, were seen in flavonoids mostly with the amino acids P465, K566, V509, and L562. In addition, flavonoids exerted \( \pi \)-cation and \( \pi \)-anion interaction with R331 and E563, E559, E524 in STAT1, respectively. Finally, only rhamnetin and cyanidin had van der waals and amine - \( \pi \) -Stacked interaction, respectively.

Molecular dynamics assays were developed to validate the docking results. For it, we worked with the flavonoids that presented the best free energy (quercetin, diosmetin, and fisetin). The results obtained were compared with those of the reference ligand. Figure 9 shows the different trajectory analyses employed to assess stability (RMSD), fluctuations (RMSF), area of solvent accessibility (SASA), and compaction with the radius of gyration.

The RMSD values of the evaluated systems ranged from 2.5 to 15 Å in the first 20 ns, except for fisetin, which remained constant throughout the 100 ns of testing, with a value of 2.5 Å, which was the same for the rest of the systems after stabilization (native STAT, reference ligand, quercetin, and diosmetin), thus denoting adequate stability (Fig. 9a). Behavior remained similar in the studies where STAT3 was evaluated against quercetin, luteolin and wogonin, whose RMSD variations were minimal with stabilities in all systems throughout the simulation time and concluding that the molecules anchor to the protein. no notable alteration in the binding site [73]

The RMSF results show that all complexes maintained a similar behavior according to the fluctuation of residues compared to the native protein, starting at residue 125. However, the STAT1-fisetin complex presents lower mobility and flexibility in the residues between positions 1–125 with RMSF values between 2 and 4 Å (Fig. 9b).

The solvent accessible area (SASA) of each assessed complex is shown in Fig. 9c. The obtained SASA values ranged from 34000 to 39000 Å. They decreased throughout time and settled into a steady range after 30 ns of simulation. However, the degree of compaction of the complexes in relation to the native receptor is determined by their radius of gyration, as seen in Fig. 9d. The complexes including STAT1-diosmetin and STAT1-reference ligand showed values that were quite comparable to those of native STAT1, the rest of the complex exhibited highest values regarding STAT1 native, varying between 36 and 38 Å.

Free energy binding analysis was conducted utilizing 1755 complex frames from the trajectory in a 100 ns simulation, employing MMGBSA calculations. Table 5 reveals STAT1-quercetin, STAT1-diosmetin, STAT1-fisetin, and STAT1-reference ligand were $-30.1605$, $-20.9186$, $-28.6466$, and $-7.7690$ Kcal/mol, respectively. The free energies of the complexes between STAT1 and flavonoids show a greater energy affinity compared to the reference ligand of the protein, validating the results obtained by molecular docking.
Table 5
Binding free energy components (kcal/mol) of STAT1 against ligands and control using the GBSA method.

<table>
<thead>
<tr>
<th>Energy Component</th>
<th>Quercetin</th>
<th>Diosmetin</th>
<th>Fisetin</th>
<th>Reference Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEL</td>
<td>-100.7583</td>
<td>-55.3592</td>
<td>-15.1823</td>
<td>-124.6605</td>
</tr>
<tr>
<td>EGB</td>
<td>101.5923</td>
<td>61.8841</td>
<td>24.8570</td>
<td>129.0466</td>
</tr>
<tr>
<td>ESURF</td>
<td>-4.3005</td>
<td>-3.1143</td>
<td>-3.8935</td>
<td>-2.2244</td>
</tr>
<tr>
<td>Δ Ggas</td>
<td>-129.4523</td>
<td>-79.6883</td>
<td>-49.6101</td>
<td>-134.5912</td>
</tr>
<tr>
<td>Δ Gsolv</td>
<td>97.2918</td>
<td>58.7698</td>
<td>20.9635</td>
<td>126.8222</td>
</tr>
<tr>
<td>Δ Total ± SD</td>
<td>-32.1605</td>
<td>-20.9186</td>
<td>-28.6466</td>
<td>-7.7690</td>
</tr>
</tbody>
</table>

The anti-inflammatory properties of the flavonoids discussed in this article, as well as their possible therapeutic use in the treatment of SCA, are supported by many studies. The main mechanism associated with the protective effect of these flavonoids is the capture of free radicals and reactive oxygen species. [74, 75]

Thangaswamy et al. (2021) evaluated the therapeutic benefits of quercetin in a SCD transgenic sickle mouse model (NY1DD) subjected to 18 h of hypoxia followed by 3 h of reoxygenation. The administration of this flavonoids in a dose of 200 mg/kg to NY1DD sickle mice, attenuates hypoxia-induced pathophysiology and brings parameters close to wild type control mice during reoxygenation. [76]

On the other hand, using nuclear magnetic resonance and high-resolution mass spectroscopy, Adeniyi et al. (2022) showed that quercitrin (quercetin 3-rhamnoside), a glycoside derivative of quercetin is the active ingredient in *Alchornea cordifolia* Müll. Arg., also known as Christmas Bush, a plant traditionally used in Africa to treat SCA. In their study Methanol extracts of *A. cordifolia* leaves and its sub-fractions showed >70% suppression of HbSS erythrocyte sickling in blood samples treated with sodium metabisulphite or through incubation in 100% N₂. Moreover, at 0.4 mg/mL, the purified quercitrin showed 93.1 ± 2.69% erythrocyte sickling inhibition and 87.2 ± 2.39% substantial anti-sickling action, which prevented the polymerization of isolated HbS and stabilized sickle erythrocyte membranes. The comparison of blood samples metabolome by flow-infusion electrospray-high-resolution mass spectrometry showed that quercitrin could change the metabolomes of HbSS erythrocytes to resemble those of HbAA by pathways associated with the alteration of arachidonic acid metabolism, anaerobic bioenergy, and antioxidants process. [77]

*Woodfordia fruticosa* (L.) Kurz is another plant that is often used in traditional medicine to treat symptoms associated with SCA, particularly in the tribal populations of Amarkantak, Madhya Pradesh. S. Mishra et al. (2022) tested *W. fruticosa* extracts for phytoconstituents, antioxidants, anti-inflammatory,
and anti-sickling activities. The study found that the plant's methanolic extract effectively reversed sickle red blood cells (66 ± 1%) and prevented Hb polymerization. The extract also inhibited lipoxygenase. Similarly, they demonstrated the presence of flavonoids such as kempferol and quercetin along with numerous of their derivatives such as quercetin 3-O-(600-galloyl)-D galactopyranoside, quercetin 3-O-(600-galloyl)-D galactopyranoside, quercetin, quercetin 3-O-D-galactoside, quercetin 3-L-arabinoside, quercetin 3-rhamnoside, quercetin 3-L-arabinoside, quercetin 3-rhamnoside. [78]

Muhammad et al. (2019) investigated quercetin's anti-sickling effects on human sickle erythrocytes using in vitro models that altered deoxyhemoglobin and redox homeostasis. The study indicated that quercetin treatment efficiently avoided sickling at 5.0 µg/mL, reversed it at 7.5 µg/mL, and significantly protected erythrocyte membrane integrity (P < 0.05). The above-mentioned mechanisms are connected to quercetin's capacity to reduce lipid peroxidation and enhance GSH and CAT levels (P < 0.05). [79]

Balushi et al. (2019) found that antioxidant compounds like quercetin may improve sickle cell membrane permeability. Increased cation permeability in these cells promotes HbS polymerization by dehydrating RBCs and externalizing the prothrombotic aminophospholipid phosphatidylserine (PS). Quercetin inhibited three key cation routes involved in dehydration: deoxygenation-induced cation conductance, Ca²⁺ and K⁺ channel, and K⁺-Cl⁻ cotransporter. It also decreased Ca²⁺-induced PS exposure and hemolysis. [80]

There are also reports in the literature of glycosides derived from diosmetin (another flavonoid with the lowest binding energy with STAT1) with anti-sickle properties like diosmin. Gwozdzinsk et al. (2023) discovered that diosmin slightly reduced total carbonyl levels and slightly increased the total non-enzymatic antioxidant capacity of red blood cells. In turn, diosmin caused a big rise in the amounts of total thiols and glutathione in these cells. Researchers looked at the rheological properties of red blood cells and found that diosmin slightly lowers the internal viscosity of red blood cells. This, along with raising the levels of glutathione and thiol compounds, helps protect red blood cells from oxidative stress and keeps the cell membrane stable, which may be helpful for people with SCA. [81]

Chrysin is yet another flavonoid that has garnered significant attention for its anti-sickling properties. The compound has been linked to many mechanisms, including the modification of deoxy-haemoglobin, 2,3-bisphosphoglycerate mutase, redox homeostasis, and functional chemistry in human sickle erythrocytes. Muhammad et al. (2020) report that chrysin can reverse sickling by 66.5% and 69.6% at 12.5 µg/mL and prevent sickling to the maximum extent possible at 2.5 µg/mL. The observed proportion of hemolysis in comparison to induced erythrocytes indicates that treatment with chrysin considerably restored the integrity of the erythrocyte membrane. Lipid peroxidation was also considerably inhibited and reversed by chrysin. [82]

Conclusions
The use of network pharmacology, which incorporates bioinformatics methodologies, facilitated the discovery of ten CHGs associated with proinflammatory pathways in SCA (MX1, FIT1, IFIT3, STAT1, ISG15, GBP1, OAS1, OAS2, OAS3, and RSAD2) through the examination of a dataset obtained from the Gene Expression Omnibus (GEO).

All CHGs were linked to the INF-I signaling pathway, and based on their study, the signal transducer and activator of transcription 1 (STAT1) was selected as a biological target for molecular docking tests due to its importance in this pathway.

Text mining in the natural product databases Supernatural III, Ambinter-Greenpharma natural composite library, and AntiBase allowed the extraction of more than 100 phenolyc compounds. Nevertheless, for the docking studies, the most reported 15 flavonoids in the scientific literature were: acacetin, cyanidin, chrysin, diosmetin, epicatechin, fisetin, formononetin, gallolatechin, kaempferol, luteolin, morin, naringenin, peonidin, quercetin, and rhamnetin.

Docking studies showed that all assessed flavonoids have the lowest binding energy in comparison to the reference ligand, STOCK-1N-69677. Quercetin, diosmetin, and fisetin were the flavonoids having the lowest binding energy with STAT1. Among the interactions exerted by these compounds could be hydrogen bonds (V461, E524, S560, and S583), alkyl interactions (P465, K566, V509, and L562), and cation and anion interactions (R331 and E563, E559, and E524).

The docking findings were confirmed through molecular dynamics experiments, showing acceptable stability, comparable mobility, and flexibility across complexes, and stable SASA values. The radius of gyration measured compaction with the native receptor, resulting in similar results.

Flavonoids reported in this study have been found to have anti-inflammatory properties and potential therapeutic use in treating SCA. The main mechanism of their protective effect is the capture of free radicals and reactive oxygen species. Quercetin and its glycoside derivates, such as quercitrin, have been the most reported flavonoids with anti-sickling properties, normally used in traditional medicine in extracts from plants such as *Alchornea cordifolia* and *Woodfordia fruticosa*. Diosmetin glycosides and Chrysin are other flavonoids highly reported for their anti-sickling properties, whose mechanisms could be attributed to the reduction of total carbonyl levels and increasing the total non-enzymatic antioxidant capacity of red blood cells, as well as the modification of deoxy-haemoglobin, 2,3-bisphosphoglycerate mutase, redox homeostasis, and functional chemistry in human sickle erythrocytes.

**Declarations**

The authors declare no competing interests.

Lila Rosa Maturana Pérez: https://orcid.org/0009-0001-4727-5234

Johana Márquez Lázaro: https://orcid.org/0000-0001-6642-0874
Neyder Contreras Puentes: https://orcid.org/0000-0003-0974-8894
Antístio Álvis Amador: https://orcid.org/0000-0002-7324-2487
Albeiro Marrugo Padilla: https://orcid.org/0000-0003-1843-4782

Author Contribution

Lila Rosa Maturana Pérez: Formal analysis; Investigation; Writing - original draft. Johana Márquez Lázaro: Writing - original draft; Data curation; Writing - review & editing, Supervision. Neyder Contreras Puentes: Data curation; Formal analysis; Conceptualization. Antístio Álvis Amador: Funding acquisition; Project administration, Writing - review & editing, Supervision. Albeiro Marrugo Padilla: Writing - original draft; Data curation; Formal analysis; Investigation; Methodology; Conceptualization; Funding acquisition; Project administration.

References


42. Liu Y (2023) Integrative network pharmacology and in silico analyses identify the anti-omicron SARS-CoV-2 potential of eugenol, Heliyon. 9. https://doi.org/10.1016/j.heliyon.2023.e13853


76. Thangaswamy S, Branch CA, Ambadipudi K, Acharya SA Quercetin completely ameliorates hypoxia–reoxygenation-induced pathophysiology severity in ny1dd transgenic sickle mice: intrinsic mild steady state pathophysiology of the disease in ny1dd is also reversed. Biomolecules 11(10). https://doi.org/10.3390/biom11101473


Figures
**Figure 1**

**Identification of DEGs in the dataset GSE53441.** a) GEO2R volcano plots demonstrate the change in gene expression. The fold change and the P-value (log-scaled) are shown by the X and Y axes, respectively. Each symbol indicates a separate gene: red denotes upregulation and blue represents downregulation. c) The heatmap displayed log FC from DEGs.
Figure 2

Results of the functional enrichment analysis of the identified DEGs in DAVID database. The figure shows the enrichment percentages in terms of biological process, molecular function, cellular component, and pathway analysis.
**Figure 3**

**PPI-Net.** a) Features of PPI-Net build STRING and Cytoscape with DEGs. b) Central cluster of the PPI-net (MCODE score: 24.333).

<table>
<thead>
<tr>
<th>Score</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.91</td>
<td>PARP14</td>
</tr>
<tr>
<td>19.56</td>
<td>SAMD9L</td>
</tr>
<tr>
<td>19.76</td>
<td>IFI27</td>
</tr>
<tr>
<td>19.91</td>
<td>EPST11 – EIF2AK2</td>
</tr>
<tr>
<td><strong>20.83</strong></td>
<td>CMPK2</td>
</tr>
</tbody>
</table>
Figure 4

a) Central hub genes identified by the CytoHubba algorithms, Degree (Deg), Maximum Neighborhood Component (MNC), Maximal Clique Centrality (MCC), EcCentricity (EC) and Closenes. b) Venn diagram with the intersection analysis of central hub genes. c) Interaction between central hub genes.
Comparison of the central hub genes' levels of expression identified in the PPI-Net's main cluster. The expression levels were derived from the GEO2R analysis; all genes, except for IFI3, were substantially overexpressed in SCA patients (different letters on the bar, P < 0.05).
Figure 6

Receiver operator characteristic curves of central hub genes.
Type I interferons (IFN-I) activate the classic JAK–STAT pathways via the interferon receptors. Tyrosine kinase 2 (TYK2) and JAK1, two Janus activated kinases (JAKs), are linked to the two subunits of the type I IFN receptor, IFNAR1 and IFNAR2. The two subunits of this receptor, IFNR1 and IFNR2, are also linked to JAK1 and JAK2, respectively. Tyrosine phosphorylation of STAT1 (signal transducer and activator of transcription 1) and STAT2 is caused by activation of the JAKs linked to the type I IFN receptor. This
results in the formation of STAT1–STAT2–IRF9 (IFN-regulatory factor 9) complexes, also referred to as ISGF3 (IFN-stimulated gene (ISG) factor 3) complexes. To start the transcription of genes, these complexes go to the nucleus and attach to IFN-stimulated response elements (ISREs) in DNA. Figure designed in Biorender (https://app.biorender.com).

**Figure 8**

Principal interactions of flavonoids with the lowest binding energy with STAT1.
Figure 9

Molecular dynamic simulation of flavonoids with the best free energy in docking studies (quercetin, diosmetin, and fisetin), ligand of reference (STOCK-1N-69677), and STAT1. a) RMSD; b) RMSF. c) Radius of gyration. d) SASA. Red line: black line: STAT1 native; red line: ligand of reference-STAT1, green line: fisetin-STAT1, blue line: quercetin-STAT1, and yellow line: diosmetin-STAT1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.docx
- Supplementarytables.xlsx