Expression of Interferon-Stimulated Genes in Insulitic Pancreatic Islets of Patients Recently Diagnosed with Type 1 Diabetes

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Short running title: ISGs in T1D islets

Word count: 2669

Number of tables: 0

Number of figures: 4 (+2 supplementary)
Abstract

A primary insult to the pancreatic islets of Langerhans, leading to activation of innate immunity, has been suggested as an important step in the inflammatory process in type 1 diabetes (T1D). The aim of this study was to examine whether interferon-stimulated genes (ISGs) are overexpressed in human T1D islets affected with insulitis. By employing laser capture microdissection and qPCR array, 23 out of 84 examined ISGs were found to be overexpressed at least 5-fold in insulitic islets from living patients with recent onset T1D, participating in the Diabetes Virus Detection (DiViD) study, compared to in islets from non-diabetic organ donors. Most of the overexpressed ISGs, including GBP1, TLR3, OAS1, EIF2AK2, HLA-E, IFI6 and STAT1, showed higher expression in the islet core compared to in the peri-islet area containing the surrounding immune cells. In contrast, the T cell attractant chemokine, CXCL10, showed an almost 10-fold higher expression in the peri-islet area than in the islet, possibly explaining partly the localization of T cells mainly to this region.

In conclusion, insulitic islets from recent onset T1D subjects show overexpression of ISGs, with an expression pattern similar to that seen in islets infected with virus or exposed to IFNγ/IL1β or IFNα.
Introduction

Activation of the innate immune response in the islets of Langerhans has been suggested to be an important step in development of type 1 diabetes (1-3). In particular, viral infection of the beta cells leading to the production of type I interferon and induction of several hundred interferon-stimulated genes (ISGs) has been suggested (4-6). ISGs constitute a group of genes that are up-regulated in response to interferon (IFN) and put the surrounding cells in an antiviral state, protecting them from being infected. Being induced by virus infection (7), signs of their expression have been interpreted as ‘viral footprints’ but, importantly, many of these genes are overexpressed also under other conditions, in the absence of viral infection (8; 9).

Studies of human pancreatic islets at onset of type 1 diabetes are very rare. Although immunohistochemical studies have suggested expression of type I IFN (10; 11) and some ISGs (12; 13) in type 1 diabetic islets, other studies have failed to detect type I IFN in pancreata from diabetic subjects (14). End-point PCR, with no or limited quantitative value, has suggested IFNα expression to be more prevalent in T1D than control pancreata (15; 16), but this has not been confirmed by quantitative methods. Whole transcriptome analysis of pancreatic tissue demonstrated upregulation of some ISGs in a pancreas soon after T1D onset, but this upregulation was not found in isolated islets from the same donor (17).

In this study, freshly frozen and cultured pancreatic tissue from living patients with recent onset type 1 diabetes collected within the Diabetes Virus Detection (DiViD) study (18) were examined. We have published previously that islets isolated from these patients showed mainly background levels of cytokine/chemokine release (19). However, the enzymatic digestion of the pancreas and isolation of islets may lead to induction of inflammatory
markers also in the controls. In this present study, we used laser capture microdissection (LCM) to extract RNA from islets with insulitis directly from the frozen pancreatic tissue and compared the expression of 84 ISGs with that in non-insulitic islets from non-diabetic organ donors. By separately microdissecting the islet core and the peri-insulitis leukocytes in insulitic T1D islets, we aimed to determine the origin of the detected overexpression. In addition, RT-PCR was used to search for the presence of enterovirus in the microdissected samples.
Research Design and Methods

Human samples

Pancreatic biopsies from a total of five patients recruited to the DiViD study, and five multi-organ donors procured within the Nordic Network for Islet Transplantation, were included in the study. The DiViD samples were collected by pancreatic tail resections performed 3-9 weeks after the diagnosis of type 1 diabetes and the patient characteristics have been described previously in detail (18-21). Good quality RNA, suitable for use in this present study, was possible to extract from laser-captured islets from DiViD case 2-6. The multi-organ donors were previously healthy, without known pancreatic disease, and age-matched to the DiViD cases (mean age 25.8, range 20-32). The DiViD study was approved by the Norwegian Government’s Regional Ethics Committee and informed consent was obtained from the patients after oral and written information from the diabetologist and surgeon separately. The use of pancreatic tissue from deceased organ donors for research was obtained verbally from the deceased’s next of kin by the attending physician and documented in the medical records in accordance with Swedish law and as approved by the Regional Ethics Committee in Uppsala (Dnr 2015/444).

Laser-capture microdissection

Frozen tissue samples from the pancreatic tail region were sectioned and mounted on Superfrost Plus glass (Menxel-Gläser, Braunschweig, Germany) or Arcturus PEN Membrane Glass Slides (Life Technologies, Carlsbad, CA, USA) for IHC and LCM respectively. Consecutive sections were stained for CD3 or used for LCM to microdissect islets with insulitis (≥15 CD3+ cells) from the diabetic samples and islets without insulitis from the non-diabetic samples. The procedure was as described previously (19).
In order to microdissect peri-insulitis and non-insulitic islet cores separately, slides for IHC were double-stained for CD45 (mouse mAbs 2B11+PD7/26, dilution 1:75, DAKO) and insulin (polyclonal guinea pig anti-insulin, dilution 1:140, DAKO). CD45 was chosen as a marker instead of CD3 in this case to allow exclusion of any islet core affected by non-T cell insulitis. Insulin-containing islets with peri-insulitis, but without insulitis in the islet core, were localized, first on the IHC slides, and then on the unstained consecutive membrane slides by their autofluorescence. In DiViD subject 3, several islets fulfilled these criteria in multiple sections per islet and, from these, the non-insulitic islet core, the peri-insulitis, and a non-infiltrated peri-islet region could be microdissected and pooled in separate tubes for RNA extraction and expression analysis. The procedure is described in detail in Fig. 1.

**RNA isolation and expression analysis from microdissected tissue**

RNA isolation, cDNA synthesis, pre-amplification of cDNA, and expression analysis was performed with kits from Qiagen (Sollentuna, Sweden) as described (19). A pathway-specific primer mix (Human Type I Interferon Response, PBH-016Z, Qiagen) was used for the pre-amplification and a PCR array (Human Type I Interferon Response, PAHS-016ZC, Qiagen) was used for the expression analysis of 84 known ISGs. Beta actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and 60S acidic ribosomal protein P0 (*RPLP0*) were chosen for normalization. Genes with a Cq >35 were regarded as non-detected and assigned a Cq of 35 to calculate fold induction. RNA from the same samples was analyzed for the presence of Enterovirus by a semi-nested RT-PCR amplifying a part of the 5’UTR region of the Enterovirus genome as described previously (21; 22).

**Analysis of isolated islets**
Total RNA extracted from islets isolated by collagenase digestion from the tip of the tail was subjected to whole transcriptome sequencing as reported (20). Normalized reads per kilobase per million mapped reads (RPKM) for the genes included in the RT2 Profiler PCR array for ISGs are presented in this study and the full data set (reads) is openly available in the BILS DOI repository (http://doi.bils.se/) under doi: 10.17044/BILS/g000002.

**Statistical analysis**

$P$ values were calculated for each gene using Mann-Whitney signed rank test and a rank-based volcano plot was created based on the raw $P$ values and the fold expression relative to the mean of the controls.
Results

Interferon-stimulated genes are overexpressed in T1D islets compared to non-diabetic controls

Expression of 41 of the 84 analyzed genes was detected in all samples of laser-captured islets from all donors. Four genes (SH2D1A, CD80, HLA-G, TLR7) had detectable expression levels in insulitic T1D islets but were not detected in islets from non-diabetic control subjects. 23 of the 84 genes showed an at least 5-fold overexpression in insulitic T1D islets compared to non-diabetic controls whereas only one showed overexpression in the controls (Fig. 2A and 2B). Interestingly, type I IFN (IFNA2, IFNB1, IFNA4, IFNA1) was rarely detected and was not overexpressed in the T1D islets.

The overexpression of some ISGs (e.g. GBP1, TLR3, OAS1, CXCL10, CCL5, and CASP1) was detected also in islets from T1D subjects that were isolated by collagenase digestion and handpicked (Fig. 3 and Suppl. Fig. 1). However, compared to islets isolated from non-diabetic subjects, less ISGs were overexpressed in isolated islets than in laser-captured islets, suggesting that the overexpression is most evident in islets with insulitis and/or that it is affected by the islet isolation process itself.

Separate analysis of ISGs in peri-insulitis and non-insulitic islet cores

To distinguish islet expression from expression in the infiltrating immune cells, in islets affected by peri-, but not intra-, insulitis, the non-insulitic islet core, the surrounding peri-insulitis, and the non-infiltrated peri-islet areas were microdissected separately in sections from DiViD subject 3. Due to a lower number of microdissected cells compared to when all insulitic islets were captured, genes with a relatively low expression were not detected. However, 38 of the 84 ISGs were detected in the islet core, peri-islet area, or both (Fig. 4).
Most of these, including GBP1, TLR3, OAS1, EIF2AK2, HLA-E, IFI6 and STAT1, showed higher expression in the islet core compared to in the peri-islet area containing the surrounding immune cells. In contrast, the T cell attractant chemokine, CXCL10, overexpressed in T1D islets, showed an almost 10-fold higher expression in the peri-islet area than in the islet core. Most genes were detected also in non-infiltrated peri-islet areas, but their expression levels were generally lower than within the islet core (Fig. 4).

No evidence of virus infection in insulitic islets from subjects with T1D

No enterovirus RNA was detected in any of the laser-captured insulitic islets from the T1D subjects using highly sensitive PCR.
Discussion

In the present study, we demonstrate the overexpression of ISGs in the islets of patients with recent onset T1D. Since the number of cases available were limited, it is difficult to draw firm conclusions for each individual gene, especially when compensating for multiple comparisons. However, the finding that 26 of the 84 genes were either overexpressed at least 5-fold or were overexpressed and had rank-based p-values below 0.05 (fig. 2B), indicates that a pathway leading to the induction of ISGs is indeed active in the islets of patients with recent onset T1D. Whether this activation is due to interferon in these T1D patients remains to be investigated.

When comparing the PCR array data of insulitic laser-captured islets with RNA sequencing data from isolated islets, the overexpression of **GBP1, TLR3, OAS1, STAT1, CXCL10,** **CCL5**, and **CASPI** was confirmed, increasing the likelihood that these genes are truly overexpressed in T1D islets. **CXCL10** and **CCL5** encode the T cell recruiting chemokines also known as Interferon gamma-induced Protein 10 (IP-10) and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), respectively. Expression of CXCL10/IP-10 is induced by enterovirus infection of human pancreatic islets in vitro (23), a scenario that has been suggested to occur in virus-induced T1D (14). However, CXCL10/IP-10 can also be induced by IFN\(\gamma\) under inflammatory conditions and is secreted from islets obtained from subjects with type 2 diabetes (24). Also CCL5/RANTES has been demonstrated to be upregulated in islets upon enterovirus infection (7; 25; 26). **GBP1** encodes a GTPase, Guanylate-binding protein 1 (GBP1), that is induced by type I and II IFN and has anti-angiogenic effects (27). It has been shown to be increased in sera of patients with rheumatic autoimmune diseases characterized by chronic inflammatory vessel activation (28), but to our knowledge it has never before been associated with T1D. The finding of increased GBP1 expression in T1D
islets in this study may suggest the involvement of endothelial activation in T1D pathogenesis.

Importantly, the RNA-seq analysis of isolated islets has limitations compared to PCR array analysis of laser-captured islets; the islet isolation procedure and islet culture may alter gene expression levels and we possess no knowledge concerning whether the islets used for analysis were insulin-positive and/or affected by insulitis. Thus, it is likely that several of the genes found to be overexpressed in insulitic islets by PCR array analysis, but not by RNA-seq analysis of isolated islets, represent true positive findings reflecting actual differences between islets from T1D subjects close to onset of disease and matched non-diabetic controls.

The pattern of ISG overexpression is in line with that demonstrated in isolated islets infected in vitro with an enterovirus, CVB5, (7) or exposed to type I (8) or II IFN (7; 9) (suppl fig. 2). The lack of evident type I IFN expression, together with the failure to detect enteroviral genome in the analyzed islets, may argue against viral infection as a source of ISG induction in the islets of these subjects and the presence of type II IFN (IFNG) expression may in itself explain the induction of many of these genes. However, virus infection in a nearby cell, not present in the laser-captured area, cannot be excluded. The ISG overexpression in the analyzed samples could be a sign of an anti-viral state in these cells induced by infected nearby cells secreting type I IFN. In fact, presence of enterovirus was suggested in the islets of the DiViD subjects by immunostaining for the capsid protein VP1 in several endocrine cells and by trace amounts of enteroviral RNA sequences in the culture medium of isolated islets (21). However, only in one subject (case 6) could this virus be found by PCR in well-preserved frozen pancreatic tissue.
Induction of innate immunity by viruses is a complicated interplay between the virus and the infected host cell. Viruses produce proteins that efficiently inhibit the production of antiviral genes and utilize the cellular machinery for its own replication. Different strains and serotypes of a virus vary in their capacity to induce an innate immune response (29), dramatically affecting the outcome of infection. A strong innate immune response may limit viral replication, but at the same time contribute to unwanted tissue damage. It has been debated whether a ‘diabetogenic’ viral strain likely is a strong or a weak inducer of innate immunity (30). In mice that have been genetically modified to lose their ability to respond to IFN specifically in their beta cells, enterovirus infection rapidly causes diabetes (31) and a reduced expression of the IFNα-receptor associated protein TYK2, compromising the induction of ISGs by type I IFN, was recently shown to increase the susceptibility to virus-induced diabetes (32). In the present study, the ISGs were clearly overexpressed to levels comparable to when isolated human islets were cultured with high levels of IFN in vitro. These data suggest that if T1D in these cases were induced by a virus, it was not associated with weak induction of innate immunity.

Laser capture microdissection is a powerful tool to selectively study the gene expression in different tissues and cells. In this study, we used the method to quantify the gene expression specifically in islets affected with insulitis and successfully employed it to distinguish the expression in the islet core from the expression in the surrounding immune cells. Our finding of the T cell-recruiting chemokine CXCL10 in the peri-islet area, but not within the islet core, is in contrast to the IHC-based finding of this chemokine in the beta cells of recent onset T1D subjects (13; 14), and in conflict with the idea that a direct virus infection of the beta cells is the cause of its induction (14; 24; 33). However, it agrees well with the location of T cells mainly in the peri-islet area and more rarely infiltrating inside the islet core (19; 34). Future
studies are needed in order to find the underlying cause of CXCL10 induction and the recruitment of T cells to the peri-islet area. Also, this finding highlights the importance of characterizing different pancreatic regions separately, especially in a disease with a lobular pattern like T1D affecting a heterogeneous organ like the pancreas.

One weakness of this study is that it is mainly descriptive, due to the nature of the studied material. When we report ‘overexpression’ of ISGs in T1D islets, we do not know if these genes were upregulated as a part of the pathological processes leading to type 1 diabetes, are a consequence of destructive process, or even if baseline expression levels of these genes are higher in patients that develop T1D. Due to the complications associated with acquiring pancreatic tissue from live patients (18), novel imaging techniques will be required to follow ongoing pathogenic processes in the pancreas over time. Nevertheless, in this study, we utilized the pancreatic tissue taken from subjects with recent onset T1D in the DiViD study, which is the, so far, largest collection of well-preserved pancreatic tissue from subjects with recent onset T1D. This, together with a sophisticated LCM strategy, allowed us to do a molecular characterization of T1D islets not previously possible to perform. Advancing the protocols for LCM, and using them on well-characterized biopsy materials, allows high-resolution characterization of the ongoing pathogenic processes in affected pancreata.

In conclusion, we demonstrate overexpression of many ISGs in T1D islets affected with insulitis. Strategic use of laser-capture microdissection allowed us to separate islet expression from expression in the surrounding immune cells. The upstream inducer of this ISG overexpression, possibly playing an important role in the induction of T1D, remains to be defined.
Acknowledgements

The authors thank specialist nurse Trine Roald, Oslo University Hospital, Norway, whose invaluable efforts were essential to the success of the DiViD study.

Funding. This study was supported by South-Eastern Norway Regional Health Authority (Grant to KDJ), The Novo Nordisk Foundation (Grant to KDJ), the PEVNET Study Group funded by the European Union's Seventh Framework Programme [FP7/2007-2013] under grant agreement n°261441 PEVNET, the Swedish Medical Research Council (VR K2011-65X-12219-15-6, K2015-54X-12219-19-4), the Diabetes Wellness foundation, the Family Ernfors foundation, the Novo Nordisk Foundation, the Åke Wiberg Foundation, the Tore Nilsson Foundation, the Swedish Diabetes Association, Gillbergska Stiftelsen, and Barndiabetesfonden. The participants of the PEVNET consortium are described at http://www.uta.fi/med/pevnet/publications.html. Work performed at Uppsala Genome Center has been funded by RFI/VR “SNISS” Swedish National Infrastructure for large Scale Sequencing and Science for Life Laboratory, Uppsala. Human pancreatic biopsies and isolated islets were obtained from The Nordic network for Clinical islet Transplantation, supported by the Swedish national strategic research initiative EXODIAB (Excellence Of Diabetes Research in Sweden) and the Juvenile Diabetes Research Foundation.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author contributions. ML and EK performed experiments, interpreted data, and contributed to writing the manuscript. LK was responsible for clinical coordination and recruitment of patients, interpretation of data, and participated in writing the manuscript. KDJ is the principal investigator of the DiViD study and, as such, was responsible for design of the study, funding, regulatory issues, and international collaboration. He also participated in data analysis and
interpretation, and in writing of the manuscript. OS designed the experimental setup, researched and interpreted data, and wrote the manuscript. OS is the guarantor of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.


Figure legends

Figure 1. Strategy for laser capture microdissection. Frozen consecutive sections (10 µm) were mounted on regular glass slides for IHC (illustrated in white) or on PEN membrane glass slides for laser capture microdissection (illustrated in black) as depicted (A). Slides for immunohistochemistry were stained for CD45 (brown) and insulin (red) and inspected under a light microscope. Islets with peri-insulitis but without infiltrating immune cells in the islet core were localized, first on the IHC slides, and then on the unstained membrane slides where the same islets were localized by their autofluorescence. This allowed microdissection of the uninfiltrated islet core (B, dotted line), peri-insulitis (B, white arrow) and non-infiltrated peri-islet area (B, arrow) separately. First, the islet core was microdissected (C), then the peri-insulitis (D), and finally an area next to the islet, on the side not affected by peri-insulits (not shown). The same islet in consecutive sections is shown in all figures A-D. Only insulin-positive islet sections where both the previous and following IHC slide displayed the same pattern of a non-infiltrated islet core and a clear focal peri-insulitis were used and pooled for RNA extraction and analysis.

Figure 2. Expression analysis of 84 genes associated with response to interferon in insulitic islets from subjects with recent T1D onset and non-insulitic islets from non-diabetic organ donors. The expression of each gene was normalized to the expression of the reference genes (*ACTB, GAPDH, RPLP0*) and is displayed as fold up- or down regulation compared to the mean relative expression in the five non-diabetic controls (A). A rank-based volcano plot comparing the mean expression of each gene in T1D versus non-diabetic islets is shown (B). Dotted lines mark 5-fold difference (vertical) and *p*=0.05 (horizontal). Genes with *p*<0.05 or overexpressed at least 5-fold are labeled with their gene symbol. *P* values were calculated for each gene using Mann-Whitney signed rank test.
Figure 3. mRNA expression levels of ISGs that were detected as overexpressed both in laser-captured insulitic T1D islets by PCR array (A) and in isolated T1D islets by RNA sequencing (B). Genes are noted by their gene symbol. In (A) the expression of each gene is displayed relative to that of the reference genes (*ACTB, GAPDH, RPLP0*). Each data point represents the expression in islets from one subject. RPKM; reads per kilobase per million mapped reads.

Figure 4. Expression of interferon-stimulated genes in non-insulitic islet cores (black bars), in peri-insulitis (white bars), and non-infiltrated peri-islet areas (striped bars) of insulin-containing T1D islets affected with peri-insulitis. Only genes with detectable expression in either the islet or peri-islet area are shown. The expression of each gene relative to the expression of the reference genes (*GAPDH* and *RPLP0*) is shown (2^{ΔC_q}).
Figure 1. Strategy for laser capture microdissection. Frozen consecutive sections (10 µm) were mounted on regular glass slides for IHC (illustrated in white) or on PEN membrane glass slides for laser capture microdissection (illustrated in black) as depicted (A). Slides for immunohistochemistry were stained for CD45 (brown) and insulin (red) and inspected under a light microscope. Islets with peri-insulitis but without infiltrating immune cells in the islet core were localized, first on the IHC slides, and then on the unstained membrane slides where the same islets were localized by their autofluorescence. This allowed microdissection of the uninfiltrated islet core (B, dotted line), peri-insulitis (B, white arrow) and non-infiltrated peri-islet area (B, gray arrow) separately. First, the islet core was microdissected (C), then the peri-insulitis (D), and finally an area next to the islet, on the side not affected by peri-insulits (not shown). The same islet in consecutive sections is shown in all figures A-D. Only insulin-positive islet sections where both the previous and following IHC slide displayed the same pattern of a non-infiltrated islet core and a clear focal peri-insulitis were used and pooled for RNA extraction and analysis. 

Fig. 1

96x50mm (300 x 300 DPI)
Figure 2. Expression analysis of 84 genes associated with response to interferon in insulitic islets from subjects with recent T1D onset and non-insulitic islets from non-diabetic organ donors. The expression of each gene was normalized to the expression of the reference genes (ACTB, GAPDH, RPLP0) and is displayed as fold up- or down regulation compared to the mean relative expression in the five non-diabetic controls (A). A rank-based volcano plot comparing the mean expression of each gene in T1D versus non-diabetic islets is shown (B). Dotted lines mark 5-fold difference (vertical) and p=0.05 (horizontal). Genes with p<0.05 or overexpressed at least 5-fold are labeled with their gene symbol. P values were calculated for each gene using Mann-Whitney signed rank test.

Fig. 2A
317x1131mm (300 x 300 DPI)
Figure 2. Expression analysis of 84 genes associated with response to interferon in insulitic islets from subjects with recent T1D onset and non-insulitic islets from non-diabetic organ donors. The expression of each gene was normalized to the expression of the reference genes (ACTB, GAPDH, RPLP0) and is displayed as fold up- or down regulation compared to the mean relative expression in the five non-diabetic controls (A). A rank-based volcano plot comparing the mean expression of each gene in T1D versus non-diabetic islets is shown (B). Dotted lines mark 5-fold difference (vertical) and $p=0.05$ (horizontal). Genes with $p<0.05$ or overexpressed at least 5-fold are labeled with their gene symbol. P values were calculated for each gene using Mann-Whitney signed rank test.

Fig. 2B
65x47mm (600 x 600 DPI)
Figure 3. mRNA expression of ISGs that were detected as overexpressed both in laser-captured insulitic T1D islets by PCR array (A) and in isolated T1D islets by RNA sequencing (B). Genes are noted by their gene symbol. In (A) the expression of each gene is displayed relative to that of the reference genes (ACTB, GAPDH, RPLP0). Each data point represents the expression in islets from one subject. RPKM; reads per kilobase per million mapped reads.
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Fig. 4
82x37mm (300 x 300 DPI)
Suppl. Figure 1. Expression of 84 genes associated with response to interferon extracted from RNA-seq data of isolated islets from subjects with recent T1D onset and islets from non-diabetic organ donors. The expression is displayed as fold up- or down regulation compared to the mean expression in the three non-diabetic controls.
Suppl. Figure 2. Fold overexpression compared to controls of the ISGs in laser-captured T1D islets in this study (A) and what has been induced experimentally in isolated islets in other studies (B-E). The data in (B) was extracted from the RNA sequencing data of islets exposed to IL1\(\beta\) and IFN\(\gamma\) for 48 h, published by Eizirik et al. 2012 (1), Supporting information, Dataset S1, “table_RPKM.xls. The data in (C) and (D) were from microarray data on islets infected with Coxsackievirus B5 (C) or islets exposed to IL1\(\beta\) and IFN\(\gamma\) (D) for 48 h, published by Ylipaasto et al. 2005 (2), Electronic Supplementary Material 2 and 3. The data in (E) is from PCR array data of islets exposed to IFN\(\alpha\) for 6 h and published by Lind et al. 2013 (3), Supplemental file 1. In Ylipaasto et al, only genes considered significantly changed were reported and thus, in (C) and (D), genes that were not significantly changed are displayed as zero. Lind et al used an earlier version (QIAGEN, PAHS-016A) of the ISG PCR array used in this study (QIAGEN, PAHS-016ZC), and thus, only genes included in both arrays are shown in (E).