The thrombophylic network of auto antibodies in celiac disease

Aaron Lerner M.D., M.H.A\textsuperscript{1}\textsuperscript{*}, Nancy Agmon-Levin M.D\textsuperscript{2}, Yinon Shspira\textsuperscript{2}, Boris Gilburd M.D\textsuperscript{2}, Sandra Reuter M.D.\textsuperscript{3}, Idit Lavi\textsuperscript{4} Yehuda Shoenfeld M.D\textsuperscript{2}.

Pediatric Gastroenterology and Nutrition Unit\textsuperscript{1}, Carmel Medical Center\textsuperscript{1,4}, B. Rappaport School of Medicine, Technion-Israel institute of Technology\textsuperscript{1}, Haifa. The Zabludowicz Center for Autoimmune Diseases, Sheba Medical Center, Tel-Ashomer, Israel\textsuperscript{2}, Aira e.v./Aesku.Kipp Institute, Wendelsheim, Germany\textsuperscript{3} and Epidemiology and Community Medicine\textsuperscript{4}, Haifa, Israel

* The authors contributed equally to the manuscript.

Reprint and correspondence address:
Prof. Aaron Lerner
Pediatric gastroenterology and nutrition Unit
Carmel Medical Center
7, Michal St
Haifa, 34362, Israel
Tel: 972-4-8250527
Fax: 972-4-8250126
E-mail: lerner_aaron@clalit.org.il

Running title: Autoantibodies and hypercoagulability in celiac disease.
Abstract

Background: Celiac disease is a life-long autoimmune condition, affecting genetically susceptible individuals that may present thromboembolic phenomena. This thrombophilia represents a puzzle with multiple constituents: hyperhomocysteinemia, B12 and/or folate deficiency, MTHFR mutations, and protein C and S deficiency due to vitamin K deficiency. However, the well known thrombogenic factors anti phosphatidylserine/prothrombin and antiprothrombin were never and antiphospholipid antibodies were scarcely explored in celiac disease.

Methods: The serum auto antibodies levels were determined in 245 individuals, classified to 3 groups. Group 1 comprised 69 children with definitive celiac disease (age: 7.04 ± 4.3 years M:F ratio 1.06) and group 2 comprised of 86 normal children (age: 6.7 ± 4.17 years M:F ratio 0.87), representing controls. The pediatric populations were compared to group 3 including 90 adults, parents of group 1 (age: 34.6 ± 11.35 years M:F ratio 1.2). Antibodies were checked by ELISA (Aesku; Germany).

Results: Mean serum anti-phosphatidylserine/prothrombin IgG antibodies' OD levels were 32.4±19.4, 3.6±2.5, 16.1±15.8 in group 1, 2, 3 respectively (p<0.0001). 45.7%, 0% and 7.8% of group 1, 2 and 3 respectively were positive for the antibody (p<0.01).

Mean serum anti-phosphatidylserine/prothrombin IgM antibodies' OD levels were 14.1±8.7, 6.7±6.4, 11.2±10.6 in group 1, 2, 3 respectively (p<0.0001). 22.9%, 4.5% and 12.2% of group 1, 2 and 3 respectively were positive for the antibody (p<0.01, groups 1-2).

Mean serum anti prothrombin and anti phospholipid IgG bodies' OD levels were higher in group 1, 2, 3 (p<0.005) and in groups 1-3, 2-3 (p<0.01), respectively.

Group 1, 2 and 3 were positive for anti phospholipid IgG antibodies (, groups 1-3, 2-3 p<0.02). Celiac disease sera harbor a higher anti prothrombin IgG levels compared to controls.

Conclusions: It is suggested that the intestinal injury/endothelial dysfunction/platelet's abnormality/enhanced apoptosis, recently described in celiac disease, are at the origin of increased exposure of phospholipids or new epitopes representing auto antigens. Those auto antibodies might play a pathogenic role in the thrombophilia associated with celiac disease and represent markers for potential anticoagulant preventive therapy.

Key words: Anti-phosphatidylserine/prothrombin, prothrombin, phospholipid, autoantibodies, celiac disease, hypercoagulability.
Background

Celiac disease (CD) is the most common autoimmune food intolerance in the world. It is a life-long autoimmune condition [1] mainly of the gastrointestinal tract, affecting the small intestine of genetically susceptible individuals. Environmental factors are crucial for disease induction. Gluten, which is the storage protein of wheat and its alcohol soluble gliadins are the offending inducers of the disease together with structurally related molecules found in barley, rye and oat. Tissue transglutaminase is the auto antigen against which the abnormal immune response is directed to [2] and two main auto antibodies: anti endomysium and anti tTG are the most currently useful serological markers to screen for the disease [3, 4]. The sequential chain of events operating in the disease was recently unraveled, and gives the hope for future therapeutic strategies [5]. Furthermore, its epidemiology, prevalence and clinical presentation are changing constantly and with time, new clinical presentations are depicted and widen the plethora of clinical variability of CD [6].

It has been shown that the classical intestinal clinical picture of malnutrition, chronic diarrhea and nutritional deficiencies are disappearing and the extra-intestinal presentations are emerging. Skin, endocrine, skeletal, hepatic, hematological, gynecological, infertility, dental and behavioral abnormalities are often described [7-9]. Nowadays, we are witnessing epidemiological shift in the disease phenotype toward a more advanced age, and increased prevalence of latent, hypo symptomatic\asymptomatic presentations [6].
A newly explored area of CD is the hypercoagulability and the resulting thromboembolic phenomena. There is an increase risk of stroke, in adults and children with celiac disease [10-15]. Thrombophilia, pregnancy loss, deep vein thrombosis, small bowel infarction, atrial fibrillation, Budd-Chiari syndrome, portal and splenic vein thrombosis, and cardiovascular disease were described [16-21]. Even the onset of the disease may be due to a thrombotic event [11, 17, 21]. More so, hyperhomocystinemia with related vitamin deficiency in untreated CD and MTHFR variants' frequency just add to the hypercoagulable status in the patients [21-26].

In fact, there is an increased incidence of autoimmune diseases in CD [1, 7, 27,28]. Two examples associated with thrombophilia are systemic lupus erythematosus (SLE) and anti phospholipid syndrome (aPL) [29, 30]. Three autoantibodies associated with the two entities are anti-phosphatidylserine/prothrombin (anti-PS/PT), antiphospholipid and (aPL) and antiprothrombin (aPT). Anti-PS/PT and aPL autoantibodies confer increased risk for thromboembolic events and poor outcome in those diseases [31-39]. The correlation between aPS/PT antibodies and clinical manifestations of aPL syndrome and the importance of aPS/PT as a marker for this syndrome is well established. The relation between anti-PS/PT antibodies and hypercoagulability state is further strengthened by their increased incidence in cerebral infarction [40]. aPT autoantibodies are prevalent in SLE and aPL syndrome and are associated with thrombosis and pregnancy morbidity [41-44].
Despite the coexistence of CD and thromboembolic events, the aPS/PT and aPT status was never and aPL activity was scarcely investigated in CD. On the above backgrounds of aPS/PT/aPT/aPL antibodies and thrombophilia, hypercoagulability in CD, and increased incidence of SLE and aPL in CD, the presence of aPS/PT, compared to aPL, aPT and anti cardiolipin antibodies, were explored in CD children and their parents, compared to pediatric controls. Increased incidence of aPS/PT-IgG in the celiac group, intermediate incidence in their parents, compared to none in the control one was detected. Additionally, higher rates of aPS/PT-IgM and prothrombin-IgG autoantibodies' activities in the celiac patients, compared to the other two groups, was depicted. It seems that the presently studied thrombophylic autoantibodies are operative in CD, extending the hypercoagulability network in this disease.

**Materials and methods**

Study populations

Serum aPS/PT, aPT and aPL autoantibodies levels were determined in 248 individuals, divided to 3 groups. Group 1 comprised 70 Israeli children with definitive CD (age: 7+/−4 years M:F ratio 1.06) and group 2 presented by 88 normal children (age: 6+/−4 years M:F ratio 0.9), as controls. The pediatric populations were compared to group 3 include 90 adults, parents of group 1 (age: 35+/−11 years M:F ratio 1.2).

The following information was collected on the three groups: Diet: gluten containing or free diet.
Symptoms: abdominal pain, short stature, vomiting, diarrhea, anemia, failure to thrive and IgA deficiency. Familial diseases: CD, Diabetes mellitus type1\2, FMF, IBD, thyroid disease. Laboratory parameters: complete blood count, biochemical profile, IgA levels, CD serology (see Eliza essays below).

Celiac disease was diagnosed according to the revised criteria of the European Society for Pediatric Gastroenterology and nutrition, based on specific serology and duodenal biopsies [45]. All the participants were on a gluten containing diet and had physical examination, laboratory work-up, celiac serology.

Eliza assays

1. Celiac serology

Three ELISA assays are included in our celiac screening algorithm, as recently described [4, 46]. The AESKU CeliCheck Neo-epitope assay was tested on the TRITURUS analyzer (GRIFOLS SA, Barcelona Spain). The DiaSorin tTG IgA assay was tested on the Liaison (DiaSorin Saluggia, Italy), and the ORGENTEC tTG IgA+IgG assay was tested on the ETI-MAX 3000 analyzer (DiaSorin Saluggia, Italy). NEQAS is routinely used as the external quality control program.

2. Anti-cardiolipin, phospholipid, prothrombin and aPS/PT essays
Sera were tested for the above mentioned antibodies using solid phase enzyme immunoassay (AESKULISA, AESKU diagnostics, Germany), according to manufacturers' protocol.

Briefly, serum samples diluted 1:10 were incubated in the micro plates coated with the specific antigen. Binding was detected by anti-human immunoglobulins peroxidase (conjugate) and TMB-substrate. The positive sera were calculated according to the manufacture's equations for cut off value determined as specified below or utilizing other cut off as follows:

**Cardiolipin check:** The immunoassay employed highly purified cardiolipin plus native human beta2-cardiolipin 1 for the combined quantitative and qualitative detection of IgA, IgM and IgG antibodies against cardiolipin in the sera. Positive cut off >24U/ml.

**Phospholipid IgG/IgM:** As for the cardiolipin check except for the use of anti–human IgG/IgM peroxidase Positive cut offs for both antibodies: >18U/ml.

**Prothrombin IgG:** The immunoassay employed highly purified prothrombin (factor II) for the combined quantitative and qualitative detection of IgG antibodies against prothrombin in the sera. Anti–human IgG peroxidase conjugate was employed. Positive cut off >18U/ml.

**Phosphatidylserine/prothrombin IgA, IgG, IgM:** The immunoassay employed highly purified phosphatidylserine plus native human prothrombin for the combined quantitative and qualitative detection of IgA, IgM and IgG antibodies against PS/PR in the sera. Anti–human IgA/IgG/IgM peroxidase conjugates
were used. Positive cut off for PS/PR-IgA >28U/ml. The manufacture's cutoff is 18U/ml. Based on multiple determinations on 92 Israelis healthy subjects, a higher cut off of mean+2SD, was used. The ROC curve data were: area under the curve of 0.855, standard error 0.0315, 95% confidence interval 0.791-0.905, Z statistic 11.258 and P<0.0001.

**Endoscopy and intestinal histology**

All patients in group 1, underwent esophago-gastro-duodenoscopy using GIF-xp 20 endoscope (pentax, Tokyo, Japan). At least 5 biopsies were obtained: 4 from the second part of the duodenum for the diagnosis or exclusion of CD and 1 from the antrum.

The biopsies were immediately fixed in buffered formalin and embedded on edge in paraffin. Sections were stained with hematoxylin-eosin and Giemsa, analyzed by the pathologist and graded according to Marsh criteria, as previously described (3). On the day of endoscopy 5ml of peripheral blood was withdrawn, centrifuge 5000 c/sec for 10 minutes and the serum was frozen in -80°Celsius until assayed for serology.

The ethical committee of Carmel Medical Center approved the study and a written informed consent was obtained from the parents or guardians of the children.

**Statistical analysis**

Data analysis was performed using the PASW 18 statistical package (PASW, Chicago IL).
Comparison the levels of the autoantibodies: Anti-cardiolipin, phospholipid, prothrombin and aPS/PT between the three study group was performed by Kruskal Wallis test.

For multiple comparisons between any two study groups Mann Whitney test was used.

For examining the association between the positive cut offs for all antibodies, with the study groups Chi square test or exact test for small sample were used.

All p values were two-sided, and statistical significance was defined as p<0.05.

Results

No epidemiological statistical difference between the pediatric groups was detected.

Table No 1 shows the mean±S.D.and (median) of the different autoantibodies in pediatric celiac disease (PCD), their parents (P) and pediatric control (PC), presently studied.

Table 1: Mean and median of autoantibodies' activity in celiac children, their parents compared to pediatric controls.

<table>
<thead>
<tr>
<th>Autoantibodies/groups Mean±S.D.and (median)</th>
<th>PCD No=70</th>
<th>P No=90</th>
<th>PC No=88</th>
<th>PCD/P</th>
<th>PCD/PC</th>
<th>P/PC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPS/PT-IgA</td>
<td>7.9±9.5 (5.6)</td>
<td>10.7±13.6 (8.2)</td>
<td>ND</td>
<td>**</td>
<td>ND</td>
<td>ND</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>aPS/PT-IgG</td>
<td>32.4±19.5 (27.7)</td>
<td>16.1±15.9 (12.3)</td>
<td>3.6±2.5 (3.3)</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>aPS/PT-IgM</td>
<td>14.2±8.7 (12.9)</td>
<td>12.4±15.5 (8.6)</td>
<td>6.7±6.4 (4.9)</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 2: % positivity of autoantibodies in celiac children, their parents compared to pediatric controls.

<table>
<thead>
<tr>
<th>Autoantibodies positivity/groups</th>
<th>O.D cut-off levels</th>
<th>Pediatric celiac disease No=70</th>
<th>Celiac disease parents No=90</th>
<th>Pediatric controls No=88</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPS/PT-IgA</td>
<td>&gt;28</td>
<td>4.3</td>
<td>3.3</td>
<td>N.D.</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>aPS/PT-IgG</td>
<td>&gt;28</td>
<td>45.7***</td>
<td>7.8***</td>
<td>0 ***</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>aPS/PT-IgM</td>
<td>&gt;28</td>
<td>7.1</td>
<td>9.9</td>
<td>3.4</td>
<td>&lt;0.23</td>
</tr>
<tr>
<td>Protrombin-IgG</td>
<td>&gt;18</td>
<td>17.1</td>
<td>11</td>
<td>14.9</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Phospholipid-IgG</td>
<td>&gt;18</td>
<td>11.4</td>
<td>3.3*</td>
<td>12.5*</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td>Phospholipid-IgM</td>
<td>&gt;18</td>
<td>1.4</td>
<td>2.2</td>
<td>N.D.</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cardiolipin check</td>
<td>&gt;24</td>
<td>0</td>
<td>1.1</td>
<td>0</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*p<0.05,**p<0.01,***p<0.0001.
Discussion

The main result of the present study is the increased incidence of aPS/PT-IgG in the celiac group, intermediate incidence in their parents, compared to none in the control one. Secondary results are the increased rates of aPS/PT-IgM and prothrombin-IgG autoantibodies in the celiac patients, compared to the other two groups. Of notice is the constantly, parallel, gradual decrease of the aPS/PT-IgG and IgM, along the three groups: celiac children/their parents/pediatric controls, respectively. The fact that none of the parents had positive celiac serology, although potential CD can't be rule out, point to a potential genetic influence on the aPS/PT autoantibodies presence. In fact, being an autoimmune disease with a well established genetic susceptibility and increased familial predisposition, increased presence of autoantibodies and autoimmune diseases in first degree relatives of CD patients is well known [47-49] and aPS/PT should be added to the list. Additionally, aPS/PT should be added to the increased list of associated autoantibodies in CD affected patients [27, 28].

Despite the fact that many of the autoantibodies might present an epiphenomenon, it is suggested that mainly aPS/PT, but also anti thrombin and aPL autoantibodies are pathogenic and play an active role in CD pathogenesis and complications. The presence of aPS/PT is directly relayed to thromboembolic events in aPL and SLE and cerebral infarction [31-40]. The thrombogenic properties of aPS/PT correlate with increased thrombin generation in aPL syndrome, thus contribute to the understanding of the pathophysiology of
thrombophilia in those patients [36]. Those autoantibodies are strong risk factors for venous thromboembolism in SLE patients by inducing activated protein C resistance [37]. The other two IgG-autoantibodies, namely; anti thrombin and aPL, are also associated with thrombotic events in aPL syndrome and SLE [32,33,50,51], and are risk of myocardial infarction in middle-aged men [52,53].

The pathphysiology of the thromboembolic phenomena associate with CD [10-26] represent a puzzle with multiple constituents: Hyperhomocysteinemia, B12 and/or folate deficiency, MTHFR mutations, and protein C and S deficiency due to vitamin K deficiency [21-26,54]. The present study unravels a serie of autoantibodies: aPL, anti prothrombin and mainly aPS/PT to this puzzle's constituents that are suggested to play a pathogenic role in the thrombogenicity of CD.

Phosphatidylserine is a regular constituent of the inner leaflet of the cell membrane, which is exposed only on the outside of the cell membrane during apoptosis or by damaged endothelial cells [55]. It is known that prothrombin and aPL antibody binds specifically to the surface of apoptotic cells [56,57]. More so, recently, Ieko et al [58] reported that IgG-aPS/PT recognizes prothrombin bound to phosphstidylserine on platelets and endothelial cells and, directly or via Fc-gamma receptors, activates a variety of procoagulant agents. On the other hand, the CD complementary aspects are the endothelial dysfunction [59], platelet's abnormalities [60, 61] and increased apoptosis [62] reported in CD. Thus, it is suggested that the intestinal injury\endothelial dysfunction\platelet's abnormality\enhanced apoptosis are at the origin of increased exposure of
phospholipids or new epitopes, which are at the origin of anti prothrombin/ aPL and aPS/PT autoantibodies. Those antibodies might play a pathogenic role in the thrombophilia associated with CD.

A new light was shed, recently, on the "inflammation coagulation crosstalks" [63]. Recent studies have unveiled molecular underpinnings of the intimate interconnection between both systems. Being a classical inflammatory state, CD, can present such crosstalks, resulting in enhanced coagulability in the intestinal arena and on the systemic level. Due to the several pathways of mucosal injury, autoantigens like: phospholipids, phosphatidylserine, prothrombin are exposed, inducing aPS/PT, aPL, aPT antibodies production. With their thrombogenic capacities, those autoantibodies can present the first or an additional hit in the thrombogenic background operating in CD. Due to the increased coagulability in CD and the harmful potential consequences, the patients positive for those antibodies should be considered to receive preventive anticoagulant therapy.

Conclusions

Increased incidence of aPS/PT-IgG in the pediatric celiac group, intermediate incidence in their parents, compared to none in the control one was detected. Additionally, higher rates of aPS/PT-IgM and prothrombin-IgG autoantibodies' activities in the celiac patients, compared to the other two groups, were observed. Based on the extensive literature of thromboembolic phenomenon described in CD, it seems that the presently studied thrombophylic autoantibodies are operative in CD, extending the hypercoagulability
network of the disease. The place of the auto antibodies, presently described, as potential markers for thromboembolic manifestations in CD is a subject for future exploration.

The authors declare that they have no competing interests

References


2010, 9:144-147.


40. Okuma H, Kitagawa Y, Ishikawa T, Takagi S. Study of phosphatidylserine-dependent anti-prothrombin


Additional files provided with this submission:

Additional file 1: Cover letter BMC Medicine.doc, 30K
http://www.biomedcentral.com/imedia/4389312058542462/supp1.doc