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AUTOIMMUNE PANCREATITIS

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AUTOIMMUNE PANCREATITIS.

Distinct pathophysiological profiles for discrimination of autoimmune pancreatitis subtypes.

Keywords:

Type 1 and type 2 autoimmune pancreatitis, chronic pancreatitis and pancreatic cancer.

Introduction :

Autoimmune pancreatitis (AIP) is a new nosological entity that was first reported by Sarles et al. in 1961(1) in Marseilles and then named by Yoshida et al. in 1995(2) in Japan. Many Western researchers then ignored it, but over the last decade, interest in AIP has grown, its recognition has increased and the disease has been intensively studied around the world.

AIP is an immune mediated fibro-inflammatory form of pancreatitis that has a unique histopathologic pattern: a) a periductal lymphoplasmocytic infiltration, b) storiform fibrosis and c) obliterative phlebitis that makes it distinguishable from other pancreas disease. Moreover, clinically AIP is the only type of pancreatitis that responds to steroid administration.

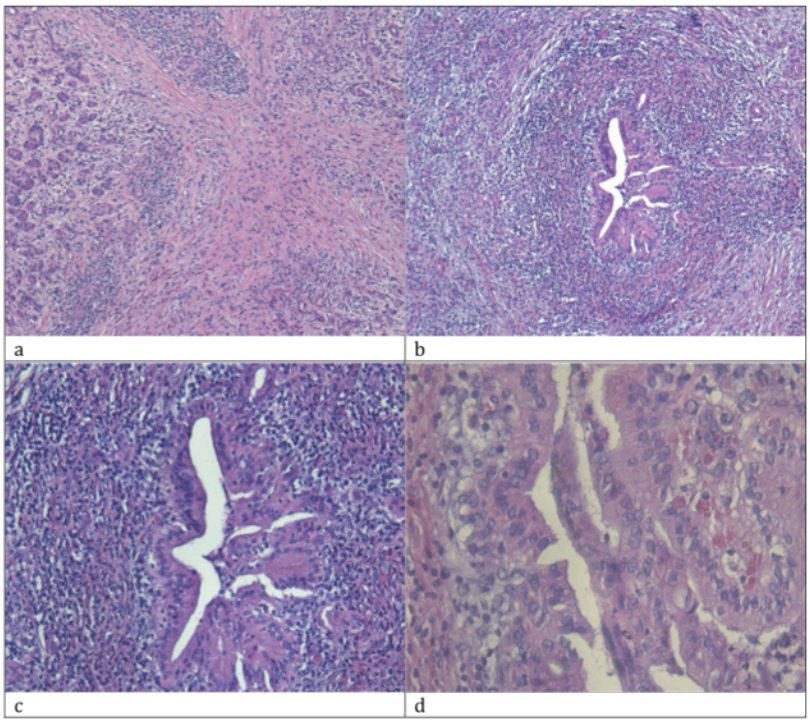

Figure 1 : Hematoxylin and eosin stain:

a) AIP type 1, low magnitude, cellular fibrosis replacing periductal acinar tissues and small duct with lymphoplasmocytic infiltration

b) AIP type 2, low magnitude and c) intermediary magnitude showing periductal lymphoplasmocytic infiltration and storiform fibrosis characteristic to AIP

d) AIP type 2, high magnitude, pancreatic duct showing granulocytic epithelial lesions (GEL) which is the destruction of the epithelium by invading granulocytes, specific to type 2 AIP.

The Honolulu consensus document, recently published by Chari et al., clarifies the histopathologic and clinical subtypes of AIP(3). It appears that there are two types of AIP, with distinct clinical profiles including differences in age of appearance, sex ratio, geographic distribution and also in their histological and immunological signature (table 1).

Type 1 AIP (the most common type in Asia), was also named lymphoplasmocytic sclerosing pancreatitis (LPSP) because of its histological features its association with elevated IgG4 serum levels and IgG4 positive cells infiltration as well as various other autoantibodies such as anti-nuclear antibodies, anti-lactoferrin and anti-carbonic anhydrase II. Furthermore, this latter type involves other organs than the pancreas, indicating that it is more a systemic disease.

Type 2 AIP, called idiopathic duct centric pancreatitis (IDCP), is recognized by its specific histological feature: granulocytic epithelial lesions (GEL). These lesions consist of a focal disruption and destruction of the duct epithelium caused by the invasion of neutrophilic granulocytes, which makes some people call it AIP with GEL. This later subtype is more common in Western countries.

	Type 1 AIP	**Type 2 AIP**
Clinical presentation	Obstructive jaundice 75% Acute pancreatitis 15%	Obstructive jaundice 17-40% Acute pancreatitis 50%
Mean age	60	40
Sex ratio	Male>>Female	Male=Female
Geographic distribution	Asia> USA and EU	EU>USA>Asia
Serology	High serum IgG/IgG4 Presence of autoantibodies	Normal serum IgG/IgG4 No autoantibodies
Other organs involvement	1) sclerosing sialadenitis 2) IgG4+ associated cholangitis 3) retroperitoneal fibrosis 4) IgG4+ associated tubulo-interstitial nephritis	1) Inflammatory bowel disease

Table 1: Main differences between AIP subtypes.

Because the main differential diagnosis of AIP is pancreatic cancer, it is important to differentiate them before surgery. Although it would be detrimental to treat a patient with a resectable pancreatic carcinoma with steroid, an unnecessary surgery in AIP patients should be avoided.

Furthermore, the awareness that AIP may be part of a new clinicopathological entity of an IgG4-related disease, together with retroperitoneal fibrosis, sclerosing sialadenitis and sclerosing

cholangitis has recently progressed(4). This entity may also incorporate pulmonary, renal and other extrapancreatic sites and is only linked with type I AIP.

However, the etiology and the pathophysiological mechanisms of AIP remain still unknown. Several findings suggest that an autoimmune mechanism might be involved. Mainly because AIP is associated with 1) hypergammaglobulinemia and 2) increased levels of IgG4 in type I AIP, but also because of 3) the presence of a particular common human leukocyte antigen (HLA) in some populations, 4) high titers of circulating immune complexes, and of an elevated number of regulatory T (Tregs) cells in tissue and blood of affected individuals, and 5) the presence of autoantibodies such as anti-nuclear antibodies (ANA), anti-lactoferrin (aLA) and anti-carbonic anhydrase II (ACA II) (mostly unspecific to the disease but still more prevalent in AIP patients vs. non AIP patients). Finally, 6) the common association with other autoimmune diseases and 7) a positive response to steroid therapy strengthen this hypothesis.

Findings regarding the etiology of AIP are various and heterogeneous, and point as well to possible alterations in cellular and humoral immune responses. Regarding cellular immune response in AIP, Okazaki et al. suggested that an elevation of the T helper 1 (Th1) over the T helper 2 (Th2) type immune response may be required for AIP pathogenesis(5). Conversely, Zen et al. showed a significant involvement of Th2 in AIP pathogenesis(6). This later study involved AIP patients with positive IgG4 immunostaining of extrapancreatic organs indicating that they included principally patients with type 1 AIP. These studies were performed at a time when the subtypes of AIP had not been individualized yet. Thereby, we can speculate that different findings could result from the fact that the pathogenesis of AIP subtypes may be distinct. With better recognition of these two subtypes, additional studies are required to demonstrate if both types of AIP with their differences in clinical profiles, age of appearance, sex ratio, geographic distribution, involvement of other organs and immunological signature have distinct pathogenic mechanism and by there distinct etiology. For this reason, as far as it is possible, it is important to always individualize both AIP subtypes whenever a question is assessed in the field of AIP.

However, to explain the heterogeneous findings, several groups, including Park et al. suggested a biphasic model to explain AIP etiology, with first an initiation then a progression of the disease with the support of T regs (7) . This model is based on two main observations.

1) Immunization with carbonic anhydrase II or lactoferrin of the thymectomized AIP mouse model induces an infiltration of CD4+ T cells over B cells in different organs such as pancreas, salivary glands, and bile ducts that mimics type 1 AIP(8). The mouse model develops a depletion of naïve T regs in the periphery and MHC class II restricted autoreactive CD4+ T cells, indicating that these two are crucial in the induction of systemic organ lesions. These CD4+T cells may activate macrophages and proinflammatory reactions during the early stage of AIP with a direct cytotoxic effect through Fas ligand expression(9). Furthermore, the WBN/Kob rat, that has a

congenital decreased in peripheral T regs levels, develops spontaneously sialadenitis, thyroiditis, sclerosis cholangitis and tubulointerstitial nephritis. This observation strengthens the hypothesis that T regs depletion and CD4+ T cells are important in the phase of initiation of AIP.

2) Zen et al. showed a predominance of Th2 over Th1 immune response in AIP with the presence of a large number of T regs. They hypothesized that this large amount of Tregs is induced in a second step to inhibit an initial Th2 immune reaction. Indeed, it is known that T regs are activated by excessive immune reactions in certain types of infections or allergic disorders and are known to prevent a Th2 type immune response(10,11).

Hence, the "biphasic model" would speculate that first, an immune-inflammatory response to self-autoantigens (aLF, ACA II,…) or to molecular mimicry (H.Pylori) induces the disease by activating antigen-presenting cells and macrophages stimulation. Then, in the presence of decreased naïve T regs, Th1 cells activate, release their cytokines and induce the cellular immune reaction. After and in response to Th1 cells activation, T regs are stimulated and induce Th2-cytokines release and activate the humoral response with production of IgG and IgG4 autoantibodies. This concept suggests that the pathophysiology of AIP might resemble the Sjögren's syndrome in which Th1 cytokines may be needed for the initiation and/or maintenance of the disease, and Th2 cytokines are important for disease progression.

The lack of identification of patients with type 1 and type 2 AIP in these previous series make interpretation of these studies very difficult. This mixture of patients might be one of the reasons why findings about AIP are heterogeneous.

The question we address in these experiments is to define the cytokine expression in sera and pancreatic tissues of type 1 and type 2 AIP, in order to see if there is a difference in these two groups. To be able to have a comparison in the amount of expression of these cytokines and to have relevance in clinical diagnosis, we decided to compare AIP groups with pancreatic cancer (PDAC) and chronic pancreatitis (CP), both difficult differential diagnosis of AIP.

Patients and samples:

Patients were recruited from Heidelberg Hospital Clinic (Germany) and chosen according to the patient's list we had in the European pancreatic center (EPC). There were selected depending on the availability and amount of samples (sera or pancreatic tissues) at the time of experiments and as the first in the list we had. Particular attention was paid in order to have as much as possible similar patients between the three different experiments. All pancreatic tissues were reviewed by a pathologist to confirm disease diagnosis (type 1 and type 2 AIP, PDAC and CP).

1. Bio-Plex® system in sera:

The experiment included 24 different AIP patients from Heidelberg Hospital. Among patients, 18 had surgery between 2004 and 2009 and 6 patients had no operations. 21 were men and 3 were women with an average of 51.79 (SE=3.23) years old at the time of the operation for those who underwent surgery and at the time of diagnosis for those who had no surgery. The diagnostic was confirmed on the histology for patients who had surgery. For those you had no surgery, diagnosis was made according to the HISORts Mayo clinic criteria and effectiveness of steroid treatment. Eleven patients were type 1 AIP, 6 were type 2 AIP and 7 had no subtype defined. We had respectively 21 and 22 patients suffering of PDAC and CP.

	Total	AIP				CP	PDAC
			AIP I	AIP II	AIP unknown		
Number of patients	67	24	11	6	7	22	21
Number of observa-tions	76	33	16	9	8	22	21
Male #(%)	50 (74.6%)	21 (87.5%)	11 (100%)	5 (83.3%)	5 (71.4%)	16 (72.7%)	13 (61.9%)
Mean age at operation time (standard error)[1]	57.49 (1.97)	51.79 (3.23)	55.18 (3.21)	34.67 (2.79)	61.14 (6.70)	53.00 (3.26)	68.71 (2.53)

Table 2: Sample used in the first experiment

2. Bio-Plex® system in pancreatic tissues:

In this experiment, pancreatic tissues from 14 AIP patients (4 type 1, 5 type 2 and 5 undetermined type), 12 CP and 12 PDAC were evaluated. In order to increase precision of results each sample was assessed twice and observations are mean values of two observed values. Gender distribution and mean age at the time of operation among the patients are reported in table 3.

[1] For 6 observations the age corresponds to the time of the diagnostic as no operation was done.

	Total	AIP				CP	PDAC
			AIP I	AIP II	AIP unknown		
Number of patients	38	14	4	5	5	12	12
Male #(%)	30 (78.9%)	13 (92.9%)	4 (100%)	4 (80.0%)	5 (100%)	8 (66.7%)	9 (75.0%)
Mean age at operation time (standard error)	55.78 (2.68)	52.71 (4.45)	59.75 (5.02)	42.00 (7.07)	57.80 (8.38)	48.75 (4.56)	66.42 (3.52)

Table 3: Sample used in the second experiment

3. Immunohistochemistry(IHC) in pancreatic tissues:

The study included 29 patients who had undergone surgery during the years of 2004 to 2008. There are 8 AIP patients (5 type 1 and 3 type 2), 10 patients with CP and 11 patients with PDAC. 22 were men and 7 women with an average age of 60.86 (SE 2.45, minimum age of 39 years old to a maximum age of 80 years old) at the time of surgery. Gender distribution and mean age at operation time for each groups are reported in table 4.

	Total	AIP			CP	PDAC
			AIP I	AIP II		
Number of patients	29	8	5	3	10	11
Male #(%)	22 (75.9%)	7 (87.5%)	5 (100%)	2 (66.7%)	7 (70.0%)	8 (72.7%)
Mean age at operation time (standard error)*	60.86 (2.45)	57.75 (4.59)	63.20 (3.75)	48.67 (9.17)	55.50 (4.84)	68.00 (2.48)

Table 4: Sample used in the third experiment

Methods:

Bio-Plex® system:

For the two first experiments, cytokine levels in our samples were determined using a Bio-Plex human cytokine 17 plex panel kit (Bio-Rad) that was analyzed on the Bio-Plex workstation of the

European Pancreatic Center(EPC). Bio-Plex cytokine assay is a bead-based assay that quantifies 17 human cytokines in diverse materials such as serum or plasma. Using a 96 well microplate format it quantitates at a same time in a same liquid and under constant and equal conditions 17 cytokines over a broad dynamic range. These cytokines are IL-2, 4, 5, 6, 7, 8, 10, 12 (p70), 13, 17, 1β, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β and TNF-α. The kit includes a cytokine assay that contains antibody-conjugated beads, detection antibody and antigen standards, a cytokine reagent kit with assay buffer, wash buffer, detection antibody diluent, streptavidin-PE, 96-well filter plate, sealing tape and instruction manual and a diluent kit for sample and standard dilution.

The concept of Bio-Plex relies on assays on beads. Reactions take place on these beads while they are in suspension. Using a two-dye method, luminex produce 100 distinct bead sets. Bio-Plex uses these uniquely color-coded bead sets to identify multiple assays in a single well. While suspended in a sample, the bound bind targeted molecules. Fluorescently labeled reporter tags (streptavidin PE) bind to the sample molecules. Precision fluidics align the beads in single file and pass them through the lasers one at a time. A red classification and green reporter laser illuminate individual beads to identify each bead's spectral address and associated reporter signal. Concentrations (pg/ml) are calculated by Bio-Plex Manager software using a standard curve derived from a recombinant isotype standard and are then reported into an excel file.

1. Bio-Plex® system in sera:

Sera from patients were pull out from main freezers of the EPC, depending on the list we had and were diluted 1 to 4 in assay buffer. Using the Bio-Plex human cytokine 17 plex panel kit, we disposed on the 96 microplate 8 standard in duplicate, 2 blank solutions, 34 AIP, 22 PDAC and 22CP.

We first prewet the filter plate and add beads. We washed 2 times and add 50µl of standard, blank or sample depending on the wall and incubate 30 min. Then, we washed and filtered the plate 3 times, add 25 µl of detection antibody and incubate it during 30 minutes. Again, we washed and filtered the plate 3 times. 50 µl of streptavidin-PE were added to stop the reaction and the plate was incubated 10 minutes. We washed and filtered it 3 more times and beads were resuspended with 125µl of assaybuffer. After a strong shake the plate was read using the Bio-Plex reader computer.

2. Bio-Plex® system in pancreatic tissues:

First all tissues were cut in slides of 9 µm and mixed in a cell lysis solution containing lysis buffer, factor 1, factor 2 and a protease inhibitor (phenylmethylsulfonyl fluoride, PMSF) from the Bio-Plex cell lysis kit product insert of Bio-Rad. Each tube was then incubated during a period of minimum 24 hours in -80°C. Using the SonoPuls mini 20 Bandelin®, an ultrasonic homogenizer,

each solution was homogenate and then centrifuged at 4° during 15 minutes. The supernatant was collected.

The protein concentrations in the supernatant were quantified using the ELISA system in order to be adjusted to 600 µg/ml in each sample of our assays.

On the 96 microplate we disposed 8 standard, 1 blank solution, 14 AIP, 13 PDAC and 12 CP all else in duplicate. Then, the experiment was run as described above with the sera.

3. IHC in pancreatic tissues:

Distribution and intensity of 9 cytokines were evaluated on immunohistological stained pancreatic tissues. These cytokines were endocan, TNF-α, IFN-γ, IL-6, IL-8, IL-10, IL-12, IL-17 and IL-23.

For this, pancreatic tissues fixed in formalin and embedded in paraffin (using ASP 300 Leica®) were collected from the storage of the European Pancreatic Center and cut into 4µm thick. They were deparaffinized into decreasing concentration of ethanol. To break the methylene bridges and expose the antigenic sites to allow the antibodies to bind, we used a heat-induced epitope retrieval method with citrate buffer that we heated in the microwave (at 60°C). Dilutions of the primary and secondary antibodies were determined by testing a range according to the recommended concentration in the datasheets and are listed in table 5. To estimate the contribution of the non-specific interaction and Fc receptor binding, control has been made in parallel for each slide (reagent used are listed in the table 5). Time of coloration was determined under the microscope for each cytokines at the chosen concentration. All tissues were stained by hand as soon as all antibodies parameters were known and tested. Some tissues were detached from the slide or were not stained well; for this reason they were not taken into consideration for the statistical analyze.

Hematoxylin and eosin (H&E) staining was performed for each tissue to confirm the diagnosis. Furthermore, each slide was stained with an anti-CD3 antibody. These slides were used as a reference to the distribution and localization of T cells in each tissue. Then, immunohistochemistry for each cytokine was performed using the peroxidase-anti-peroxidase (PAP) method well known in the laboratory.

All slides were conserved in the European Pancreatic Center of Heidelberg. Dr Gaida from the Pathology department of Heidelberg Hospital, a specialist in pancreatic tissues lecture, read the slides and evaluated the distribution and the intensity of each cytokines grading each of them from 0 to 3 subjectively. Mean grade of intensity and distribution for each cytokines in each disease are reported in tables 7 and 8 respectively.

First antibody		anti-CD3	anti-TNFa	anti-IFNg	anti-IL 6	anti-IL 8	anti-IL 10	anti-IL 12	anti-IL 17	anti-IL 23 P19	anti-Endocan
	Katalognr.	ab 16669	ab 1793	ab 9657	ab 9324	ab 18672	ab 34843	ab 9992	ab 79056	ab 110630	LIA-0901
	Company	abcam	abcam	abcam	abcam	abcam	abcam	abcam	abcam	abcam	LungInnov
	Optimal dilution	1:100	1:100	1:200	1:100	1:300	1:400	1:50	1:100	1:100	1:200
	Negatif control	Universal Neg.Ko. 1:2	Universal Neg.Ko. 1:2	Universal Neg.Ko. 1:2	Universal Neg.Ko. 1:2	Universal Neg. Control 1:2	Universal Neg. Control 1:2	Neg.Control Goat IgG	Universal Neg. Control 1:2	Universal Neg. Control 1:2	Universal Neg. Control 1:2
Second antibody		anti-rabbit	anti-mouse	anti-rabbit	anti-mouse	anti-mouse	anti-rabbit	anti-goat IgG	anti-rabbit	anti-mouse	anti-mouse
	Company	Dako	Dako	Dako	Dako	Dako	Dako	Dako	Dako	Dako	Dako
	Katalognr.	K 4003	K 4001	K 4003	K 4001	K 4001	K 4003	F 0250	K 4003	K 4001	K 4001

Table 5: Antibodies used for IHC staining

Cytokine	Total	AIP			CP	PDAC
			AIP I	AIP II		
endocan	4 (IQR=2; n=27)	4 (IQR=1.5; n=7)	4 (IQR=0; n=5)	2 (IQR=0.5; n=2)	3.75 (IQR=1.62; n=10)	5.5 (IQR=1.5; n=10)
IFNg	4 (IQR=1.5; n=25)	4.75 (IQR=2; n=8)	5.5 (IQR=3.5; n=5)	4 (IQR=1; n=3)	4.75 (IQR=3.5; n=8)	4 (IQR=1.5; n=9)
TNFa	2.5 (IQR=2.5; n=29)	1.5 (IQR=0.25; n=8)	1.5 (IQR=0; n=5)	1.5 (IQR=0.5; n=3)	2.5 (IQR=1.12; n=10)	2.5 (IQR=5; n=11)
IL-6	4 (IQR=1.5; n=29)	4 (IQR=1.5; n=8)	4 (IQR=1.5; n=5)	4 (IQR=0.75; n=3)	4 (IQR=1.5; n=10)	7.5 (IQR=2.75; n=11)
IL-8	4 (IQR=1.5; n=29)	4 (IQR=1; n=8)	4 (IQR=1.5; n=5)	4 (IQR=1.25; n=3)	4 (IQR=0; n=10)	2.5 (IQR=3.25; n=11)
IL-10	3.5 (IQR=2.5; n=25)	2.5 (IQR=2.5; n=7)	4 (IQR=1; n=4)	1.5 (IQR=0.5; n=3)	2 (IQR=1.38; n=8)	4.75 (IQR=2; n=10)
IL-12	2.5 (IQR=2.5; n=27)	1.5 (IQR=0.5; n=7)	1.5 (IQR=0; n=5)	3.25 (IQR=0.75; n=2)	4 (IQR=1.5; n=9)	3.5 (IQR=1.5; n=11)
IL-17	4 (IQR=1.5; n=29)	4 (IQR=1; n=8)	4 (IQR=0; n=5)	4 (IQR=2; n=3)	4.75 (IQR=1.5; n=10)	4 (IQR=2.25; n=11)
IL-23	2.5 (IQR=1; n=29)	1.5 (IQR=0.25; n=8)	1.5 (IQR=1; n=5)	1.5 (IQR=0; n=3)	2.5 (IQR=1; n=10)	2.5 (IQR=0; n=11)

Table 6: Mean grade of combined intensity and distribution (IQR: interquartile range, n: number of observation)

Cytokine	Total	AIP			CP	PDAC
			AIP I	AIP II		
endocan	2 (IQR=1; n=27)	2 (IQR=1; n=7)	2 (IQR=0; n=5)	1 (IQR=0; n=2)	1.5 (IQR=1; n=10)	2 (IQR=0; n=10)
IFNg	2 (IQR=1; n=25)	2.5 (IQR=1; n=8)	2 (IQR=1; n=5)	3 (IQR=0.5; n=3)	2.5 (IQR=1; n=8)	2 (IQR=0; n=9)
TNFa	1 (IQR=1; n=29)	1 (IQR=0; n=8)	1 (IQR=0; n=5)	1 (IQR=0; n=3)	1 (IQR=0.75; n=10)	1 (IQR=2; n=11)
IL-6	2 (IQR=1; n=29)	2 (IQR=0; n=8)	2 (IQR=0; n=5)	2 (IQR=0; n=3)	2 (IQR=1; n=10)	3 (IQR=1; n=11)
IL-8	2 (IQR=1; n=29)	2 (IQR=0.25; n=8)	2 (IQR=0; n=5)	2 (IQR=0.5; n=3)	2 (IQR=0; n=10)	2 (IQR=1; n=11)
IL-10	1 (IQR=1; n=25)	2 (IQR=1; n=7)	2 (IQR=0.5; n=4)	1 (IQR=0.5; n=3)	1 (IQR=0.25; n=8)	2 (IQR=1; n=10)
IL-12	1 (IQR=1; n=27)	1 (IQR=0; n=7)	1 (IQR=0; n=5)	1.5 (IQR=0.5; n=2)	2 (IQR=1; n=9)	2 (IQR=1; n=11)
IL-17	2 (IQR=0; n=29)	2 (IQR=0.25; n=8)	2 (IQR=0; n=5)	2 (IQR=0.5; n=3)	2 (IQR=0; n=10)	2 (IQR=0; n=11)
IL-23	1 (IQR=1; n=29)	1 (IQR=0.25; n=8)	1 (IQR=1; n=5)	1 (IQR=0; n=3)	1.5 (IQR=1; n=10)	2 (IQR=1; n=11)

Table 7: Mean grade of intensity (IQR: interquartile range, n: number of observation)

Cytokine	Total	AIP			CP	PDAC
			AIP I	AIP II		
endocan	2 (IQR=1; n=27)	2 (IQR=0; n=7)	2 (IQR=0; n=5)	1.5 (IQR=0.5; n=2)	2.5 (IQR=1; n=10)	3 (IQR=0; n=10)
IFNg	2 (IQR=1; n=25)	2 (IQR=1; n=8)	3 (IQR=1; n=5)	2 (IQR=0.5; n=3)	2 (IQR=1; n=8)	2 (IQR=1; n=9)
TNFa	2 (IQR=1; n=29)	1 (IQR=0.25; n=8)	1 (IQR=0; n=5)	1 (IQR=0.5; n=3)	2 (IQR=0; n=10)	2 (IQR=1.5; n=11)
IL-6	2 (IQR=1; n=29)	2 (IQR=1; n=8)	2 (IQR=1; n=5)	2 (IQR=0.5; n=3)	2 (IQR=0; n=10)	3 (IQR=0.5; n=11)
IL-8	2 (IQR=1; n=29)	2 (IQR=0.25; n=8)	2 (IQR=0; n=5)	2 (IQR=0.5; n=3)	2 (IQR=0; n=10)	2 (IQR=1; n=11)
IL-10	2 (IQR=2; n=25)	1 (IQR=1; n=7)	2 (IQR=0.25; n=4)	1 (IQR=0; n=3)	1.5 (IQR=1; n=8)	3 (IQR=0; n=10)
IL-12	2 (IQR=1; n=27)	1 (IQR=0.5; n=7)	1 (IQR=0; n=5)	2 (IQR=0; n=2)	2 (IQR=1; n=9)	2 (IQR=1; n=11)
IL-17	2 (IQR=1; n=29)	2 (IQR=0.5; n=8)	2 (IQR=0; n=5)	2 (IQR=1; n=3)	2 (IQR=1; n=10)	2 (IQR=1; n=11)
IL-23	1 (IQR=0; n=29)	1 (IQR=0; n=8)	1 (IQR=0; n=5)	1 (IQR=0; n=3)	1 (IQR=0; n=10)	1 (IQR=0.5; n=11)

Table 8: Mean grade of distribution (IQR: interquartile range, n: number of observation)

Statistic methods:

1. Bio-Plex® system in sera:

Results on the Bio-Plex Manager software were converted into an excel file. Two walls (X40, X19) could not be interpreted because of an error of manipulation (as agglutination) and aberrant concentration respectively. For this reason they have been deleted. For some patients several measurements are made over time and to increase the precision of results all measurements are kept and used in following analyses; thus some patients contribute to our analyses several times. Number of patients and observations are reported in table 2.

Cytokines are measured over an operational detection range and their mean concentrations are reported in table 12. Measurements are made using Bio-Plex kit as described above over an operational detection range. In other words if some observations are higher or lower than operational detection limits then the measured values are not reliable and should be considered as censored values.

Fitting a model to censored interdependent response variable

The complicated nature of response variable under study imposes some sort of difficulty for analyzing them. To fit an ordinary linear model to a response variable we need to have independent observations; the lack of independence is apparent in our current sample as we have several measurements made on the same individual. The other difficulty arises when we notice the fact that some observations are actually lower than the detection limit of measurement tool. Thus, the response variable is censored below a certain limit and can't be measured correctly.

We chose to use the so called "tlmec" function of R[i2] which can be used to fit linear mixed effect models on response variables which are censored below a certain level. The mixed effect linear model is a more general case of linear models where a random effect is also added to the model, mixed linear models are widely used when some interdependence is present in the sample structure. The inclusion of random effects in the patient level is necessary in our analyses as several patients contribute to fit models with more than one observation and so observations cannot be considered as independent.

By fitting a linear mixed model (with capability of handling censored response in our case) to a response variable versus a categorical factor, with P factors for example, the model has to choose a reference category and compare other categories of the factor versus the reference category by estimating P-1 parameters. The comparison among other categories however cannot be performed; to do so a separate set of comparisons should be performed.

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[2] Larissa Matos, Marcos Prates and Victor Lachos (2012). tlmec: Linear Student-t Mixed-Effects Models with Censored Data. R package version 0.0-2. http://CRAN.R-project.org/package=tlmec

After fitting the "tlmec" model several times (to be able to make necessary comparisons) we adjusted our results using the Bon-Ferroni correction for multiple comparisons in order to avoid inflation in the first error rate whenever the model is refitted. All comparisons on different cytokines were done twice, once comparisons were done comparing AIP versus PDAC and CP patients and then all models were re-fitted to compare among type 1 and type 2 AIP, PDAC and CP groups. In the first series the reference category is always AIP while in the second series AIP group is subdivided into two categories type 1 and 2 AIP, each one of these sub-categories is once considered as the reference category and all desired analyses were performed twice (once with type 1 AIP and once with type 2 AIP as the reference category); the results of the second series are thus adjusted for multiple comparisons using Bon-Ferroni correction.

2. Bio-Plex® system in pancreatic tissues:

The concentration of each cytokine in this experiment is measured twice for each individual sample and the mean observation is used as the only observation made on that sample; this leads to a more precise estimate of the cytokine concentration with less variation; from the statistical point of view this loss of variation might be equivalent to a loss of information but instead we will be able to use a less complicated statistical analysis method as each individual contributes once to the analysis at hand.

To avoid the out of practical range observations different dilutions have been tested, however, some observations are still lower than the practical observation range of machines we have used. These out of range values should not be considered as missing values and are contributing to our analysis as censored data. On the other hand, because of an important number of out of range values in IL-5 concentrations, they could not be interpreted and they were hence deleted.

Censored Regression model

The linear regression is perhaps the basis of modern statistics and plays an important role in statistical modeling of data. We however cannot use the ordinary linear regression model in comparing cytokine concentrations among different groups of our sample. The censored regression (12) is a generalization of ordinary linear regression in which some observations can be censored (from left or right).

Individuals have contributed only once to each model and thus there is no need to add the individual random effects for each patient.

Two series of models are fitted to compare the concentration of each cytokine among groups. The first series of models compare these concentrations among CP and PDAC versus AIP without distinguishing among type 1 and type 2 AIP patients. The AIP group is the reference category for making comparisons, consequently no multiple comparisons adjustment is necessary to be applied in this case as no comparison is made among CP and PDAC groups.

In the second series of analyses we have compared all groups versus type 1 AIP and then the same comparison is done comparing all groups versus type 2 AIP. Results for these analyses were adjusted for multiple comparisons as two models were fitted for each series of comparisons. Again no comparison is made between CP and PDAC groups.

3. IHC in pancreatic tissues:

We first analyzed each cytokine distribution and intensity using a Kruskall-Wallis test to compare AIP patients and reciprocally type 1 and type 2 AIP patients to CP and PDAC patients. Kruskall-Wallis is a non-parametric equivalent to the one-way ANOVA (Analysis of Variance) test which is used to assess the influence of a factor with more than two categories (group in our case) on a continuous variable, however the nonparametric nature of the Kruskal-Wallis test makes it robust compared to the ANOVA.

Furthermore, to study the observed intensity and distribution for each cytokine simultaneously one may suggest combining two variables into one overall variable. A simple choice would be to use the sum of two variables as a general summary but this strategy results in similar values for cases, which are considered to be far apart from each other. The other possibility would be to use the multiplication of intensity and distribution but this inflates the range of combined variable dramatically. Thus by mixing these ideas we decided to use the combined variable described below as the summary of both variables:

$$combined\ variable = \frac{\{(intensity + distribution) + (intensity \times distribution)\}}{2},$$

which combines two variables more adequately. Then, we analyzed this combined information using same statistical test. Whenever, more than one test is run to described the comparison between a group we corrected the p value using the Bonferroni-correction for multiple comparison test.

Results:

P-value lower than 0.05 are considered to be statistically meaningful (or significant). Results for each experiment are listed in table 9, 10 and 11. The cytokine name and the performed comparison are listed in the first two columns. The third column corresponds to the estimated coefficient of the model, which can be interpreted as the controlled average difference among compared categories. The fourth columns report original or corrected p-values whenever a multiple comparison correction has been necessary.

Regarding the results, relevant findings are represented using graphical tools. We have chosen to use the box-plots of observations inside the operational measurement range to show the centrality and variation of observed values simultaneously. These graphics are represented in figure 1 and 2.

Results should be interpreted with caution whenever too many observations are outside the operational measurement range and hence the number of observation (n) is too low.

Mean cytokine concentrations in the sera and pancreatic tissues are reported in table 12 and 13 (Annexes).

1. Bio-Plex® system in sera:

IL-8 and MIP-1b are highly expressed in the sera of patients suffering of type 2 AIP than type 1 AIP. On the other hand, cytokine as IL-6, G-CSF, MCP-1 are significantly more expressed in PDAC.

Cytokine	Description	Coefficient	P-value
IL-6	PDAC vs AIP	59.73	0.0001
	PDAC vs AIP1	64.79	0.0017
	PDAC vs AIP2	59.41	0.0126
IL-8	AIP2 vs AIP1	44.04	0.0535
	AIP1 vs AIP2	-44.04	0.0535
G-CSF	PDAC vs AIP	23.69	0.0001
	PDAC vs AIP1	24.41	0.0018
	PDAC vs AIP2	21.44	0.0210
MCP-1.MCAF	PDAC vs AIP	42.15	0.0006
	PDAC vs AIP1	48.41	0.0023
	PDAC vs AIP2	37.65	0.0476
MIP.1b	AIP2 vs AIP1	100.16	0.0179
	AIP1 vs AIP2	-100.16	0.0179
	PDAC vs AIP2	-94.84	0.0191
	CP vs AIP2	-90.42	0.0244

Table 5: Relevant findings in the first experiment

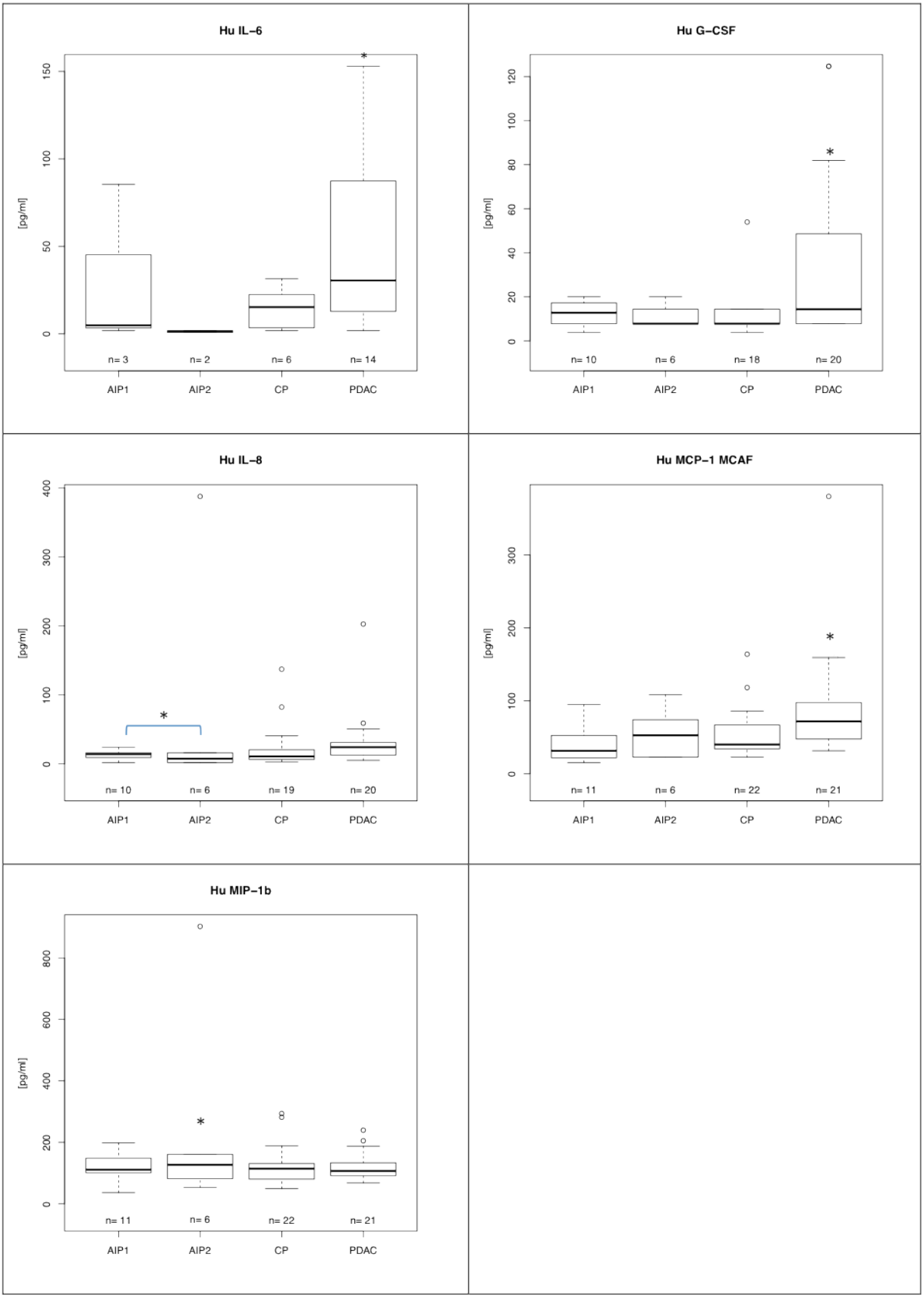

Figure 2: Comparison of the concentrations of different cytokines in the sera, n = number of observations per group in the operational measurement range

2. Bio-Plex® system in pancreatic tissues:

IL-8 and MCP-1 are highly expressed in the pancreatic tissues of type 2 compared to type 1 AIP. IL-1b is more expressed in AIP than CP and PDAC while IL-13 is less expressed in AIP compared to CP and PDAC. Interestingly, concentration of IL-17 is higher in type 2 AIP than CP while IL-10 is more expressed in CP than type 1 AIP.

Cytokine	Description	Coefficient	P-value
IL-1 b	CP vs AIP	-2.66	0.0206
	PDAC vs AIP	-1.85	0.1074
	CP vs AIP1	-3.59	0.0701
IL-8	AIP1 vs AIP2	-320.78	0.0294
	CP vs AIP2	-268.28	0.0203
IL-10	CP vs AIP	0.34	0.0795
	CP vs AIP1	0.60	0.0190
IL-13	CP vs AIP	4.18	0.0048
	PDAC vs AIP	3.10	0.0368
	CP vs AIP1	5.33	0.0243
	PDAC vs AIP1	4.24	0.0900
	CP vs AIP2	4.62	0.0398
IL-17	CP vs AIP	-12.25	0.0161
	CP vs AIP2	-16.30	0.0437
MCP-1	CP vs AIP	-222.96	0.0291
	AIP2 vs AIP1	389.65	0.0368

Table 6: Relevant results in the second experiment

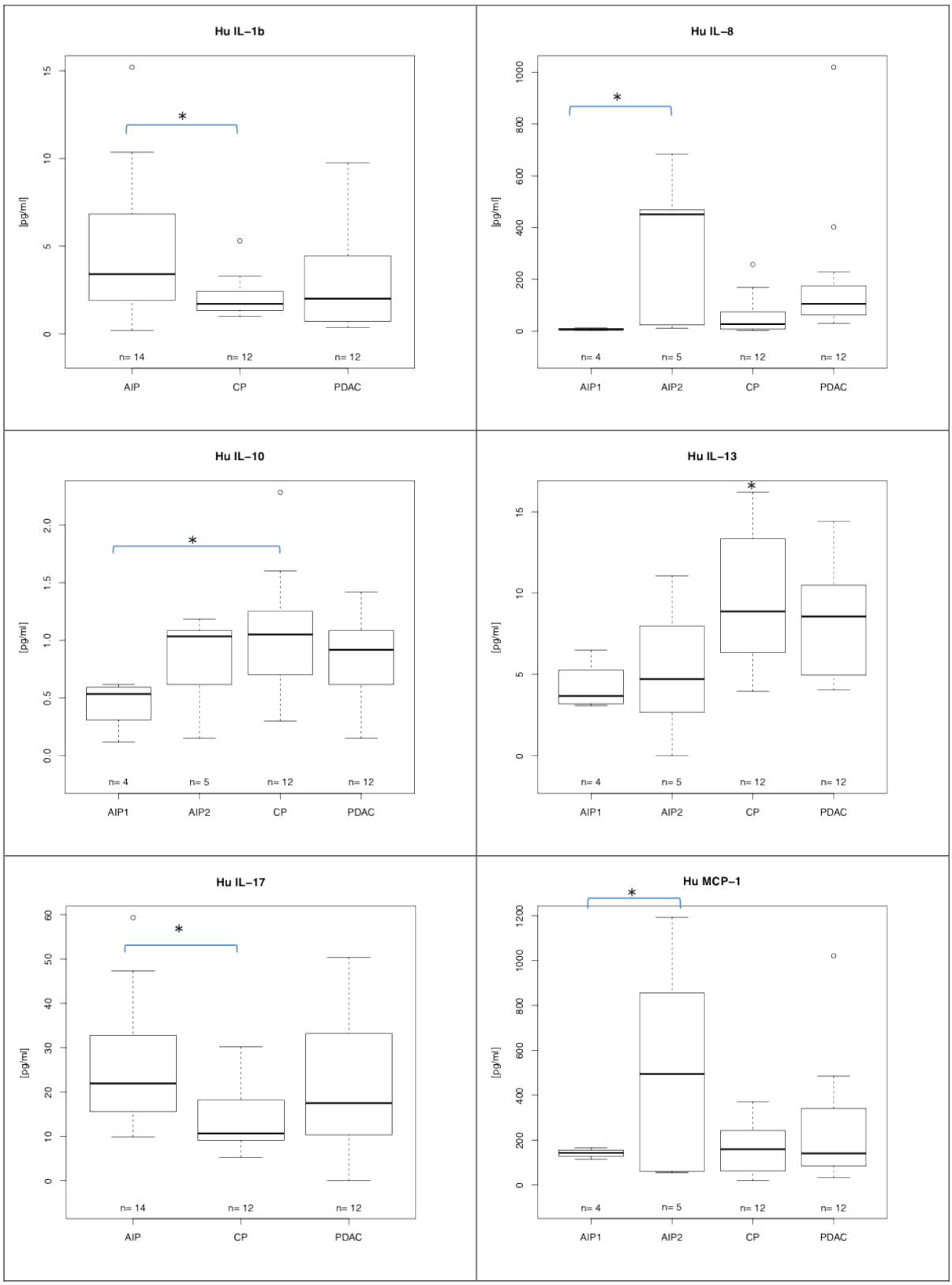

Figure 3 : Comparison of the concentrations of different cytokines in the pancreatic tissues, n = number of observations per group in the operational measurement range

3. IHC in pancreatic tissues:

	Description	P-value
Endocan distribution	AIP vs PDAC	0.0030
	AIP1 vs PDAC	0.0232
	AIP2 vs PDAC	0.0967
Endocan combined distribution and intensity	AIP vs PDAC	0.0409
TNFa distribution	AIP vs CP	0.0126
	AIP1 vs CP	0.0534
TNFa intensity	AIP vs PDAC	0.0603
TNFa combined distribution and intensity	AIP vs PDAC	0.0932
	AIP vs CP	0.0106
	AIP1 vs CP	0.0596
IL-6 distribution	AIP vs CP	0.0473
IL-6 intensity	AIP vs PDAC	0.0685
IL-6 combined distribution and intensity	AIP vs PDAC	0.0843
IL-10 distribution	AIP vs PDAC	0.0007
	AIP1 vs PDAC	0.0098
	AIP2 vs PDAC	0.0089
IL-10 combined distribution and intensity	AIP vs PDAC	0.1025
	AIP2 vs PDAC	0.0467

Table 7: List of cytokine-related variables where a significant (or important) difference is observed among groups

Mainly, endocan and IL-10 were more distributed in the pancreatic tissues of PDAC patients than AIP patients (Fig.3), while distribution of IL-6 and TNF-α, pro-inflammatory cytokines were more important in CP than AIP (Fig.4).

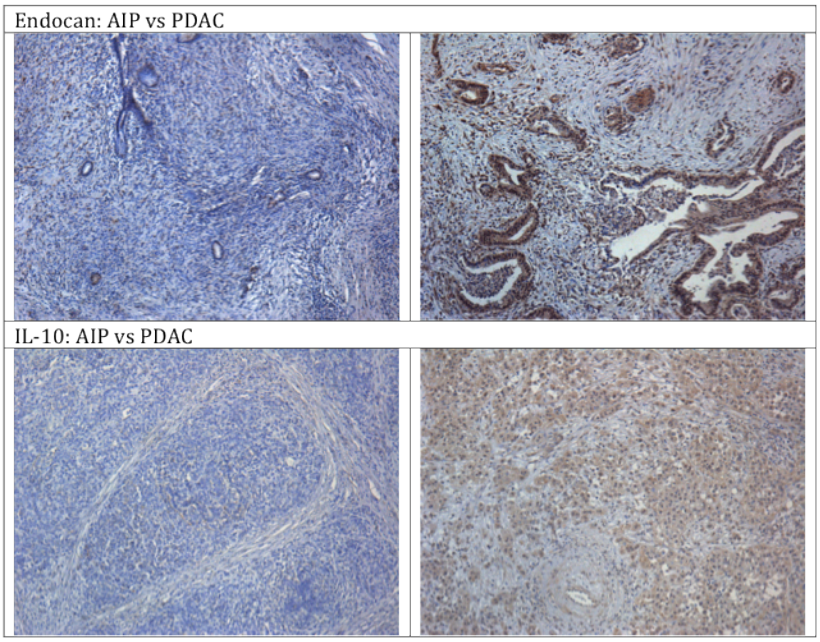

Figure 4 : P-IHC staining of endocan and IL-10 in AIP (left side) and PDAC (right side) patients (low magnitude)

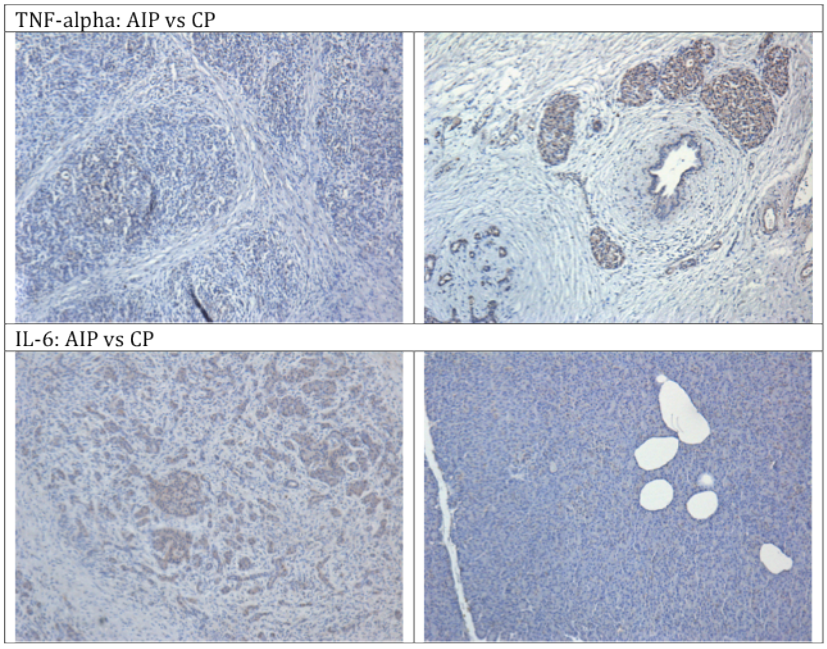

Figure 5: P-IHC staining of TNF alpha and IL-6 in AIP (left side) and CP (right side) patients (low magnitude)

Discussion:

Cytokine expressions in the sera and pancreatic tissues of both types of AIP have been evaluated. It results that three cytokines are significantly increased in type 2 AIP compared to type 1 AIP. These cytokines are interleukin-8 (IL-8), macrophage inflammatory protein-1 beta (MIP-1b) and monocyte chemotactic protein-1 (MCP-1).

In our experiment, MIP 1 beta is more expressed in the sera of patients suffering of type 2 AIP compared to type 1 AIP, PDAC or CP. Macrophage Inflammatory Protein is a chemotactic cytokine that induce directed chemotaxis in nearby stimulated cells; in other word it is a CC-chemokine. Its function is to activate human granulocytes (neutrophils, eosinophils and basophils), which can lead to acute neutrophilic inflammation. MIP-1a together with MIP-1b induce synthesis and release of other pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and TNF-α from fibroblasts and macrophages. These chemokines induce chemotaxis and adhesion of circulating leukocytes for their extravasation that occurs with destruction of the pancreatic epithelial ducts. However, since no correlation has been proved, it is likely that this chemokine is only one among several factors responsible for the recruitment of macrophages in acini lumen. However, this recruitment depends not only on the gradient of chemokines, but also on several successive steps, including adherence, rolling and extravasion, regulated by proinflammatory cytokines. Schrum et al. observed that MIP-1b are secreted by T lymphocytes particularly Th1 phenotype (13). Moreover, Bystry et al. (14)showed that activation of B cells and antigen presenting cells (APCs), induce the expression of MIP-1b that recruit regulatory T cells (CD4+ CD25+Tcell population) that are also reported to be increased in AIP. Indeed, they showed that, depletion of either regulatory Tcells or MIP-1b result in the disregulation of humoral response that leads to the production of autoantibodies and then autoimmune activation. Bustry et al. showed that activated B cells express factors that strongly attracted regulatory T cells (that express both TGF-β and CTLA-4 and unable to respond to anitCD3 stimulation) and that MIP-1b one of the chemokines that are strongly induced upon stimulation was sufficient to confer this effect. Furthermore, they found that CCR5 that is though to be the receptor for MIP-1b was expressed on these CD4+CD25+T cells. Given the role of regulatory T cells in suppressif cytotoxic T cell autoreactivity, the recruitement of regulatory T cells by APCs via these chemokines could be a general mechanism for regulating immune responses and contra-regulating autoimmunity.

Regarding the function of MIP 1beta, these results support an involvement of the CC-chemokines in the inflammatory reaction or maintenance of inflammation in type 2 AIP. We hypothesize that those T lymphocytes in type 2 AIP might be of the Th1 subset and that neutrophilic epithelial lesions described as GELs in this subtype of AIP may be induced by MIP-1b.

Monocyte chemotactic protein-1 (MCP-1) is another CC-chemokine secreted by monocytes, macrophages and dendritic cells and its function is to recruit monocyte, memory T cells and natural killer cells to the site of inflammation in time of infection or tissue injuries(15). MCP-1 has long been described as having a chemotactic activity for monocytes and basophils but not for neutrophils and eosinophils. Furthermore, it is a strong recruiter of lymphocytes from the bloodstream into inflammatory lesions. Carr et al. (16)showed that this chemoattractant function is dose-dependant and due to chemotaxis rather than chemokinesis. This chemokine was also reported to be implicated in several disease characterized by monocytic infiltrates, such as multiple sclerosis(17), psoriasis, rheumatoid arthritis(18), artherosclerosis (19) and insulin-resistant diabetes(20). Not only MCP-1 act on recruiting and directing leukocytes movement but it also influence T-cell immunity. Indeed, it is though that MCP-1 may play a role in the polarization of Th0 cells toward a Th2 phenotype via activation of the IL-4 promoter(21,22). In our study, MCP-1 was significantly more expressed in pancreatic tissues of type 2 AIP patients. On the other hand, its concentrations was not more elevated in the sera of type 2 AIP patient or other AIP groups (type 1 or total

AIP patients) while it was increased in PDAC. It is interesting to see that because MCP-1 function is to recruit T cells, one could imagine that it would be increased in pancreatic tissues of both AIP subtypes since they are characterized by a lymphoplasmocytic infiltration in the pancreatic parenchyma. To our knowledge the lymphocytes infiltrates have not been described in a higher amount in type 2 AIP than type 1. Hence, increased MCP-1 concentration in the pancreas of type 2 AIP can not be explained by a higher T cell infiltrations. Moreover, because MCP-1 is associated with a Th2 polarization, it would mean that Th2 cells over Th1 is required in the pathogenesis of type 2 AIP.

IL-8, also known as neutrophil chemotactic factor, is a neutrophil-activating cytokine released via chemotaxis and exocytosis of granule enzyme by phagocytes and different tissue cells such as epithelial cells or neutrophils itself when they are exposed to inflammatory environments(23). IL-1 and TNF-α are the most important stimuli that induce monocytes and macrophages to generate IL-8(24). It is though that IL-8 plays more a role of chemotactic agonist rather than a cytokine. It is the main tissue-derived chemoattractants for neutrophils but also for other granulocytes. Moreover, IL-8 dependant surface membrane remodeling during exocytosis leads to the expression of adhesion molecules such as CD11b/CD18, CD11c/CD18 and complement receptor type 1(25,26) that upregulate neutrophils to adhere to endothelial cells and to the extracellular matrix. Regarding IL-8 function it is not surprising that its concentration is increased in both sera and pancreatic tissues of patients suffering of type 2 AIP since the principal characteristic of type 2 AIP is the presence of important neutrophils in the pancreatic parenchyma.

Interestingly, we failed to find increase in IL-13 and IL-10 expressions in the pancreatic tissues of patients suffering of AIP as Zen et al. reported(6). Indeed, they speculated that IL-10 may a have a potential role in directing B cells to produce IgG4 and then be responsible of the elevation of serum IgG4 concentrations and IgG4-positive plasma cell infiltration. Because, AIP patient has also increase serum IgE level and sometimes eosinophilic infiltration (more in type 2 AIP for this latter) it was though that increased IL-13 might have also a role in the pathogenesis of AIP.

Conclusion:

Autoimmune pancreatitis is a newly described form of pancreatitis. Two different subtypes with distinct clinical presentation have been reported including differences in geographic distribution, age of appearance, sex ratio, histological and immunological features and rate of relapse after steroid treatment. As both subtypes have been recently individualized few studies compared both types in term of pathogenic mechanism. Furthermore, diagnosis of AIP remains still a challenge and discrimination between both subtypes is only possible on histological specimen. For these reasons, we proposed here to assess cytokine expression in both type 1 and type 2 AIP. These two latter groups were compared to PDAC and CP, both difficult differential diagnosis of AIP.

Mainly, we showed that interleukin-8 (IL-8), macrophage inflammatory protein-1 beta (MIP-1b) and monocyte chemotactic protein-1 (MCP-1) are significantly more expressed in type 2 AIP than type 1 AIP.

MIP-1b and MCP-1 are produced by macrophages and have a crucial role for immune responses towards infection and inflammation. IL-8, MCP-1 and MIP-1b are pro-inflammatory cytokines that induce

infiltration and activation of leukocytes-mainly neutrophils into the sites of inflammation of pancreas. This results into a continuous activation and amplification of the cytokine cascade that might be the origin of the specific histological characteristic of type 2 AIP that is the invasion and destruction of the pancreatic duct epithelium by granulocytes (GELs) mainly neutrophils.

These three cytokines may help to distinguish both types of AIP. However, further studies are required to explain the role of activated macrophages in type 2 AIP and the origin of this activation.

Annexes:

Cytokine mean(SE; n)	Total	AIP	AIP unknown	AIP1	AIP2	CP	PDAC
Hu.IL.2	11.14(SE=8.36;n=4)	17.96(SE=17.74; n=2)	35.7(SE=--;n=1)	0.22(SE=--;n=1)	--(SE=--;n=0)	4.31(SE=3.36;n=2)	--(SE=--;n=0)
Hu.IL.4	3.41(SE=1.87;n=5)	3.97(SE=2.31; n=4)	5.99(SE=4.81;n=2)	2.72(SE=--;n=1)	1.18(SE=--;n=1)	1.18(SE=--;n=1)	--(SE=--;n=0)
Hu.IL.5	35.49(SE=9.38;n=3)	17.4(SE=--;n=1)	17.4(SE=--;n=1)	--(SE=--;n=0)	--(SE=--;n=0)	44.53(SE=4.32;n=2)	--(SE=--;n=0)
Hu.IL.6	34.59(SE=7.55;n=28)	23.68(SE=11.41; n=8)	30.73(SE=27.39; n=3)	1.11(SE=0.35;n=3)	46.97(SE=3.39;n=2)	14.97(SE=4.79;n=6)	49.23(SE=12.58; n=14)
Hu.IL.7	18.05(SE=3.17;n=25)	12.33(SE=1.6; n=12)	12.61(SE=2.56; n=7)	10.31(SE=4.26;n=2)	13.04(SE=1.68;n=3)	27.89(SE=7.75;n=9)	13.07(SE=1.19;n=4)
Hu.IL.8	26.19(SE=6.33;n=70)	24.11(SE=12.19; n=31)	11.61(SE=1.61; n=15)	52.37(SE=41.98;n=9)	14.56(SE=3.55;n=7)	23.48(SE=7.68;n=19)	31.98(SE=9.53; n=20)
Hu.IL.10	21.06(SE=6.87;n=10)	61.59(SE=--;n=1)	61.59(SE=--;n=1)	--(SE=--;n=0)	--(SE=--;n=0)	13.38(SE=--;n=1)	16.95(SE=6.57;n=8)
Hu.IL.12.p70.	46.06(SE=35.58;n=3)	67.2(SE=49.58; n=2)	67.2(SE=49.58; n=2)	--(SE=--;n=0)	--(SE=--;n=0)	3.78(SE=--;n=1)	--(SE=--;n=0)
Hu.IL.13	29.29(SE=13.88;n=5)	84.49(SE=--;n=1)	84.49(SE=--;n=1)	--(SE=--;n=0)	--(SE=--;n=0)	15.49(SE=1.92;n=4)	--(SE=--;n=0)
Hu.IL.17	10.74(SE=3.64;n=6)	8.56(SE=5.33; n=4)	5.52(SE=--;n=1)	9.57(SE=7.4;n=3)	--(SE=--;n=0)	15.11(SE=0;n=2)	--(SE=--;n=0)
Hu.G.CSF	18.42(SE=2.88;n=68)	11.51(SE=0.8; n=30)	11.83(SE=1.34; n=14)	11.34(SE=1.51;n=9)	11.09(SE=1.24;n=7)	11.56(SE=2.61;n=18)	34.95(SE=8.49; n=20)
Hu.GM.CSF	31.81(SE=15.37;n=3)	42.57(SE=--;n=1)	42.57(SE=--;n=1)	--(SE=--;n=0)	--(SE=--;n=0)	--(SE=--;n=0)	26.44(SE=24.95; n=2)
Hu.IFN.g	368.36(SE=314.53; n=4)	449.28(SE= 429.84;n=3)	664.2(SE=644.75; n=2)	19.44(SE=--;n=1)	--(SE=--;n=0)	125.6(SE=--;n=1)	--(SE=--;n=0)
Hu.MCP.1. MCAF.	62.31(SE=5.75;n=76)	49.3(SE=4.91; n=33)	43.04(SE=5.92; n=16)	53.8(SE=11.07;n=9)	56.75(SE=11.13; n=8)	54.02(SE=7.22;n=22)	91.45(SE=16.49; n=21)
Hu.MIP.1b	132.93(SE=12;n=76)	147.59(SE=25.41; n=33)	114.1(SE=11.1; n=16)	214.26(SE=87.07; n=9)	139.57(SE=29.21; n=8)	123.85(SE=13.66;n=22)	119.42(SE=9.86; n=21)
Hu.TNF.a	306.78(SE=--;n=1)	306.78(SE=--; n=1)	306.78(SE=--;n=1)	--(SE=--;n=0)	--(SE=--;n=0)	--(SE=--;n=0)	--(SE=--;n=0)
IL.1b	14.31(SE=2.41;n=2)	14.31(SE=2.41; n=2)	16.72(SE=--;n=1)	11.9(SE=--;n=1)	--(SE=--;n=0)	--(SE=--;n=0)	--(SE=--;n=0)

Table 8: Mean cytokine concentrations in sera (pg/ml), SE: standard error, n: number of observation in the operational measurement range

	Total	AIP				CP	PDAC
Cytokine mean(SE;n)			AIP I	AIP II	AIP unknown		
Hu.IL.2	2.57 (SE=0.41; n=22)	3.32 (SE=0.93;n=8)	2.54 (SE=0.19;n=2)	4.53 (SE=2.04;n=3)	2.62 (SE=1.63; n=3)	2.12 (SE=0.44;n=8)	2.16 (SE=0.58;n=6)
Hu.IL.4	0.57 (SE=0.06; n=35)	0.65 (SE=0.11;n=11)	0.42 (SE=0.17;n=4)	1.07 (SE=0.2;n=3)	0.58 (SE=0.04; n=4)	0.52 (SE=0.1;n=12)	0.54 (SE=0.11;n=12)
Hu.IL.6	44.49 (SE=10.52; n=37)	35.63 (SE=10.87;n=13)	15.71 (SE=4.42;n=4)	31.27 (SE=25.11;n=4)	55.04 (SE=18.81;n=5)	25.95 (SE=8.53;n=12)	72.62 (SE=28.17; n=12)
Hu.IL.7	4.52 (SE=0.46; n=37)	3.95 (SE=0.79;n=13)	2.84 (SE=0.56;n=4)	2.95 (SE=0.43;n=4)	5.64 (SE=1.86; n=5)	4.68 (SE=0.69;n=12)	4.98 (SE=0.92;n=12)
Hu.IL.8	132.43 (SE=34.69;n=38)	138.6 (SE=60.19;n=14)	7.01 (SE=1.83;n=4)	327.79 (SE=133.07;n=5)	54.68 (SE=32.92;n=5)	59.51 (SE=22.62; n=12)	198.17 (SE=80.2; n=12)
Hu.IL.10	0.89 (SE=0.08; n=37)	0.78 (SE=0.15;n=13)	0.45 (SE=0.11;n=4)	0.81 (SE=0.19;n=5)	1.08 (SE=0.4;n=4)	1.05 (SE=0.15;n=12)	0.86 (SE=0.1;n=12)
Hu.IL.12.p70.	11.92 (SE=0.92; n=38)	12.84 (SE=1.68;n=14)	14.69 (SE=3.1;n=4)	12.42 (SE=3.93;n=5)	11.78 (SE=1.86; n=5)	10.58 (SE=1.04;n=12)	12.19 (SE=1.94;n=12)
Hu.IL.13	7.92 (SE=0.65; n=37)	5.91 (SE=0.95;n=13)	4.22 (SE=0.78;n=4)	6.6 (SE=1.85;n=4)	6.71 (SE=1.93; n=5)	9.55 (SE=1.22;n=12)	8.46 (SE=1.05;n=12)
Hu.IL.17	21.08 (SE=2.23; n=37)	25.91 (SE=3.97;n=14)	26.98 (SE=6.88;n=4)	29.96 (SE=9.54;n=5)	21.02 (SE=3.67; n=5)	13.66 (SE=2.29;n=12)	23.03 (SE=4.31;n=11)
Hu.G.CSF	7.82 (SE=1.71; n=30)	5.88 (SE=1.62;n=9)	3 (SE=0;n=2)	3.87 (SE=1.37;n=4)	10.49 (SE=3.39; n=3)	10.7 (SE=3.82;n=10)	6.78 (SE=2.89;n=11)
Hu.GM.CSF	98.08 (SE=3.85; n=38)	94.92 (SE=8.79;n=14)	86.74 (SE=19.8;n=4)	98.52 (SE=11.45;n=5)	97.85 (SE=17.85;n=5)	104.68 (SE=4.54;n=12)	95.17 (SE=5.03;n=12)
Hu.IFN.g	26.39 (SE=2.91; n=31)	25.17 (SE=6.68;n=9)	9.3 (SE=2.28;n=3)	43.99 (SE=12.01;n=3)	22.23 (SE=8.83; n=3)	25.44 (SE=3.13;n=12)	28.64 (SE=6.05;n=10)
Hu.MCP.1. MCAF.	274.35 (SE=45.38; n=38)	388.4 (SE=92.23; n=14)	141.4 (SE=10.36; n=4)	531.05 (SE=222.73; n=5)	443.36 (SE=98.46; n=5)	165.44 (SE=33.43; n=12)	250.18 (SE=81.13; n=12)
Hu.MIP.1b	245.03 (SE=55.41; n=38)	296.42 (SE=85.9; n=14)	284.73 (SE=68.9; n=4)	422.66 (SE=230.53; n=5)	179.53 (SE=61.07; n=5)	110.77 (SE=22.54; n=12)	319.35 (SE=140; n=12)
Hu.TNF.a	11.76 (SE=1.8;n=38)	5.84 (SE=0.58;n=14)	6.39 (SE=1.65;n=4)	5.49 (SE=0.92;n=5)	5.74 (SE=0.67;n=5)	12.88 (SE=2.06;n=12)	17.54 (SE=4.79;n=12)
IL.1b	3.34 (SE=0.52;n=38)	4.76 (SE=1.1;n=14)	5.69 (SE=3.17;n=4)	3.62 (SE=1.8;n=5)	5.17 (SE=1.12;n=5)	2.1 (SE=0.35;n=12)	2.91 (SE=0.81;n=12)

Table 9: Mean cytokine concentrations in pancreatic tissues (pg/ml), SE: standard error, n: number of observation in the operational measurement range

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