DETECTION OF ABERRANT INTRA-EPITHELIAL LYMPHOCYTES IN REFRACTORY CELIAC DISEASE

BSAC, 21-10-2016

N. BOECKX, MD., PHD.
LABORATORY MEDICINE, UZ LEUVEN
CELIAC DISEASE: WHAT?

- **Auto-immune** disorder that chronically affects the **small intestine**

- Induced by **dietary gluten** in genetically predisposed individuals (alleles encoding HLA-DQ2 or DQ8)

- Worldwide **prevalence ~1%**
GASTRO-INTESTINAL signs and symptoms
- chronic diarrhea and abdominal pain
- steatorrhea
- weight loss, failure to thrive, growth failure, anorexia
- bloating
- vomiting, ...

EXTRA-INTESTINAL signs and symptoms
- iron-deficiency anemia and other nutritional deficiencies (vitamin B12, vitamin D, folate, zinc, vitamin B6)
- fatigue, ...

ASSOCIATED (AUTOIMMUNE) CONDITIONS
- type 1 diabetes
- autoimmune thyroid / liver disease
- Sjögren syndrome, ...

all associated with HLA risk alleles (HLA haplotypes DQ2 and/or DQ8)
CELIAC DISEASE: DIAGNOSIS

1. Serologic markers of celiac disease
   - IgA/IgG against tissue transglutaminase (tTG)
   - Endomysial antibody (IgA)
   - IgA/IgG against deamidated gliadin peptide (DGD)

2. Intestinal biopsies
   - Mucosal injury, more pronounced in proximal intestine, mild or absent distally
   - Microscopic findings: atrophic villi, crypt hyperplasia, increase in number of intraepithelial lymphocytes (NOT specific for CD)

3. Genetics
   - Class II HLA types DQ2 and DQ8 (in almost all CD patients, but also in 30-40% of Western Caucasian population, only 3% of individuals with these haplotypes develop CD (high negative predictive value))
CELIAC DISEASE: DIAGNOSIS

1. Serologic markers of celiac disease
   - IgA/IgG against tissue transglutaminase (tTG)
   - Endomysial antibody (IgA)
   - IgA/IgG against deamidated gliadin peptide (DGD)

2. Intestinal biopsies
   - Mucosal injury, more pronounced in proximal intestine, mild or absent distally
   - Microscopic findings: atrophic villi, crypt hyperplasia, increase in number of intra-epithelial lymphocytes (IEL) (NOT specific for CD)
CELIAC DISEASE: DIAGNOSIS

1. Serologic markers of celiac disease
   - IgA/IgG against tissue transglutaminase (tTG)
   - Endomysial antibody (IgA)
   - IgA/IgG against deamidated gliadin peptide (DGD)

2. Intestinal biopsies
   - Mucosal injury, more pronounced in proximal intestine, mild or absent distally
   - Microscopic findings: atrophic villi, crypt hyperplasia, increase in number of intra-epithelial lymphocytes (IEL) (NOT specific for CD)

3. Genetics
   - Class II HLA types DQ2 and DQ8 (in almost all CD patients, but also in 30-40% of Western Caucasian population; only 3% of individuals with these haplotypes develop CD)
the only treatment for celiac disease is a strict gluten-free diet

- reduces symptoms, mortality and risk for malignancy
- lifelong diet (expensive, socially isolating)
- avoiding
  - wheat (‘tarwe’)
  - rye (‘rogge’)
  - barley (‘gerst’)

OBVIOUS SOURCES OF GLUTEN:

bread, bagels, cakes, cereal, cookies, pasta, noodles, pastries, pies, rolls
REFRACTORY CELIAC DISEASE (RCD)

- persisting or recurring symptoms despite strict adherence to gluten-free diet
  - diarrhea, abdominal pain, involuntary weight loss, …
  - severe malnutrition, protein-losing enteropathy, ulcerative jejunitis, ….

- patients are nearly always adults (50 years or thereafter)

- affects less than 1% of CD patients, but significant morbidity and mortality

- subdivided into 2 types of RCD
  - RCD type I
  - RCD type II
## RCD TYPE I AND II

<table>
<thead>
<tr>
<th>RCD type I</th>
<th>RCD type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>no increased risk for enteropathy-associated T-cell lymphoma (EATL)</td>
<td>increased risk to develop EATL</td>
</tr>
<tr>
<td></td>
<td>risk for other gastro-intestinal cancers is not substantially increased</td>
</tr>
</tbody>
</table>
### RCD TYPE I AND II

<table>
<thead>
<tr>
<th>RCD type I</th>
<th>RCD type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>no increased risk for enteropathy-associated T-cell lymphoma (EATL)</td>
<td>increased risk to develop EATL risk for other gastro-intestinal cancers is not substantially increased</td>
</tr>
<tr>
<td>normal 5-year survival</td>
<td>poor 5-year survival (~50%)</td>
</tr>
</tbody>
</table>
## RCD TYPE I AND II

<table>
<thead>
<tr>
<th>RCD type I</th>
<th>RCD type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>no increased risk for enteropathy-associated T-cell lymphoma (EATL)</td>
<td>increased risk to develop EATL risk for other gastro-intestinal cancers is not substantially increased</td>
</tr>
<tr>
<td>normal 5-year survival</td>
<td>poor 5-year survival (~50%)</td>
</tr>
<tr>
<td>low numbers of aberrant intra-epithelial lymphocytes (IELs)</td>
<td>high(er) numbers of aberrant IEL</td>
</tr>
</tbody>
</table>
### RCD TYPE I AND II

<table>
<thead>
<tr>
<th>RCD type I</th>
<th>RCD type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>no increased risk for enteropathy-associated T-cell lymphoma (EATL)</td>
<td>increased risk to develop EATL</td>
</tr>
<tr>
<td></td>
<td>risk for other gastro-intestinal cancers is not substantially increased</td>
</tr>
<tr>
<td>normal 5-year survival</td>
<td>poor 5-year survival (~50%)</td>
</tr>
<tr>
<td>low numbers of aberrant intra-epithelial lymphocytes (IELs)</td>
<td>high(er) numbers of aberrant IEL</td>
</tr>
<tr>
<td><strong>BENIGN</strong> =&gt; often responds to treatment with eg. topical steroids</td>
<td><strong>PRE-MALIGNANT</strong> (indolent lymphoma (pre-EATL)) =&gt; requires cytotoxic chemotherapeutic therapy, eg. 2-CDA</td>
</tr>
</tbody>
</table>
**PHENOTYPE OF IELs**

**Normal IELs**

- Majority (>70%) of IELs are sCD3+ T-cells
  - TCRab (80%)
    - >85% CD8+
    - only ~10% CD4+
  - TCRgd (5-15%) with variable expression of CD8 (40-80%)

- 10-20% of IELs are CD3- cells
PHENOTYPE OF IELs

Normal IELs

- Majority (>70%) of IELs are sCD3+ T-cells
  - TCRab (80%)
    - >85% CD8+
    - only ~10% CD4+
  - TCRgd (5-15%) with variable expression of CD8 (40-80%)
- 10-20% of IELs are CD3- cells

Aberrant IELs

- T-cells
  - surface CD3-
  - surface CD8-
  - cytoplasmatic CD3+

Clonal expansion of this population is only found in a subgroup of RCD patients and EATL patients
- RCD type I: <20% aberrant IELs
- RCD type II: 20-100% aberrant IELs

1. Immunohistochemistry: CD3 and CD8 staining

2. TCR gene rearrangement studies (γ, β, δ)

3. Flowcytometric immunophenotyping
## METHODS TO IDENTIFY ABERRANT IELs

<table>
<thead>
<tr>
<th>Method</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunohistochemistry CD3 and CD8 staining</td>
<td>IHC and TCR-clonality studies:</td>
<td>no differentiation between cyCD3 and sCD3 lower sensitivity: high cut-off (&gt;50% CD3+CD8- of CD3+ IELs) high interobserver variability</td>
</tr>
<tr>
<td></td>
<td>• reliable tools to identify dominant aberrant IEL populations</td>
<td>fails to identify clonal IELs in patients with 20-25% aberrant IELs</td>
</tr>
<tr>
<td>TCR gene rearrangement studies (γ, β, δ)</td>
<td>• BUT fails to identify a moderate increase of these cells</td>
<td></td>
</tr>
</tbody>
</table>
## METHODS TO IDENTIFY ABERRANT IELS

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Immunohistochemistry CD3 and CD8 staining | IHC and TCR-clonality studies:  
• reliable tools to identify dominant aberrant IEL populations | no differentiation between cyCD3 and sCD3  
lower sensitivity: high cut-off (>50% CD3+CD8- of CD3+ IELs)  
high interobserver variability |
| TCR gene rearrangement studies (γ, β, δ) |  
• BUT fails to identify a moderate increase of these cells | fails to identify clonal IELs in patients with 20-25% aberrant IELs |
| Flowcytometric immunophenotyping |  
• can differentiate between cyCD3 and sCD3  
• can also identify patients with only a moderate increase in aberrant IELs (sCD3-CD8-CD7+cyCD3+) | in 95% of non-refractory CD and control patients, the highest % aberrant T-cells in duodenal biopsy specimens is 20% |

GOLDEN STANDARD
# T-CELL CLONALITY ANALYSIS VERSUS FCM ANALYSIS

## Detection of aberrant IELs

<table>
<thead>
<tr>
<th></th>
<th>RCD evolving to EATL, N = 10</th>
<th>RCD without EATL, N = 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;20% aberrant IELs</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>&lt;20% aberrant IELs</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

## T-cell clonality analysis

<table>
<thead>
<tr>
<th>Clonality</th>
<th>FCM</th>
<th>Molecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal</td>
<td>7†</td>
<td>7</td>
</tr>
<tr>
<td>Polyclonal</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

* Poor quality DNA, clonality analysis inconclusive

<table>
<thead>
<tr>
<th></th>
<th>FCM</th>
<th>Molecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>100%</td>
<td>78%</td>
</tr>
<tr>
<td>Specificity</td>
<td>46%</td>
<td>46%</td>
</tr>
<tr>
<td>NPV</td>
<td>100%</td>
<td>75%</td>
</tr>
<tr>
<td>PPV</td>
<td>59%</td>
<td>50%</td>
</tr>
</tbody>
</table>

LYMPHOCYTE SUBSETS IN DUODENAL BIOPSY SPECIMENS AS % OF INTESTINAL LYMPHOCYTES (BY FCM)

<table>
<thead>
<tr>
<th>Subset</th>
<th>Controls without CD n= 49</th>
<th>Untreated CD n = 17</th>
<th>CD on GFD n = 60</th>
<th>RCD I n = 16</th>
<th>RCD II n = 17</th>
<th>Primary EATL n = 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ T-cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (10th-90th percentile)</td>
<td>86 (78-93)</td>
<td>94 (80-97)</td>
<td>90 (78-97)</td>
<td>93 (81-98)</td>
<td>43 (16-63)</td>
<td>90 (55-94)</td>
</tr>
<tr>
<td>CD4+ T-cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (10th-90th percentile)</td>
<td>24 (10-44)</td>
<td>19 (9-32)</td>
<td>18 (7-38)</td>
<td>13 (5-29)</td>
<td>13 (3-17)</td>
<td>19 (11-21)</td>
</tr>
<tr>
<td>CD8+ T-cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (10th-90th percentile)</td>
<td>56 (39-76)</td>
<td>67 (41-81)</td>
<td>61 (42-79)</td>
<td>70 (52-88)</td>
<td>20 (2-31)</td>
<td>63 (25-64)</td>
</tr>
<tr>
<td>CD7+ lymphocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (10th-90th percentile)</td>
<td>96 (88-98)</td>
<td>95 (85-99)</td>
<td>96 (88-98)</td>
<td>95 (91-99)</td>
<td>96 (90-98)</td>
<td>94 (58-96)</td>
</tr>
<tr>
<td>CD16/56+ NK cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (10th-90th percentile)</td>
<td>7 (3-14)</td>
<td>3 (1-7)</td>
<td>5 (1-12)</td>
<td>3 (1-10)</td>
<td>5 (1-17)</td>
<td>4 (0.4-5)</td>
</tr>
<tr>
<td>CD19+ B-cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (10th-90th percentile)</td>
<td>0.5 (0.1-3)</td>
<td>2 (0.4-12)</td>
<td>1 (0.1-6)</td>
<td>1 (0.01-3)</td>
<td>1 (0.2-8)</td>
<td>2 (0.01-13)</td>
</tr>
<tr>
<td>CD7+CD3-cyCD3+ aberrant T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (10th-90th percentile)</td>
<td>4 (1-9)</td>
<td>1 (0.07-4)</td>
<td>2 (0-5)</td>
<td>2 (0.5-10)</td>
<td>52 (34-89)</td>
<td>2 (0.4-7)</td>
</tr>
</tbody>
</table>

LYMPHOCYTE SUBSETS IN DUODENAL BIOPSY SPECIMENS AS % OF INTESTINAL LYMPHOCYTES (BY FCM)

⇒ Percentage aberrant T-cells (CD7+ surface CD3− cytoplasmic CD3+) in duodenal biopsy specimens of each disease category. There were significantly more aberrant T-cells in the RCD II group as compared to all other groups, in all cases $p < 0.0001$.

⇒ Percentage CD8+ lymphocytes in duodenal biopsy specimens of each disease category. There were significantly less CD8+ T-cells in RCD II as compared to all other groups, in all cases $p < 0.0001$.

FCM ANALYSIS: ISOLATION AND STAINING OF IELS

- 4 – 8 biopsies (stored in PBS at 0-4°C)
- isolation of IELs from intestinal biopsies
  - no chemical or enzymatic treatment
  - done by vigorous shaking: 60 min at 37°C (can also be done at room temperature)

- calcium chelants (DTT, EDTA): induces the disassembly of inter-epithelial junctions and the release of epithelial cells and IELs
- ~100,000 IELs per cubic millimeter small bowel biopsies (1 x 1 x 1 mm): enough for staining of IELs required for diagnosis and monitoring of CD (IELs will constitute ~5% (1-10% range) of the released cells)

- IELs in supernantant
FCM ANALYSIS: PANEL AND GATING STRATEGY

- CD7 – cy isotype – CD45 – sCD3
- CD7 – cy CD3 – CD45 – sCD3
## CLINICAL CASE 1

<table>
<thead>
<tr>
<th>Case 1 M, 57y</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main clinical problem(s)</strong></td>
</tr>
<tr>
<td><strong>Pre treatment FCM % aberrant IEL</strong></td>
</tr>
<tr>
<td><strong>RCD: type I or II?</strong></td>
</tr>
<tr>
<td><strong>Post Cladribine/ Everolimus FCM % aberrant IEL</strong></td>
</tr>
</tbody>
</table>
### CLINICAL CASE 1

| Case 1  
M, 57y |
|----------|
| Main clinical problem(s) | 2011: celiac disease, R/ GFD  
6-2015: vomiting, anorexia, weight loss, diarrhea, …. => RCD |
| Pre treatment FCM % aberrant IEL | 11-2015: FCM on intestinal biopsy <1% |
| RCD: type I or II? | type I |
| Post Cladribine/ Everolimus FCM % aberrant IEL | NA |
### CLINICAL CASE 2

**Case 2**  
M, 58y

| Main clinical problem(s) | 2002: celiac disease, R/ GFD  
2013: dysphagia, weight loss, vomiting, …despite GFD => **RCD**  
2013-2015: multiple gastroscopies: no macro- / microscopic evidence of progression towards lymphoma |
|---|---|
| Pre treatment FCM % aberrant IEL | 7-2015: FCM on intestinal biopsy  
**96%** |
| RCD: type I or II? | **type II** => R/ Cladribine + Everolimus |
**CLINICAL CASE 2**

<table>
<thead>
<tr>
<th>Case 2</th>
<th>M, 58y</th>
</tr>
</thead>
</table>
| **Main clinical problem(s)** | 2002: celiac disease, R/ GFD  
2013: dysphagia, weight loss, vomiting, …despite GFD => **RCD**  
2013-2015: multiple gastroscopies: no macro- / microscopic evidence of progression towards lymphoma |
| **Pre treatment** | 7-2015: FCM on intestinal biopsy  
96% |
| **RCD: type I or II?** | **type II** => R/ Cladribine + Everolimus |
| **Post Cladribine / Everolimus** | 3-2016: FCM on intestinal biopsy  
94% |
| Main clinical problem(s) | 2002: celiac disease, R/ GFD  
11-2014: weight loss, diarrhea, … despite strict GFD => RCD |
|-------------------------|-----------------------------------------------------------------|
| Pre treatment FCM % aberrant IEL | I-2015: FCM on intestinal biopsy  
73% |
| RCD: type I or II? | type II => R/ Cladribine |
## CLINICAL CASE 3

<table>
<thead>
<tr>
<th>Case 3</th>
<th>M, 78y</th>
</tr>
</thead>
</table>
| **Main clinical problem(s)** | 2002: celiac disease, R/ GFD  
11-2014: weight loss, diarrhea, … despite strict GFD => RCD |
| **Pre treatment FCM % aberrant IEL** | 1-2015: FCM on intestinal biopsy  
73% |
| **RCD: type I or II?** | type II => R/ Cladribine |
| **Post Cladribine FCM % aberrant IEL** | 3-2016: FCM on intestinal biopsy  
9% |
TAKE HOME MESSAGES

- RCD type II patients are at risk for development of EATL
- FCM is well suited for the identification of RCD type II patients
- A cut-off value of 20% aberrant IELs appears reliable for early risk stratification and targeted therapeutic options in RCD patients
- Quantification of aberrant IELs is useful for subsequent follow-up of treated RCD II patients
ACKNOWLEDGEMENTS

Dept. of Laboratory Medicine
S. Govers
N. Van den Panhuyzen

Dept. of Gastroenterology
M. Hiele
T. Vanuytsel

Dept. of Hematology
P. Vandenberghe
G. Verhoef

Dept. of Gastroenterology
K. Nys
J. Cremer
S. Vermeire

T. van Gils
HJ. Bontkes
CJJ. Mulder
G. Bouma