Diagnosis, treatment and long-term effects of venous thromboembolism
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Chapter 12

Toll-like receptor 9 gene expression in the post-thrombotic syndrome, residual thrombosis and recurrent deep venous thrombosis: a case-control study

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Abstract

Objective: Animal models suggest that toll-like receptor 9 (TLR9) promotes thrombus resolution after acute deep venous thrombosis (DVT). We hypothesised that TLR9 expression is lower in patients with post-thrombotic syndrome (PTS) and investigated the role of TLR9 in residual thrombosis (RT) and recurrence.

Methods: Patients with a history of DVT with PTS (cases, n=30) and without PTS after minimal 24 months follow-up (controls, n=30) were selected. Healthy individuals (HI, n=29) without DVT were included as reference. TLR9 mRNA expression in leukocytes was determined by qPCR and normalised to the housekeeping succinate dehydrogenase subunit A gene using the ΔCt method. Sub analyses were performed to explore the TLR9 expression in patients with and without RT and multiple DVT episodes.

Results: The median TLR9 expression was 0.45 (interquartile range 0.31 to 0.93), 0.39 (0.25 to 0.69) and 0.62 (0.32 to 0.75) in cases, controls and HI respectively (p=0.61). The median TLR9 expression was 0.39 (0.26 to 0.51) in patients with RT compared to 0.55 (0.30 to 0.86, p=0.13) in those without. The median TLR9 expression was significantly lower in patients who had one DVT compared to patients with recurrent DVT, 0.37 (0.23 to 0.63) versus 0.55 (0.43 to 0.96) respectively (p<0.01).

Conclusion: No significant difference in TLR9 expression was found between cases, controls and HI. However TLR9 expression seems lower in individuals with DVT and RT, albeit not significant. Interestingly, TLR9 might play a role in recurrent DVT, as the TLR9 expression was significantly higher in patients with recurrent DVT.
Introduction

Post-thrombotic syndrome (PTS) is a common complication of deep venous thrombosis (DVT), which occurs in 20-50% of patients after DVT.\(^1\)\(^4\) The clinical presentation may vary from minor signs including skin discoloration, venous ectasia, discomfort and swelling, to severe manifestations such as chronic pain, intractable edema or leg ulcers impairing daily activities.\(^5\)\(^6\) Due to its high prevalence, severity and chronicity, PTS has a significant impact on quality of life and is associated with considerable socio-economic consequences for both the patient and the health care system.\(^4\)\(^6\)

The pathogenesis of PTS is not yet fully understood. It has been proposed that the symptoms of PTS are caused by the end-organ manifestation of venous hypertension, as a result of several processes such as tissue remodeling, impaired thrombus resolution and continued inflammation.\(^7\)\(^9\) Besides Virchow’s triad, there are strong indications that inflammation also plays a role in the pathogenesis of DVT.\(^10\) During an inflammatory process multiple procoagulant mechanisms are stimulated, whereas anticoagulant mechanisms are inhibited. Although immune-mediated inflammation plays a role in the thrombotic process, little is known about the role of innate immunity.

Toll-like receptor 9 (TLR9) is a member of the toll-like receptor family mainly expressed on the membrane surface of endosomes and lysosomes of leukocytes.\(^11\) TLR9-mediated recognition of (viral or bacterial) unmethylated CpG dinucleotides triggers a T-lymphocyte helper 1 (Th1) immune response directed against the intracellular pathogen. TLR9 has also been involved in thrombus formation and resolution.\(^10\)\(^12\)\(^13\) In animal studies, deletion of TLR9 was associated with significantly increased thrombus size, decreased neovascularisation, fibrosis and thrombus resolution. This may be due to the fact that deletion of TLR9 lowers the levels of Th1 cytokines (INF\(\alpha\), IL-1\(\alpha\), IL-2), whereas inflammation is needed in the process of thrombus resolution. Therefore decreased cytokine levels could account for impaired thrombus resolution.\(^13\) In a prospective study in humans, TLR9 expression was down-regulated in patients with a DVT at the time of diagnosis compared to healthy controls.\(^10\)

So far, the association between TLR9 and PTS in humans has not been investigated. As deletion or low levels of TLR9 are associated with larger thrombi and less thrombus resolution, we hypothesised that TLR9 expression is lower in patients with PTS. Furthermore we investigated the role of TLR9 in residual thrombosis (RT) and recurrent DVT.
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Materials and methods

Patients and study design

A case-control study was performed, including 30 patients with a history of DVT who developed PTS (cases) and 30 patients with a history of DVT without PTS (controls). Thirty healthy individuals (HI) without a history of venous thromboembolism were invited to participate as a reference population. The cases and controls were selected from a cohort of patients that was followed prospectively after acute DVT. Patients were recruited from the Maastricht University Medical Centre or the Flevohospital in Almere, the Netherlands. Patients with a history of DVT and PTS development (Villalta ≥5) were defined as cases5,14. Patients with a history of DVT and without PTS development (Villalta ≤4) after a minimal follow-up of 2 years after DVT, were defined as controls. Subjects or patients with known venous insufficiency were excluded from the study because of possible interference with the endpoints. Cases, controls, and HI were similar for gender, age and body mass index.

RT was determined with ultrasonography 3-12 months after treatment with anticoagulation.

The medical ethical committee of the Maastricht University Medical Centre approved the study and all patients gave written informed consent.

Measurement of TLR9 gene expression

Venous blood was drawn from all subjects in EDTA polypropylene tubes for plasma. The EDTA tubes were centrifuged for 5 minutes at 2500 g (3790 rpm, room temperature). One ml of buffy coat was separated and 10 ml RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) were added to the buffy coat and mixed well. The samples were stored at -20°C until analysis.

Total RNA was purified from the preserved buffy coats with the High Pure RNA isolation kit (Roche Diagnostics). After quantification at the NanoDrop, total RNA was subjected to reverse transcription with random primers at 37°C for 120 minutes (High Capacity cDNA Reverse-Transcription kit, Life Technologies, Bleiswijk, the Netherlands). TLR9 mRNA expression in leukocytes was determined by qPCR on a LightCycler 480 (Roche Diagnostics) using validated TLR9-specific primers and hybridisation probes (RealTime-ready assay, Roche Diagnostics) according to the manufacturer’s instructions. Similar Real-Time-ready assays were used to quantify the mRNA expression of the housekeeping genes mitochondrial succinate dehydrogenase subunit A (SDHA) and beta-actin (ACTB)15. TLR9 expression was normalised to the expression of SHDA and ACTB using the ΔCt method. Relative expression was calculated as

\[ 2^{-\Delta\Delta C_t}} \]

where \( C_t \) being the threshold cycle.
Statistical analyses
SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The non-parametric Kruskal-Wallis test was performed to test for differences in TLR9 expression levels between cases, controls and HI. Post-hoc Mann-Whitney U test was performed on significant (p<0.05) Kruskal-Wallis test results. Sub analyses with the Mann-Whitney U test were performed to compare the TLR9 expression in patients with and without RT and in patients with one and multiple DVT episodes. TLR9 expression levels were described as medians and interquartile ranges (IQR). Multivariate regression analyses were performed to determine whether the use of statins, aspirin and anticoagulation affects the TLR9 expression. Spearman correlation was performed to test the association between the two housekeeping genes.

Results
The total study population consisted of 89 subjects; 30 cases, 30 controls and 29 HI were included. Ninety subjects were originally selected, but one HI was found ineligible because of the presence of venous insufficiency. The median age was 64 years (IQR 46 to 76) for cases, 67 years (IQR 58 to 76) for controls and 61 years (IQR 59 to 68) for the HI (Table 12.1). Blood collection was performed at a median follow-up of 85 months (IQR 58 to 122) after the first DVT in the cases and 86 months (IQR 50 to 99) after the first DVT in the controls (Table 12.1). Median Villalta score was 7 (IQR 6 to 9) for the cases and 2 (IQR 1 to 3) for the controls (Table 12.1). Use of aspirin was similar in cases, controls and HI (p=0.91). Use of statins seemed higher in cases and controls, but this was not statistically significant (p=0.22). Use of oral anticoagulants was significantly higher in cases as compared to controls and HI (p<0.01).
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Table 12.1 | Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Cases (n=30)</th>
<th>Controls (n=30)</th>
<th>HI (n=29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (IQR)</td>
<td>64 (46-76)</td>
<td>67 (58-76)</td>
<td>61 (59-68)</td>
</tr>
<tr>
<td>Male, no (%)</td>
<td>15 (50%)</td>
<td>14 (47%)</td>
<td>15 (52%)</td>
</tr>
<tr>
<td>BMI, median (IQR)</td>
<td>28 (25-32)</td>
<td>25 (24-31)</td>
<td>26 (24-29)</td>
</tr>
<tr>
<td>Villalta score*, median (IQR)</td>
<td>7 (6-9)</td>
<td>2 (1-3)</td>
<td></td>
</tr>
<tr>
<td>Recurrent DVT</td>
<td>14 (47%)</td>
<td>9 (30%)</td>
<td></td>
</tr>
<tr>
<td>Follow-up after first DVT, median months (IQR)</td>
<td>85 (58-122)</td>
<td>86 (50-99)</td>
<td></td>
</tr>
<tr>
<td>Follow-up after most recent DVT, median months (IQR)</td>
<td>51 (31-73)</td>
<td>64 (41-89)</td>
<td></td>
</tr>
<tr>
<td>Oral anticoagulant use</td>
<td>16</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Acenocoumarol</td>
<td>13</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Phenprocoumon</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Statin use</td>
<td>11</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Aspirin use</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Abbreviations: HI, healthy individuals; IQR, Interquartile range; BMI, body mass index; DVT, deep venous thrombosis.

*Average of 1-4 measurements of Villalta score
**TLR9 expression in cases, controls and HI**

In line with data on record\(^1\), ACTB mRNA (median \(C_t\) 15.5) was more abundant than SHDA mRNA (median \(C_t\) 24.5) in leukocyte total RNA, but the mRNA expression of these housekeeping genes was not influenced by the disease status (case, control or HI). The median \(C_t\) of TLR9 mRNA over the whole population was 25.3.

The median TLR9 expression relative to SHDA was 0.45 (IQR 0.31 to 0.93) in cases, 0.39 (IQR 0.25 to 0.69) in controls and 0.62 (IQR 0.32 to 0.75) in HI (\(p=0.61\), Figure 12.1). Multivariate regression analysis did not show an effect of statins, aspirin or anticoagulant use on the expression of TLR9. The correlation (Spearman’s coefficient) between SHDA-normalised and ACTB-normalised TLR9 mRNA expression levels in the whole population was 0.60 (\(p<0.01\)). Overall, SHDA is considered a more reliable housekeeping gene for mRNA studies in leukocytes.\(^1\)

**TLR9 expression and residual thrombosis**

Information on RT was available for all the patients with a history of DVT, irrespective of the development of PTS. RT was present in 25 (42%) patients with a history of DVT. The median TLR9 expression relative to SHDA was 0.39 (IQR 0.26–0.51) in patients with RT compared to 0.55 (IQR 0.30–0.86, \(p=0.13\)) in patients without RT (Figure 12.2).

**TLR9 expression and recurrent DVT**

Recurrent DVT occurred in 23 (38%) of patients with a history of DVT. The median TLR9 expression relative to SHDA was significantly lower in patients who had one DVT compared to patients with recurrent DVT, 0.37 (IQR 0.23–0.63) versus 0.55 (IQR 0.43–0.96) respectively (\(p<0.01\), Figure 12.3). Multivariate regression analysis did not show an effect of statins, aspirin or anticoagulant use on the expression of TLR9 in patients with one DVT versus patients with recurrent DVT.

**TLR9 expression and follow-up duration**

Patients with a follow-up ≤ 36 months showed a tendency to have a higher median TLR9 expression relative to SHDA than patient with a follow-up > 36 months, 0.67 (IQR 0.26–1.71) versus 0.43 (IQR 0.27–0.70) respectively, \(p=0.07\) (Figure 12.4).
**Figure 12.1** | TLR9 gene expression in cases, controls and HI

Abbreviations: HI, healthy individuals; SDHA, succinate dehydrogenase complex subunit A; TLR9 expression: SDHA, TLR9 expression relative to SHDA.

**Figure 12.2** | TLR9 gene expression and residual thrombosis

Abbreviations: RT, residual thrombosis; SDHA, succinate dehydrogenase complex subunit A; TLR9 expression: SDHA, TLR9 expression relative to SHDA.
**Figure 12.3** | TLR9 gene expression and recurrent DVT

Abbreviations: DVT, deep venous thrombosis; SDHA, succinate dehydrogenase complex subunit A; TLR9 expression: SDHA, TLR9 expression relative to SHDA.

**Figure 12.4** | TLR9 gene expression and follow-up duration

Abbreviations: FU, follow-up; SDHA, succinate dehydrogenase complex subunit A; TLR9 expression: SDHA, TLR9 expression relative to SHDA.
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Discussion

We sought to assess whether less thrombus resolution was associated with a lower expression of TLR9 and consequently to PTS development. Patients with RT seem to have a lower TLR9 expression. This implies that TLR9 might affect thrombus resolution, which is in line with an earlier animal study where mice with TLR9 deletion had less thrombus resolution.\textsuperscript{13} In contrast to the expectation, we found no difference in the TLR9 expression between patients with the PTS, patients without PTS and HI. This could be due to the fact that a long time has expired since the occurrence of the last DVT (median 63 months), whereby the inflammatory reaction diminishes. Although we deliberately chose to select patients who had an acute DVT more than 2 years prior to inclusion to ascertain the PTS diagnosis and to rule out the acute phase reaction, we observed a lower TLR9 expression in patients with a follow-up of more than 36 months as compared to patients with a follow-up of 36 months or less, suggesting extinguished inflammation. This is also supported by our previous findings where no indication of enhanced inflammation was found in our PTS cohort, as levels of CRP, Interleukin-6 (IL-6) and Interleukin-8 (IL-8) were not different between the cases, controls and HI after a median follow-up of 63 months.\textsuperscript{16} Other studies also showed that inflammatory markers like CRP, IL-6, IL-8 collected more than 12 months after the DVT episode are not predictive for PTS development, while earlier blood sampling of inflammation markers yielded conflicting results.\textsuperscript{17-21} Another variable that might have affected the results could be the low Villalta score in our PTS group. The median Villalta score of the patients with PTS was 7. One can speculate that if the patients would have had more severe PTS, the difference in TLR9 expression between the cases, controls and HI could have been more evident.

Strikingly, our results suggest that TLR9 might play a role in recurrent DVT. Patients with one DVT episode had a lower TLR9 expression relative to SHDA as compared with patients with recurrent DVT. This result was not influenced by the use of statins, aspirin or oral anticoagulants as regression analysis did not show an effect of these drugs on the TLR9 expression. This finding was unexpected as we hypothesised that a lower TLR9 expression is associated with larger thrombi and less thrombus resolution, consequently leading to higher risk of PTS development. Therefore, we would expect patients with recurrent DVT also to have lower TLR9 expression. As venous thrombosis is a sterile inflammatory process the high TLR9 expression could be a manifestation of enhanced inflammation in patients with recurrent DVT. The pathophysiology behind TLR9 expression and recurrent DVT is uncertain and further investigation is needed to address this and to evaluate whether TLR9 could play a role in the prediction of recurrent DVT.
Conclusion

Firstly, we did not find a significant difference in TLR9 expression between DVT patients with PTS as compared to those without this syndrome and HI. TLR9 expression seems lower in individuals with DVT and RT, albeit not statistically significant. Interestingly, TLR9 might play a role in recurrent DVT as the TLR9 expression was significantly higher in patients with recurrent DVT compared to patients with one DVT episode. However, the pathophysiology behind TLR9 expression and recurrent DVT is unclear and needs further investigation.
Reference list


