Title page

Title of paper: Potential protective effect of a G>A SNP in the 3’UTR of HLA-A for Chlamydia trachomatis symptomatology and severity of infection

Short running title: HLA-A SNP effect on risk for Chlamydia trachomatis infection

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Abstract

The inter-individual differences in response to *Chlamydia trachomatis* (CT) infections are for an important part based on the differences in our host genetic make-up. In the past, several genes and pathways have been identified and linked to protection against or risk for CT infection (i.e. susceptibility), and/or the severity of infection, with a major emphasis on the development of tubal pathology, one of the main causes of female infertility.

In the current study we analyzed in Dutch Caucasian women whether the carriage of HLA-A G>A SNP (rs1655900) was related to the susceptibility of CT infection in a STD cohort (n= 329) and to the severity of infection in a subfertility cohort (n=482). We also investigated if this A-allele was linked to increase in severity of symptoms, from mild symptoms (lower genital infection) to lower abdominal pain (upper genital tract infection) to the most severe late complication of tubal pathology, including double sided tubal pathology.

We showed that the carriage of HLA-A SNP rs1655900 studied is not associated with the susceptibility to CT infection based on the data from the STD cohort, but might be protective to the development of late complications (p=0.0325), especially tubal pathology could be relevant.

**Key words:** *Chlamydia trachomatis*; SNP; HLA; host genetic factors; tubal pathology; susceptibility
Introduction

Chlamydia trachomatis infection is the most prevalent sexually transmitted disease (STD), and the prevalence of the infection is on the rise globally, with roughly 100 million new infections occurring each year (Starnbach & Roan, 2008, Vasilevsky et al., 2014). Untreated C. trachomatis can lead to pelvic inflammatory disease, ectopic pregnancy and infertility due to tubal pathology (Wizel et al., 2008). However, remarkable differences in the clinical course of infection with C. trachomatis are observed between different individuals (Morré et al., 2009). For these differences, environmental factors such as co-infections may play a role (Hillis et al., 1994), but the differences can also be attributed to immunogenetic characteristics of the host. Understanding the immune mechanisms that underlie the pathogenesis of C. trachomatis infection has major implications for diagnostic and therapeutic approaches.

During a C. trachomatis infection adaptive immune responses are initiated, activating CD4+ and CD8+ T cells (Geisler, 2010, Neefjes et al., 2011). The role of CD8+ T cells has received increased interest due to the intracellular nature of C. trachomatis (Starnbach et al., 2003, Wizel et al., 2008). Pathogen-derived factors of C. trachomatis that access the host’s cytosol are explored by several studies, since intracellular proteins are presented on the cell surface by Human Leukocyte Antigen class I (HLA-I), triggering CD8+ T cell response (Kim et al., 1999, Fling et al., 2001, Starnbach et al., 2003, Gervassi et al., 2004). A CD8+ T cell response involves induction of apoptosis of the infected cell through perforin and granzyme, enabling the cytolytic potential of CD8+ T cells. However, it has been found that CD8+ T cells in the female genital tract have limited perforin expression, downgrading their cytolytic potential during an initial C. trachomatis infection (Ibana et al., 2012). Variance in CD8+ T cell functionality was not found to have a significant influence on the clearance of a C. trachomatis infection, but it was found to influence the development of pathogenesis, including infertility (Igietseme et al., 2009, Murthy et al., 2011).

Adequate pathogen recognition is essential to initiate the immune response. There is evidence that host genetic variation affects the clinical course of infection with C. trachomatis (Den Hartog et al., 2006, Bailey et al., 2009, Jiang et al., 2012, Al-Kuhlani et al., 2014). At this point these factors appear to be the most promising biological indicators of complication after a chlamydial infection (Ouburg et al., 2009, Malogajski et al., 2013, Brankovic et al., 2014). Associations with particular single nucleotide polymorphisms (SNPs) are typically confirmed for
genes coding for a range of immunological factors, such as cytokines, chemokines, and antigen presentation components (Morré et al., 2000).

Successful presentation of chlamydial antigens enables the highly selective process of triggering a lymphocytic response. Determining the role of antigen presentation and elicitation of the immune responses is of crucial significance for the currently insufficient understanding of the immunopathology of genital infection with *C. trachomatis* (Geisler, 2010). A number of alleles and sub-alleles in *HLA* genes have been found to be associated with susceptibility to chlamydial infection or associated pathologies (Morré et al., 2009). Since CD8+ T cells have been found to play an important role in complications after a *C. trachomatis* infection (Igietseme et al., 2009, Murthy et al., 2011, Ibana et al., 2012), a SNP in the gene region coding for HLA-I will be the focus of this study. A previous study presented by Kapil et al. stated that *HLA-DQB1*05 had a protective effect for reinfection (p=0.012, OR 2.6, 95% CI 1.2-5.6) (Kapil et al., 2013). In 2013 this work was presented orally at the STD & Aids World Congress and the *HLA-A* SNP rs1655900 was also presented as a possible candidate in their study in 199 African American subjects. We found the presented rs number to link to an SNP in the 3'UTR of *HLA-A* (ALFRED, 2014). We studied this SNP (*HLA-A* rs1655900) in relation to 1) the susceptibility of genital infection with *C. trachomatis*, 2) occurrence of symptoms, and 3) for the severity of symptoms to the most severe form of double sided tubal pathology in infertile women.

**Methods**

**STD cohort**

From a previous described cohort of 1150 Dutch Caucasian women, sufficient DNA was available from 329 samples to type the *HLA-A* SNP rs1655900 (Ouburg et al., 2005). In summary, between 2000-2004 data was collected from female patients who visited the STD outpatient clinic in Amsterdam, The Netherlands. Questionnaires responses were gathered about urogenital complaints, varying from increased discharge, having bloody discharge during and/or after coitus, recent lower abdominal pain (LAP) - not gastrointestinal or menstruation-related - and/or dysuria. *C. trachomatis* status was assessed by Roche Amplicor PCR as previously described (Ouburg et al., 2005). Out of the 329 women, 128 (39%) were *C. trachomatis* positive (CT+) and 201 (61%) were *C. trachomatis* negative (CT-). Out of the CT+ women, 72 (56%) were asymptomatic, 56 (44%) were symptomatic and 16 (13%) had LAP.
Subfertility cohort

From 482 serum samples from Dutch Caucasian women SNP data were available. These women were attending the fertility clinic of the University Medical Center Groningen, the Netherlands, and met the inclusion criteria for this study: *C. trachomatis* antibody test (CAT) result available, laparoscopic and/or hysterosalpingography (HSG) data available. Of the 482 serum samples, 471 had Chlamydia serology results available, and of these 413 (87.7%) were CT- and 58 (12.3%) were CT+ (pELISA, Medac Diagnostika mbH, Hamburg, Germany). Tubal Pathology (TP) was defined as extensive periadnexal adhesions and/or distal occlusion of at least one tube. From 58 women who tested positive for *C. trachomatis*, 11 (19%) were diagnosed with TP. In this group 55% were diagnosed with single sided TP (TPss) and 45% double sided TP (TPds). 378 (80%) women were diagnosed TP negative (TP-) through HSG. Of the CT+ women, 13 did not fulfill the criteria of TP and were not analyzed, of the CT- women 77 belonged to the HSG-0 group, which means that they were either not assessed, had mild HSG positivity but without reference to laparoscopy or other reasons, and were therefore not analyzed.

METC approval

The act "Medical Research Involving Human Subjects" (WMO, Dutch Law), states that anonymous spare human materials and data may be used for research purposes after patients have been informed about this possible use and they have had the opportunity to object. All patients participating in the present study had not objected and therefore no ethical approval is required (MEC Letter reference: # 10.17.0046).

DNA isolation and SNP assessment

DNA was isolated with the High Pure PCR Template Preparation Kit (HPPTP Kit) according to the manufacturer’s instructions (Roche Molecular, Mannheim, Germany). For the STD cohort 200 microliter cervical swab was resuspended in 1 ml 2SP, and for the subfertility cohort 200 microliter sera. The *HLA-A G>A* SNP rs1655900 was assessed using RT-PCR with detection on the LightCycler II (Roche Diagnostics, Basel, Switzerland) for the GG, AG and AA genotypes.

Statistical analyses
In the STD cohort CT+ and CT- women were compared to each other, and within the CT+ group women with and without symptoms were compared. In the subfertility cohort the CT-TP- and the CT+TP+ were compared to each other, as well as women with and without tubal pathology within the CT+ group. To study increased severity the following groups were compared for a trend in the occurrence of the HLA-A SNP studied: CT+ women with symptoms, to CT+ women with LAP, to women with TPss, and to women with TPds.

Data were compared between groups using Chi-square test and Fisher's Exact test when appropriate. Risk factors were described as odds ratio (OR) with 95% confidence interval (CI). P-values < 0.05 were considered statistically significant. Analyses were performed using IBM SPSS Statistics. The regression coefficient ($r^2$) for the line between CT+ women with symptoms, LAP, TPss, and TPds was calculated in Microsoft Office Excel. In general, the higher the $r^2$, the better the model fits the data. 80% explained variation is considered good, >90% is a very good fitting line and an association can be assumed.

**Results**

The HLA-A SNP G>A (RS1655900) are shown in Table 1. The STD and Subfertility cohorts were in Hardy Weinberg Equilibrium.

**STD cohort: susceptibility to infections**

There were no significant associations with the HLA-A SNP studied, between the CT+ and CT- women enrolled in this cohort or between those with and without symptoms (table 1). The distribution of the genotypes (GG, GA, AA) is comparable in the two groups.

**Subfertility Cohort: severity of infection**

Women who tested negative for C. trachomatis and who had a negative result on the HSG were compared to women with C. trachomatis and tubal pathology for the occurrence of carriage of HLA-A rs1655900 *A. In this comparison a protective trend is seen for women carrying the A-allele versus women with the G-allele (table 1), since there are relatively more women with the A-allele in the CT- group. In the CT- group there are 34% (127 GA&AA\textsubscript{CT-HSG}/378 GG&GA&AA\textsubscript{CT-HSG}) versus 9% (1 GA&AA\textsubscript{CT+TP+}/11 GG&GA&AA\textsubscript{CT+TP+}). The same protective trend was observed when solely looking at CT+ women with and without tubal
pathology 38% (13 GA&AA_{TP}/34 GG&GA^*AA_{TP}) versus 9% (1 GA&AA_{CT+TP}/11 GG&GA&AA_{CT+TP}). In all women with TP (n=11), all women with TPds (n=5) had the wildtype genotype (table 1 and figure 1).

*Increasing severity of infection among CT+ women*

The occurrence of the carriage of \textit{HLA-A} rs1655900 *A in patient groups with increasing severity - from CT+ women with symptoms to CT+ women with TPds – showed a statistically significant regression coefficient (r^2) of 0.932 (P= 0.0349) (figure 1). This suggests that there is a correlation between the mutant allele and a less severe disease progression.

**Discussion**

When analyzing the carriage of \textit{HLA-A} rs1655900 *A in patient groups with respectively an increasing degree of general and mild symptoms, LAP, TPss and TPds, we observed a decreasing trend of carriage of the A-allele in our cohorts. Our findings point to a protective role of allele A over G, especially for symptoms and late complications in women who tested positive for \textit{C. trachomatis} (r^2: 0.932; P=0.0349).

The SNP lies in the 3'UTR intergenic region of \textit{HLA-A} gene, which encodes for the heavy chain of the heterodimeric HLA-I molecule. The 3'UTR region is not translated into the protein sequence, hence the SNP is unable to lead to a change of amino acids. It may, however, work by changing the level of post-transcriptional expression. A seemingly protective effect observed in our study would correspond to a reduction of expression of \textit{HLA-A} transcript, resulting in less assembled HLA-I dimers and insufficient antigen presentation. A long-term consequence would be lower inflammation levels and less damage incurred by immune reactions to the local tissue. CD8+ T cells contribute to immune defense against \textit{C. trachomatis} (Wizel \textit{et al.}, 2008), however excessive cytolytic activity of these cells and the accompanying inflammation may be a major factor to contribute to the tubal damage and scarring (Cohen & Brunham, 1999, Vasilevsky \textit{et al.}, 2014). Nevertheless, what Reddy \textit{et al.} reported is a significant increase in the number of CD8+ T cells in Chlamydia-positive infertile women versus Chlamydia-negative women (Reddy \textit{et al.}, 2004), which does point to their role in Chlamydia-related complications. Therefore, alleles of the \textit{HLA} genes whose protein products present
chlamydial antigens more successfully are expected to lead to a more pronounced inflammation and vice versa.

Our results indicate that the number of patients carrying A-allele increases based on the presence of symptoms, LAP, and the degree of tubal damage. These findings would be congruent with a carriernship of such allele that impairs antigen presentation to CD8+ T cells. The CD8+ T cells are specifically important in the immune response leading to infertility, and less perforin and tumor necrosis factor α production could protect the host from tissue damage (Murthy et al., 2011, Iiba et al., 2012). The implications of this type of study are, however, insufficient to make firm conclusions about biological mechanisms at hand and the findings warrant further studies. In earlier studies, other immunogenetic factors such as SNPs have been showed to be associated with the course of infection of *C. trachomatis* (Den Hartog et al., 2006, Morré et al., 2009, Ouburg et al., 2009). The current study contributes to the promising implications of SNPs as biological indicators in the diagnosis of complicated chlamydial infection (Malogajski et al., 2013, Brankovic et al., 2014).

Our study has several limitations. Most importantly, the precise function of rs1655900 is unknown based on the available literature. The reported findings could well be the outcome of the base change in the region involved in regulation of translation, however we might instead be observing the effect of another polymorphism that is in strong linkage to the examined SNP. Since HLA-A is not simply an HLA class 1a locus, it is an HLA class Ia locus embedded within HLA class Ib genes and its tightest linkage to non-self is with HLA class 1b, meaning a simple explanation on neither its function nor its potential linkage is easy to be given or analyzed. Furthermore, despite the considerable size of our total cohort in comparison to previous studies, the size of our severity groups does not offer sufficient statistical power. It would therefore be valuable to attempt a reproduction of this study in a cohort with a higher number of patients with increasing symptomatology and especially tubal pathology. Also, functional analysis of the consequence of the base substitute on HLA-A expression levels would be useful to potentially corroborate these findings. In conclusion, the *HLA-A G>A* SNP studied is not associated with the susceptibility to infection but appears protective to the development of late complications, especially tubal pathology, after a *Chlamydia trachomatis* infection.

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References


Figure 1: Carriership of rs1655900 *A in *C. trachomatis* negative and positive women shows a decreasing trend in *C. trachomatis* induced complications.

CT: *C. trachomatis*; HSG: Hysterosalpingography; AS: Asymptomatic; S: Symptomatic; LAP: Lower Abdominal Pain; TP: Tubal Pathology; s.s.: Single-sided occlusion of the tubae; d.s.: double-sided occlusion of the tubae. Black line: trend based on the severity of *Chlamydia* infections (dark grey bars).

Table 1: Genotype distribution of rs1655900 in susceptibility and severity cohorts.
<table>
<thead>
<tr>
<th></th>
<th>GG</th>
<th>%</th>
<th>GA</th>
<th>%</th>
<th>AA</th>
<th>%</th>
<th>Tot.</th>
</tr>
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<tbody>
<tr>
<td>Susceptibility</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT-</td>
<td>139</td>
<td>69,2%</td>
<td>54</td>
<td>26,9%</td>
<td>8</td>
<td>4,0%</td>
<td>201</td>
</tr>
<tr>
<td>CT+</td>
<td>91</td>
<td>71,1%</td>
<td>33</td>
<td>25,8%</td>
<td>4</td>
<td>3,1%</td>
<td>128</td>
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<tr>
<td>AS</td>
<td>51</td>
<td>70,8%</td>
<td>17</td>
<td>23,6%</td>
<td>4</td>
<td>5,6%</td>
<td>72</td>
</tr>
<tr>
<td>S</td>
<td>28</td>
<td>70,0%</td>
<td>12</td>
<td>30,0%</td>
<td>0</td>
<td>0,0%</td>
<td>40</td>
</tr>
<tr>
<td>LAP</td>
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<td>75,0%</td>
<td>4</td>
<td>25,0%</td>
<td>0</td>
<td>0,0%</td>
<td>16</td>
</tr>
<tr>
<td>Severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT-</td>
<td>278</td>
<td>67,3%</td>
<td>113</td>
<td>27,3%</td>
<td>22</td>
<td>5,3%</td>
<td>413</td>
</tr>
<tr>
<td>CT- HSG-</td>
<td>251</td>
<td>66,0%</td>
<td>105</td>
<td>28,0%</td>
<td>22</td>
<td>5,8%</td>
<td>378</td>
</tr>
<tr>
<td>CT+</td>
<td>42</td>
<td>72,4%</td>
<td>14</td>
<td>24,1%</td>
<td>2</td>
<td>3,4%</td>
<td>58</td>
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<tr>
<td>CT+ TP+ s.s.</td>
<td>5</td>
<td>83,3%</td>
<td>0</td>
<td>0,0%</td>
<td>1</td>
<td>16,7%</td>
<td>6</td>
</tr>
<tr>
<td>CT+ TP+ d.s.</td>
<td>5</td>
<td>100,0%</td>
<td>0</td>
<td>0,0%</td>
<td>0</td>
<td>0,0%</td>
<td>5</td>
</tr>
<tr>
<td>TP-</td>
<td>21</td>
<td>61,8%</td>
<td>13</td>
<td>38,2%</td>
<td>0</td>
<td>0,0%</td>
<td>34</td>
</tr>
</tbody>
</table>

CT: C. trachomatis; AS: Asymptomatic; S: Symptomatic; LAP: Lower Abdominal Pain; TP: Tubal Pathology; s.s.: Single-Sided occlusion; d.s.: Double-Sided occlusion; +: positive; -: negative