2 out of 5 type 1 diabetes pancreases and one pancreatic transplant had VP1+ Islets

Dotta et al 2007
More recent evidence for enteroviral infection in Type 1 diabetes

• Epidemiological studies demonstrate that EV infection associated with:
  1. Development of autoantibodies in at risk individuals
  2. Accelerated progression from autoantibody positivity to clinical diabetes

• Type 1 diabetes is associated with enterovirus infection of the gut mucosa (Oikarinen, Diabetes, 2012)

• A recent meta-analysis showed a significant association between enterovirus infection and type 1 diabetes related autoimmunity and clinical type 1 diabetes (Yeung, BMJ 2011)
Insulitis and beta-cell loss in human type 1 diabetes displays a lobular pattern.

Foulis, Diabetologia 1984, 1986
Enteroviral VP1
Enteroviral vp-1

Dotta, PNAS, 2008; Richardson, Diabetologia, 2009
Strengths and limitations of UK cohort

• The **largest** single collection in the world.
• Many cases have residual insulin-containing islet & insulitis

• Patients died between 30-50y ago
• All collected from the UK
• Varied fixation times and techniques
• Sometimes only one block available to study
• Limited patient data
• Limited variety of other tissues available
A new initiative in the USA network of Pancreatic Organ Donors (nPOD)

Benefits include

• A variety of samples from cadaveric organ donors; including some non-diabetic individuals deemed to be at risk of developing T1D.
  • Recovery and fixation are uniform between cases.
  • Information is shared between all nPOD investigators via an online informatics system.

BUT - THERE ARE VERY FEW RECENT-ONSET CASES
## The Cohorts

<table>
<thead>
<tr>
<th></th>
<th>UK Cohort</th>
<th>JDRF nPOD cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of cases</strong></td>
<td>72</td>
<td>17</td>
</tr>
<tr>
<td><strong>Mean Age</strong></td>
<td>12.65±1.1y</td>
<td>25.7±2.9y</td>
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<tr>
<td><strong>Age Range</strong></td>
<td>1-42y</td>
<td>4-50y</td>
</tr>
<tr>
<td><strong>Mean Time Since Diagnosis</strong></td>
<td>8.2±4.1mths</td>
<td>11.9±2.3y</td>
</tr>
<tr>
<td><strong>Range Time Since Diagnosis</strong></td>
<td>0-6y</td>
<td>1-35y</td>
</tr>
<tr>
<td><strong>Geography</strong></td>
<td>Scotland, England and Wales</td>
<td>USA</td>
</tr>
<tr>
<td><strong>Sample Collection</strong></td>
<td>1959-1983</td>
<td>2007 onwards</td>
</tr>
<tr>
<td><strong>Sample type</strong></td>
<td>Autopsy and organ donors</td>
<td>Organ donors</td>
</tr>
<tr>
<td><strong>Sample Processing</strong></td>
<td>Variable fixation types and times</td>
<td>Strictly controlled and uniform</td>
</tr>
</tbody>
</table>
Enteroviral VP1 (5D8/1) is expressed in the islets of type 1 diabetes patients from both cohorts
Controls

Non-diabetic control (UK)

Non-diabetic control (nPOD)
Enteroviral VP1 (5D8/1) is expressed in the insulin-containing islets of type 1 diabetes patients in both cohorts.
Enteroviral VP1 expression co-localises with insulin in both cohorts

nPOD Cohort

UK Cohort
Specificity of the Dako VP1 (5D8/1) antibody

Hansson et al, J Pathology 2013

• Suggested that 5D8/1 can bind two mitochondrial proteins, creatine kinase B (CKB) and ATP synthase beta (ATP5B) under denaturing conditions
Detection of proteins by Western blotting in HepG2 cells

- **VP1 (5D8/1)**
- **CKB**
- **ATP5B**

Molecular weight (kDa) at different time points:

- **CVB3**
  - VP1
  - CKB
  - ATP5B

- **Actin**

Time points:
- C
- 0h
- 2h
- 4h
- 6h
- 8h

Sample types:
- C
- CVB3
Peptide ELISA confirms 5D8/1 can bind CKB but not ATP5B
Dako 5D8/1 does not label paraffin-embedded, mitochondrial rich tissues or 78 of 80 human cells and tissues on a microarray.
Detection of ATP5B and VP1 in human pancreas sections

A. Insulin  
B. ATP5B  
C. VP1

VP1

ATP5B
Detection of VP1 and CKB in PANC1 cells

Mock-infected PANC1

CVB3-infected PANC1
Dako VP1 (5D8/1) Specificity

- Under denaturing conditions, we have confirmed that 5D8/1 can recognise CKB but not ATP5B
- Under carefully optimised non-denaturing IHC conditions, 5D8/1 does not recognise CKB or ATP5B
- 5D8/1 remains an extremely useful tool for detecting enterovirus infections in FFPE tissue

The presence of virus needs to be confirmed using other methods
The nPOD-V Group

- The nPOD-V group began activities last year recognising that demonstrating a pathogenic role for one or more viruses in T1D could have very important therapeutic implications.
- This effort has culminated in a grant proposal submitted to JDRF in April 2012 and awarded in August 2012.
The nPOD-V Group

• Aim is to develop a pipeline for comprehensive and integrated understanding of the role of enteroviruses in disease pathogenesis

• The sharing of tissues and coordinated analysis by multiple investigators affords a key unifying element in science and the rare opportunity to coordinate studies that take into account multiple approaches and design input from multiple investigators
Coordinated analysis of viral antigens in 30 nPOD samples

Section Number:
1-2; UK
3 IHC-Viral protein; UK
4 IHC-Viral protein; Finland
5 ISH-Viral genome; Finland
6 Insulin/ Glucagon; UK
7 IHC Class I MHC; UK
8 IHC PKR; UK
9-10; Finland

Key Info:
1. 96% concordance with viral protein IHC staining between laboratories
2. 69% concordance of IHC and ISH (20/29).
Class I MHC

Enterovirus VP1 positive islet

Hyper expression

Normal
Viral Predictors –
1. VP1 IHC (UK)
2. VP1 IHC (Finland)
3. *In situ* hybridisation (ISH)
4. MHC hyper expression

<table>
<thead>
<tr>
<th>Predictors</th>
<th>None</th>
<th>1</th>
<th>2</th>
<th>3 to 4</th>
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<tr>
<td>Controls</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>AAb+</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
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<tr>
<td>T1Ds ICIs</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>11</td>
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<tr>
<td>T1D IDIs</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
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</table>

Based on VP1/ISH and MHC hyper expression we predicted **26 of the 30 case classifications correctly** – this included all of the T1Ds with residual ICIs and 6 of the 7 controls.
Summary of Concordance Study

- IHC correctly identified all the 12 T1D or AAb+ cases with residual ICIs
  - 6 of these were also ISH+
  - All had class I MHC hyper expression

- ISH positivity was identified in 8 T1D, 1 AAb+ and 1 control

- MHC hyper-expression was seen only in T1Ds or AAb+ cases

- ONLY 92 VP1+ cells were found among a total of almost 3000 islets examined across 29 cases
  → IT WILL BE HARD TO DETECT & IDENTIFY SPECIFIC SEROTYPE (Proteomics/ RNA analysis / virus isolation)

- 1 exception; Autoantibody+ case (ISH+ & MHC h/e)
Recruitment of immune cells

Autoimmunity

Destruction of beta cells

Viral footprint?

Pathogen Recognition Receptors

VP1

Class I MHC

CD45

Insulin

IFN alpha

Hyper-expression of Class I MHC

Cytokines/Chemokines

Recruitment of immune cells

Autoimmunity

Destruction of beta cells
Enteroviral VP1 co-localises with Protein Kinase R (PKR) in both cohorts
Summary

- Small numbers of enteroviral VP1+ cells are observed in the ICIs of type 1 diabetes cases in both the nPOD collection and in a separate (older) UK cohort.

- The presence of VP1 was dependent on the presence of beta cells, regardless of duration of disease.

- Enterovirus presence was confirmed by enterovirus specific in situ hybridisation.

- In both cohorts, enteroviral VP1 expression correlated with increased expression of the pathogen-recognition receptor, PKR and hyper expression of class I MHC.
Conclusions

These results imply that enteroviral infection occurs commonly in type 1 diabetes and that an anti-viral response is mounted in infected islet cells.

This anti-viral response has the potential to sensitise beta cells to immune-mediated destruction.
Normal ICI

Acute viral infection

Reactivation of virus

Persistent silent infection with no active virus produced

Death of β cell

Release and uptake of β cell antigens by APCs

Reduced Mcl1 expression THEREFORE Increased sensitivity to apoptosis

↑ Adhesion molecules/chemokines

Induction of autoimmunity?
Collaborators

University of Exeter (Plymouth)
Noel Morgan
Shalinee Dhayal
Pia Leete
David Hilton

Glasgow
Alan Foulis
Maura Farquharson
Andrew Hamilton

University of Brighton
Adrian Bone

nPOD V members

JDRF nPOD
Martha Campbell-Thompson
Alberto Pugliese
Mark Atkinson

University of Tampere
Heikki Hyöty
Sami Oikarinen
Maarit Oikarinen
Jutta Laiho

Uppsala University
Gun Frisk
Therese Rosenling

Karolinska Instituett
Malin Flodstrom-Tullberg
Katharina Lind
Emma Svedin

Pevnet
Diabetes Research Foundation
nPOD
JDRF
Protein Kinase R – is it active?

- Promotes anti-viral responses by inhibiting protein synthesis
- Levels of rapidly turned over proteins are quickly lost from the cell
- One such protein is the anti-apoptotic Bcl2 family member – Mcl-1
Myeloid Leukemia Sequence 1 (Mcl-1)

- Mcl-1 is a rapidly turned over protein that functions to promote cell survival
- Mcl-1 is expressed in β-cells
- Mcl-1 knockdown increases stress induced β-cell apoptosis
Mcl-1 is reduced in VP1+ β-cells
The data suggest that the PKR which is induced during enteroviral infection is activated.

Additionally, the decrease in Mcl-1 levels in infected β-cells may alter their sensitivity to pro-apoptotic stimuli released from infiltrating immune cells.
Immunodetection of enteroviral capsid protein, VP-1 in the heart and pancreas of a neonate with Coxsackie B viral pancreatitis (Dako clone 5D8/1)
but not in controls

Heart

Pancreas
## Concordance Study

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Section 3 Dako VP1 UK</th>
<th>Section 4 Dako VP1 Finland</th>
<th>Section 5 ISH Finland</th>
<th>Section 7 Class I MHC</th>
<th>No. of Indicators Positive</th>
<th>Reveal</th>
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<tbody>
<tr>
<td>PB 6070-02</td>
<td>4 islets (11 cells)</td>
<td>pos</td>
<td>neg</td>
<td>Hyper expression</td>
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<tr>
<td>PT 6070-02</td>
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<td>pos</td>
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<tr>
<td>PB 6073-04</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>Normal (exocrine +)</td>
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<td>Control</td>
</tr>
<tr>
<td>PB 6081-02</td>
<td>1 islet (1 cell)</td>
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<td>pos</td>
<td>h/e (exocrine +)</td>
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<td>T1D</td>
</tr>
<tr>
<td>PB 6084-01</td>
<td>7 islets (26 cells)</td>
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<td>neg</td>
<td>Hyper expression</td>
<td>3</td>
<td>T1D</td>
</tr>
<tr>
<td>PH 6088-03</td>
<td>1 islet (8 cells)</td>
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<td>pos</td>
<td>Hyper expression</td>
<td>4</td>
<td>T1D</td>
</tr>
<tr>
<td>PB 6088-08</td>
<td>3 islets (4 cells)</td>
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<td>pos</td>
<td>Hyper expression</td>
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<tr>
<td>PB 6095-04</td>
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<td>neg</td>
<td>neg</td>
<td>Normal (exocrine +)</td>
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<td>Control</td>
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<tr>
<td>PB 6096-01</td>
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<td>neg</td>
<td>Normal</td>
<td>0</td>
<td>Control</td>
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<tr>
<td>Poth 6121-04</td>
<td>6 islets (8 cells)</td>
<td>pos</td>
<td>pos</td>
<td>Hyper expression</td>
<td>4</td>
<td>T1D</td>
</tr>
</tbody>
</table>
Figure: Progression of Insulitis at various stages of T1D

Mean No. of cells per islet

Ins ++ve

Ins +ve

Ins -ve

Insulin Level

CD4
CD8
CD20
CD68
In 2011 there were an estimated 2.9 million patients with diabetes in the UK

This is expected to rise to **5 million people by 2025.**

Diabetes UK estimates that **Up to 15 per cent of these patients may have Type 1 Diabetes**

The prediction is that **new cases of type 1 diabetes in children younger than 5 years will double by 2020.**

(Patterson et al, 2009)