Overactivation of IL6 cis-signaling in leukocytes is an inflammatory hallmark of deep vein thrombosis

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Abstract. Inflammation is a protective response of the body to various injuries, which is strictly regulated by a variety of factors, including immune cells and soluble mediators. However, dysfunction of this defensive mechanism often results in inflammation-driven diseases, such as deep vein thrombosis (DVT). The complex relationship between inflammatory cell activity and DVT has not been fully elucidated. The present study aimed to investigate the role of interleukin-6 (IL6) signaling transduction in DVT. To this aim, the expression levels of transmembrane isoforms of the IL6 receptor (IL6R) and the glycoprotein 130 responsible for the IL6 cis-signaling were evaluated in the peripheral blood mononuclear cells of patients with DVT and of healthy controls. The results indicated that leukocytes from patients with DVT exhibited overexpression of both IL6R and gp130 membrane isoforms and that these were strongly associated with the occurrence of DVT. Overall, the present findings indicated that IL6 cis-signaling may have a direct involvement in the leukocyte activation in DVT and may serve as a predictive biomarker of DVT development.

Introduction

Deep vein thrombosis (DVT) is a pathological condition characterized by the obstruction of veins of the deep venous circle caused by a thrombotic plug that blocks the normal blood flow (1). The main causes of DVT are alterations in the blood flow, damage of the vessel wall and hypercoagulability of the blood (2). DVT and pulmonary embolism (PE), a major DVT-induced complication, are often considered two sides of the same coin leading to a pathological condition called venous thromboembolism (VTE), which represents a common cause of morbidity and mortality (3). Various risk factors are associated with the development of VTE, including obesity, abnormal platelet activation, alteration of blood oxygen concentration, genetic factors, trauma and surgery (4-6), as well as infections (7). Recent reports demonstrated that a fraction of patients with COVID-19 present with coagulation and fibrinolysis alterations leading to fatal complications (8-10). Besides these risk factors, chronic inflammation has been frequently associated with DVT. Some proinflammatory markers and cytokines, including tumor necrosis factor (TNF)α, C-reactive protein (CRP), interleukin (IL)6 and IL8, can promote a procoagulant state inducing the expression of the tissue factor (11) that actives the coagulation cascade resulting in fibrin deposition (12). Several studies have demonstrated that IL6 expression is increased in patients with DVT (13-15), and its activity may be affected by alterations of the canonical transduction signaling (cis-pathway) resulting in activation of the well-known IL6 trans-signaling pathway (16). The soluble form of the IL6 receptor (sIL6R) has a central role in activating intracellular signaling in cells that are physiologically IL6-unresponsive but express the interleukin-6 cytokine family signal transducer (IL6ST, also known as gp130) (17-19). The plasma levels of sIL6R mainly depend on the proteolytic activity of a disintegrin and metalloproteinase (ADAM) family proteins on the IL6R transmembrane (TM) isoform. IL6R cleavage is enhanced by the IL6R Asp358Ala mutation (SNP rs2228145) (16). In addition, alternative splicing of the IL6R gene is responsible for the expression of sIL6R isoforms characterized by the deletion of the TM domain (19). Similarly, the soluble form of gp130 (sgp130) is produced by ADAM cleavage and alternative splicing of its gene, IL6ST. Unlike the activating role of sIL6R, the sgp130 inhibits the IL6 trans-signaling by sequestering the IL6/sIL6R complex (20).

The dysregulation of both cis and trans IL6 signaling may affect the response to IL6 of immune cells involved in inflammation and its related diseases, including DVT. For instance, a study reported an accumulation of white blood

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cells, mainly leukocytes and neutrophils, inside the thrombus or surrounding vein wall during DVT, suggesting a possible correlation between thrombosis and inflammation (21).

In the present study, the expression levels of IL6R and IL6ST were analyzed in peripheral blood mononuclear cells (PBMCs) to investigate the involvement of IL6 signaling in DVT-associated immune responses in a cohort of 19 patients with DVT and 22 healthy individuals. The expression levels of the transcriptional isoforms of IL6R and IL6ST associated with their protein membrane retention were also evaluated. In addition, the SNP rs2228145 of IL6R was detected to examine its effect on the balance between soluble and membranous IL6R isoforms in DVT.

Materials and methods

Patients and samples. A consecutive series of 19 patients with DVT of the lower limbs (age range, 46-71 years) was included in the present study from January 2019 to March 2020. The patients were admitted to the Internal Medicine Department of G. Rodolico University Hospital (Catania, Italy). The DVT diagnosis was performed with data from ultrasound (US) examination of the venous circulation of the lower limbs, the non-compression of deep veins induced by the US probe (CUS test) was considered to diagnose lower limb DVT. Patients with pregnancy, malignancy, liver disorder and inflammatory bowel disease were excluded from the study. The control group consisted of 22 healthy subjects (age range, 41.7-62 years) with no history of any chronic disease.

Patients and controls had similar ethnic background and originated from the same geographic area. The Institutional Review Board of University Hospital 'G. Rodolico' of Catania approved all procedures. All participants gave written consent for blood collection. Venous blood obtained from all subjects was placed in tubes with or without heparin. Samples were centrifuged at 2,000 x g for 10 min at room temperature to recover plasma, serum and buffy coat fractions, then stored at -80˚C until subsequent analysis. The demographic and clinical characteristics of both patients and controls are reported in Table I.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from buffy coat samples from patients with DVT and healthy control using the TRIzol® LS Reagent (cat. no. 10296028; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. For each sample, 750 ng of tota RNA, quantified using a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific, Inc.), were treated with RNase-free DNase I (cat. no. EN0525; Thermo Fisher Scientific, Inc.) to remove possible DNA contamination. A mass of 400 ng treated RNA (final concentration 20 ng/μl) was then converted into cDNA using the SuperScript IV Reverse Transcriptase kit (cat. no. 18090050; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. The cDNA obtained from each sample was subsequently analyzed by qPCR using the Luminaris Color HiGreen qPCR Master Mix, high ROX (cat. no. K0362; Thermo Fisher Scientific, Inc.). The total and TM isoforms of both IL6R and IL6ST were amplified with a 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the primer pairs and thermocycling conditions reported in Table II. The expression levels of targets were normalized with those obtained for the GAPDH housekeeping gene. Relative fold changes in gene expression were calculated using the $2^{-\Delta\Delta Cq}$ method (22). Furthermore, the IL6R TM/IL6R and IL6ST TM/IL6R mRNA ratios were calculated by dividing the Cq value of TM isoform by the Cq value of the total mRNA of each gene.

IL6R exon 9 sequencing. Genomic DNA from buffy coat samples from both DVT patients and healthy controls was extracted using a standard phenol-chloroform method and quantified using a spectrophotometer assay (NanoDrop 1000). Exon 9 from the IL6R gene was amplified using DreamTaq DNA Polymerase (cat. no. EP0702; Thermo Fisher Scientific, Inc.) with primers and thermocycling conditions reported in Table II. After PCR amplification, the DNA amplicons were purified with GeneJET PCR Purification kit (cat. no. K0702; Thermo Fisher Scientific, Inc.), according to manufacturer's indications. The purified samples were sequenced using the Mix2Seq kit (Eurofins Genomics Italy), according to the manufacturer's instructions. Chromas Lite software version 2.6.6 (technelysium.com.au/wp/) was used to retrieve and analyze the DNA sequences.

Statistical analysis. Unpaired Student's t-test was used to compare normally distributed data. Whereas, the Mann-Whitney test was performed when data were not normally distributed(Shapiro-Wilk normality test). The contingency analysis was performed using Fisher's exact test. To assess the diagnostic performance of putative biomarkers, the receiver operating characteristic (ROC) was performed considering specificity, sensitivity and likelihood ratio (LR), and the cutoff value was reported for each biomarker. All statistical analyses were performed using GraphPad Prism software Version 8.0.2 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

The analysis of total IL6R mRNA expression levels revealed no significant difference between the DVT and control groups (Fig. 1A). By contrast, the ratio of IL6R TM to IL6R total mRNA expression was increased in patients with DVT by 1.66-fold compared with healthy controls ($P<0.01$; Fig. 1B). Moreover, a significant reduction of total IL6ST mRNA was observed in patients with DVT compared with the healthy controls ($P<0.01$; Fig. 2A). However, the ratio of IL6ST TM isoform to IL6ST total mRNA expression was 1.5-fold higher ($P<0.01$) in patients with DVT compared with controls (Fig. 2B). Mutation analysis of IL6R rs2228145 SNP revealed a high frequency of the mutated (AC and CC) genotypes in both DVT and control groups (DVT, 68.42% and CTRL, 66.67%; Table III and Fig. S1). When the samples were stratified according to the IL6R sr2228145 SNP, no significant association between the mutated genotypes and the relative expression of IL6R TM was observed in any of the groups (Fig. 3).

In order to evaluate the association between the relative expression of IL6R TM and the occurrence of DVT, all subjects were stratified according to their IL6R TM/IL6R

Table I. Demographic and clinical characteristics of the subjects involved in the present study.

Clinical characteristics	Control	DVT	P-value
Age in years, median (range)	48 (41.7-62)	56 (46-71)	0.2392[b]
Sex, number (%)			
Male	12 (54.5)	12 (63.2)	0.7600[a]
Female	10 (45.5)	7 (36.8)	
Median C-reactive protein level (range), mg/l	2.06 (0.79-6.96)	6 (3-11)	0.0400[b]
Thrombophilic patients, number (%)	Not applicable	12 (63)	
Cardiopathic patients, number (%)	Not applicable	5 (27.7)	

[a]Fisher's exact test; [b]Mann-Whitney test.

Table II. Primers and PCR conditions.

A, Expression profiling

Oligo name	Sequence (5'-3')	Thermocycling conditions
IL6R total F	GTCCCAGAAGTTCTCCTGCC	Uracil-DNA glycosylase pre-treatment at 50˚C for 2 min, followed by an initial denaturation step at 95˚C for 10 min, then 40 cycles at 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec.
IL6R total R	GGCTGCAAGATTCCACAACC	
IL6R TM F	CACGCCTTGGACAGAATCCA	
IL6R TM R	CAATGGCAATGCAGAGGAGC	
IL6ST total F	GCCTCAACTTGGAGCCAGA	
IL6ST total R	TCCCACTTGCTTCTTCACTCC	
IL6ST TM F	TGAAACTGCTGTGAATGTGGA	
IL6ST TM R	GCTAAGCAAACAGGCACGAC	
GAPDH F	AGAAGGCTGGGGCTCATTTG	
GAPDH F	AGGGGCCATCCACAGTCTTC	

B, IL6R exon 9 sequencing

Oligo name	Sequence (5'-3')	Amplification conditions
IL6R exon 9 F	TGTTGGTTGGCAGAGCTGTT	95˚C for 3 min, followed by 35 cycles of 95˚C for 30 sec, 60˚C for 3 sec, 72˚C for 1 min and final 72˚C for 5 min.
IL6R exon 9 R	CACCTAAAACACGGCTTGGC	

IL, interleukin; R, receptor; TM, transmembrane; ST, signal transducer; F, forward; R, reverse.

mRNA ratio (cut-off value, 0.315), the presence of the IL6R rs2228145(C) alleles, or both. The results from Fisher's exact tests revealed a strong association between IL6R TM expression and the occurrence of DVT [odds ratio (OR), 17.07; confidence interval (CI), 3.478-83.75; P<0.001; Table III). A similar association was observed when stratifying the subjects by both the IL6R TM/IL6R ratio and IL6R rs2228145(C) allele (OR, 15.45; CI, 2.726-87.32; P<0.001; Table III). By contrast, no significant association was observed when patients were stratified according to the presence of the IL6R rs2228145(C) allele (Table III).

Concerning the IL6ST TM/IL6ST mRNA ratio , the stratification of samples above the 75th percentile of normal values (>0.2725) highlighted a strong association between IL6ST TM/IL6ST mRNA ratio and the occurrence of DVT (OR, 10.50; CI, 2.335-47.22; P<0.003; Table III).

Finally, a ROC curve analysis was performed to evaluate the diagnostic performance of the IL6R TM/IL6R ratio in DVT. The cutoff of IL6R TM/IL6R ratio was 0.335 (sensitivity, 78.95%; specificity, 95.00%) with a LR of 16.58, indicating a significant increase in the probability of DVT given a positive test (Fig. 4A). Similarly, the IL6ST TM/IL6ST ratio (cutoff, 0.31) exhibited good diagnostic performance parameters (sensitivity, 72.00%; specificity, 90.00%) with a significant association with DVT (LR, 7.22) (Fig. 4B).

Discussion

IL6 is a pleiotropic cytokine involved in several physiological processes, including the modulation of immune responses and several vascular disorders such as peripheral arterial disease, atherosclerosis and VTE (23-27). The effects of IL6

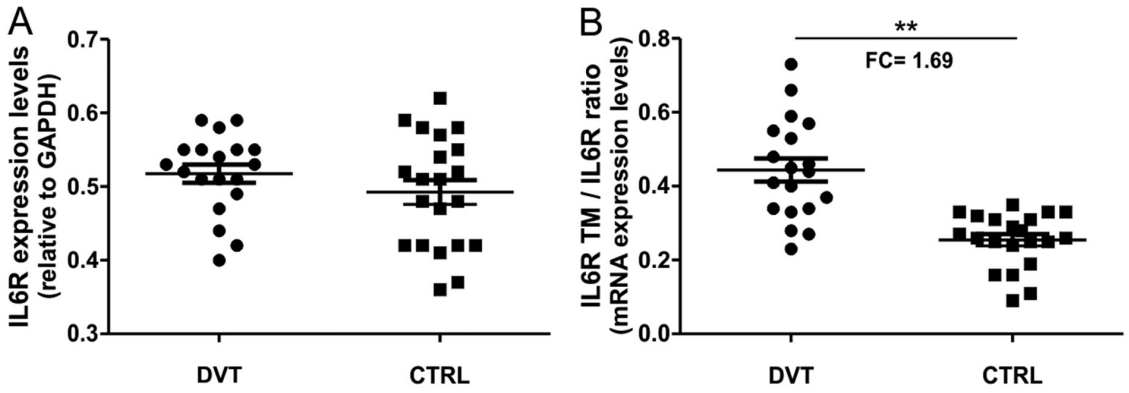

Figure 1. Quantification of IL6R expression. mRNA expression levels of (A) total IL6R and (B) IL6R TM/IL6R ratios were assessed in peripheral blood mononuclear cells from patients with DVT and from healthy controls. **P<0.01. IL, interleukin; R, receptor; TM, transmembrane; DVT, deep vein thrombosis; CTRL, control; FC, fold change.

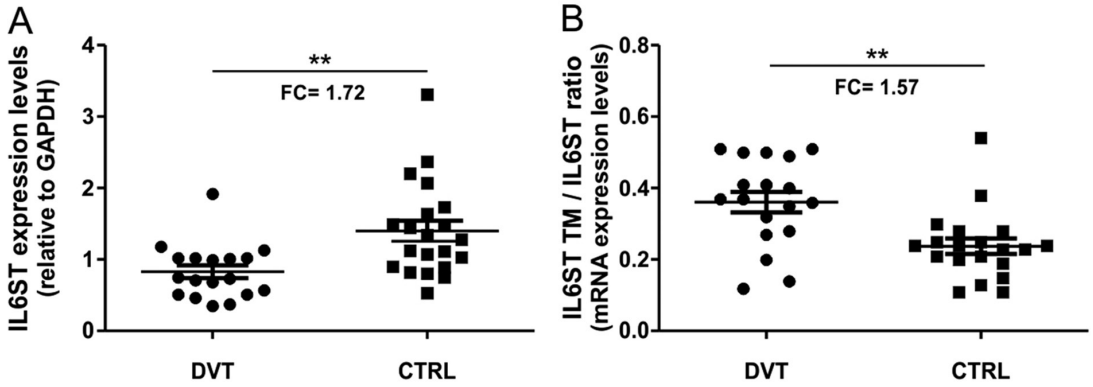

Figure 2. Quantification of IL6ST expression. mRNA expression levels of (A) total IL6ST and (B) IL6ST TM/IL6ST ratios were assessed in peripheral blood mononuclear cells from patients with DVT and from healthy controls. **P<0.01. IL, interleukin; ST, signal transducer; TM, transmembrane; DVT, deep vein thrombosis; CTRL, control; FC, fold change.

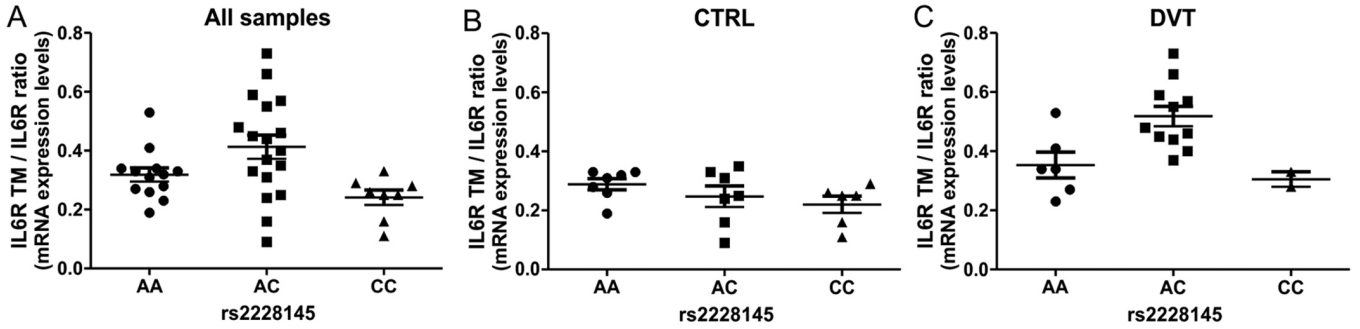

Figure 3. Stratification of IL6R TM/IL6R ratio values according to IL6R SNP rs2228145. (A) All samples. (B) Control group. (C) Patients with DVT. IL, interleukin; R, receptor; TM, transmembrane; SNP, single-nucleotide polymorphism; DVT, deep vein thrombosis; CTRL, control.

are mediated by both the IL6R and the gp130 TM receptors expressed by responsive cells (such as hepatocytes and leucocytes), activating the IL6 canonical signaling, also known as IL6 cis-signaling (16). The amount of these receptors on the membrane surface depends on the proteolytic cleavage of the IL6 binding domains by specific ADAM proteinases, as well as on the alternative splicing of the IL6R and IL6ST mRNA that results in the deletion of their transmembrane domains (16). The release of the soluble forms of IL6R and gp130 allows the activation of IL6 trans-signaling in cells lacking IL6R that are normally unresponsive to IL6 stimulation, which may be activated by IL6 if the gp130 receptor is present on their membrane surfaces (17-19). Several autoimmune (such as rheumatoid arthritis and systemic lupus erythematosus)and inflammatory diseases (such as inflammatory bowel disease and psoriasis) are sustained by the alterations at different levels of both cis- and trans-signaling of IL6 (28). Consequently, therapeutic targeting of IL6 signaling with available drug inhibitors is not always effective (28,29). Previous evidence has demonstrated the key role of inflammation in VTE/DVT pathogenesis mediated by different

Table III. Association analysis of IL6R and IL6R rs2228145 expression and the occurrence of DVT.

Parameter	Patients with DVT, number (%)	Healthy controls, number (%)	OR, 95% CI[a]	P-value[a]
IL6R TM/IL6R mRNA ratio				
≥0.315	16 (84.21)	5 (23.80)	17.07, 3.48-83.75	<0.001
<0.315	3 (15.79)	16 (76.20)		
IL6R rs2228145 genotype				
AC and CC genotypes	13 (68.42)	14 (66.67)	1.08, 0.29-4.08	1
Wild-type	6 (31.58)	7 (33.33)		
IL6R TM/IL6R mRNA ratio& rs2228145 genotype				
≥0.315 & AC and CC	12 (63.15)	2 (10.00)	15.45, 2.73-87.32	<0.001
≥0.315 WT; <0.35 WT, AC and CC	7 (36.84)	18 (90.00)		
IL6ST TM/IL6ST mRNA ratio				
≥0.2725	14 (77.78)	5 (25.00)	10.50, 2.33-47.22	<0.003
<0.2725	4 (22.22)	15 (75.00)		

[a]Fisher's exact test. DVT, deep vein thrombosis; IL, interleukin; R, receptor; TM, transmembrane; ST, signal transducer; OR, odds ratio; CI, confidence interval.

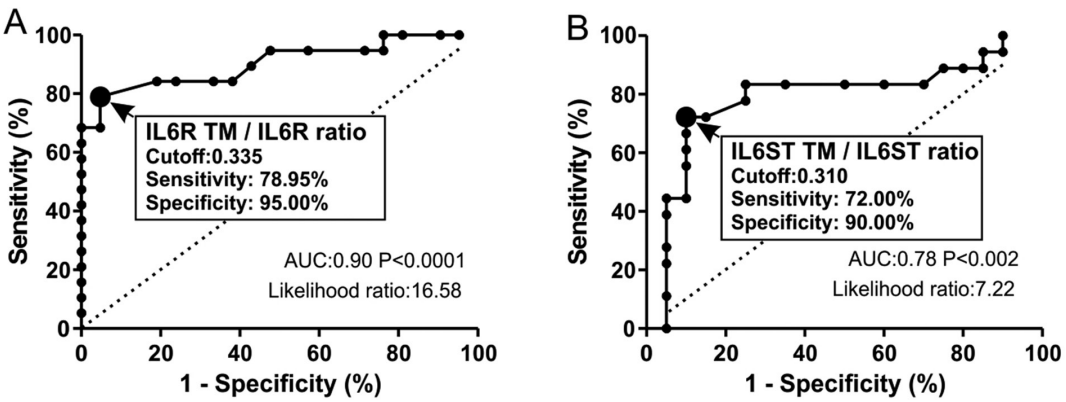

Figure 4. Evaluation of the diagnostic performance of IL6 signaling in DVT. Receiver operating characteristic for (A) IL6R TM/IL6R ratios and for (B) IL6ST TM/IL6ST ratios in DVT. IL, interleukin; R, receptor; TM, transmembrane; ST, signal transducer; DVT, deep vein thrombosis; AUC, area under the curve.

cytokines, including IL6, which is responsible for aberrant inflammatory responses (30). Several studies have highlighted how genetic polymorphisms in IL6 may be predictive of peripheral arterial disease and DVT in patients with cancer (31,14). In addition, the activation of leukocytes, as well as endothelial cells and platelets, can trigger the coagulation cascade by the formation of microparticles within intact veins (11). The role of leukocytes and their activation in both thrombus generation and vein remodeling have been debated (21).

The aim of the present study was to investigate the involvement of IL6R and IL6ST isoforms in the activation of the IL6 cis-signaling in DVT, focusing on the role of the IL6R Asp358Ala mutation (SNP rs2228145) in the alternative splicing of IL6R exon 9. In particular, the expression profiling of the different splicing isoforms of the IL6R and IL6ST receptor was analyzed in leukocytes obtained from patients with DVT and healthy donors.

The expression analysis revealed that in leucocytes from DVT patients the expression of TM transcript isoforms of both IL6R and IL6ST receptors were higher compared with those coding for the soluble isoforms. These results were supported by the strong association between higher TM transcript levels of both IL6R and IL6STand the occurrence of DVT, suggesting a crucial role of IL6 cis-signaling in DVT. Increased expression of IL6R on the membrane of leukocytes may provide a higher responsiveness to IL6, and this may be further reinforced by IL6ST being simultaneously overexpressed on the cellular membrane, thereby also activating the IL6 trans-signaling. The measurement of these molecular biomarkers may be useful to identify a subset of high-risk DVT patients that could develop a hyperinflammatory immune response associated with severe clinical outcomes (13).

The evaluation of IL6 signaling and the current results may have a fundamental impact in the prediction of DVT, as well as in the monitoring of vascular disorders due to other pathologies, and in particular during the COVID-19 pandemic. COVID-19 infection induces endothelial damage and cardiovascular disorders (10,32). Indeed, in some patients with severe COVID-19

symptomatology, abnormal expression of IL6 was observed, leading to a cytokine imbalance defined as 'cytokine storm' responsible for severe respiratory syndrome (10,33). Notably, the COVID-19 'cytokine storm' is not observed in all patients with clinical symptoms, suggesting that genetic factors affecting key cytokines or immune cells or other comorbidities, in particular diabetes and cancer, may be related to this complication (34-37). It remains unknown if IL6 polymorphisms are also associated with COVID-19 severity or with its vascular complications.

To investigate the molecular mechanisms capable of driving the expression of IL6R TM, the mutational status of IL6R was assessed, as it has been demonstrated that the rs2228145 SNP impairs IL6R transcript splicing (19). However, the present results were not consistent with this previous report (19), as no significant association between the IL6R rs2228145 variant and the IL6R TM isoform was observed, probably due to the small number of cases analyzed. Therefore, it can be hypothesized that other molecular mechanisms could affect the relative expression of either the TM or soluble IL6R isoforms. For instance, it has been demonstrated that DNA methylation can regulate the splicing mechanism, thus influencing the binding of spliceosome proteins to nascent pre-mRNA (38,39).

Altogether, the present results support the hypothesis that IL6 activates leukocytes, which in turn may be responsible for the inflammatory status in DVT through the overexpression of both IL6R and IL6ST receptors. The assessment of the IL6 receptor complex could be a hallmark of the early inflammatory conditions associated with DVT development and may be useful for the management of patients at risk of thromboembolic events. Further studies will be required to confirm the activation of IL6 cis-signaling in DVT at the protein level and in a larger patient cohort.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Authors' contributions

SSS and SC designed the project and confirm the authenticity of the raw data. BMT, RS and SC wrote the manuscript and performed the experiments. GG and RS performed acid nucleic extraction, PCR and RT-qPCR. SC and BMT performed the statistical analysis. SSS revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted according to the guidelines of The Declaration of Helsinki, and ap-proved by the Institutional Review Board of The University Polyclinic of Catania. Informed consent was obtained from all subjects involved in the study for participation and data publication.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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