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Circulating immune signatures across clinical stages of chronic pancreatitis: A pilot study

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Abstract

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Objective: This pilot study seeks to identify serum immune signatures across clinical stages of patients with chronic pancreatitis (CP).

Methods: We performed a cross-sectional analysis of prospectively collected serum samples from the PROspective Evaluation of Chronic Pancreatitis for EpidEmiologic and Translation StuDies (PROCEED)-study. CP subjects were categorised into three clinical stages based on the presence/absence of metabolic complications: 1) CP with no diabetes and exocrine pancreatic dysfunction (EPD), 2) CP with either diabetes or EPD, and 3) CP with diabetes and EPD. Blinded samples were analysed using an 80-plex Luminex assay of cytokines/chemokines/adhesion molecules. Group and pairwise comparisons were performed to characterise immune signatures across CP subgroups.

Results: A total of 135 CP subjects (evenly distributed between clinical stages) and 50 controls were studied. Interleukin 6 (IL-6), interleukin 8 (IL-8), and soluble intercellular adhesion molecule 1 (sICAM-1) were significantly elevated in CP subjects compared to controls. The levels of IL-6 and IL-8 increased with advancing disease stages, with the highest levels observed in CP with diabetes and EPD (clinical stage 3). Furthermore, hepatocyte growth factor (HGF) and macrophage-derived chemokine (MDC) were significantly increased in clinical stage 3 compared to controls.

Conclusion: Our study reveals a progressive elevation in pro-inflammatory cytokines and chemokines with advancing clinical stages of CP. These findings indicate potential targets for the development of disease-modifying interventions.

Keywords

Chronic Pancreatitis; Immune Signatures; Metabolic Complications

Introduction

Chronic pancreatitis (CP) is a fibro-inflammatory disease with irreversible replacement of the pancreatic tissue with fibrosis [1]. Complications of CP develop as the disease progresses, including exocrine pancreatic dysfunction (EPD) and diabetes [2–4]. Recent research has increasingly recognised the pivotal role of immunological mechanisms in the progression of CP, as evident in pre-clinical animal and pilot studies [3,5,6]. Crosstalk between immune and non-immune cells is a key driver of this process [3]. Notably, the dynamic interplay between macrophages and pancreatic stellate cells (PSCs) through immune signalling has emerged as a focal point of investigation, given that PSCs are central promoters of fibrogenesis in the pancreas [3,5,7,8]. This activation of quiescent PSCs by cytokines and external mediators not only fuels fibrogenesis but also influences the polarisation of macrophages, leading to a complex cross-talk between these two cell types [5,8].

The varying levels of immune mediators in the systemic circulation throughout the disease course may be closely associated with macrophage polarisation and PSC activation [5]. Our prior work has examined circulating immune signatures in human CP and other pancreatic diseases, including pancreatic cancer and recurrent acute pancreatitis, comparing them to healthy controls [3,9]. However, to date, no study has explored immune signatures

across different clinical stages of CP. Establishing associations between immune signature levels and distinct CP stages holds the potential to shed further light on the underlying pathophysiology of CP progression. This understanding could serve as a valuable tool for identifying targets for novel treatments capable of altering the disease course [10].

We hypothesised that CP patients have different circulating immune signatures across different clinical stages of CP that can be detected with in-depth immune profiling. This study aimed to 1) investigate immune signatures in CP patients versus healthy controls and 2) investigate immune signatures across clinical stages of CP.

Methods

Study design and subjects

We conducted an IRB-approved cross-sectional analysis investigating circulating immune signatures across clinical stages of CP (defined by the presence of metabolic complications) and controls. Human samples were obtained from the biorepository of the Prospective Evaluation of Chronic Pancreatitis for Epidemiologic and Translational Studies (PROCEED), which is a prospective cohort study conducted by the Consortium for the Study of Chronic Pancreatitis, Diabetes, and Pancreatic Cancer (CPDPC) [11,12]. Cases with CP were selected for the current study amongst those with a definitive diagnosis of CP defined as conclusive changes in cross-sectional imaging or histology according to the PROCEED criteria [11].

A total of 135 patients were selected for the current analysis. Being an exploratory study to evaluate immune markers of different clinical stages of CP, we aimed to have a roughly equal distribution of subjects among our study groups; hence, all participants with available samples in the planned study groups were randomly selected until reaching a maximum of approximately 50 samples. The clinical staging of CP patients was based on the presence of metabolic complications (i.e., EPD and diabetes) and comprised three distinct subgroups: stage 1: CP patients with normal pancreatic function (n=40), stage 2: CP patients with partly impaired pancreatic function (diabetes or EPD) (n=46), and stage 3: CP patients with fully impaired pancreatic function (diabetes and EPD) (n=49).

Controls with no pancreatic disease (n=50) were included for comparison. This group comprised volunteers who had no upper abdominal pain symptoms, no personal or family history of pancreatic disease, and no history of cancer, inflammatory diseases requiring medical treatment, and organ transplant or human immunodeficiency virus / acquired immunodeficiency syndrome.

Demographics, anthropometrics, and clinical parameters

Information on clinical and demographic variables, including sex, age, body mass index (BMI), race, CP aetiology, age at CP diagnosis and duration of CP, as well as history of acute pancreatitis (AP) and recurrent acute pancreatitis (RAP), was collected from the PROCEED study database. BMI, at the date on which the blood sample was drawn, was stratified into the following subgroups according to the World Health Organization

classification: underweight BMI (< 18.5 kg/m^2), normal BMI (18.5 – 24.9 kg/m^2), overweight BMI (25.0 – 29.9 kg/m^2), and obese BMI (>30 kg/m^2) [13].

EPD and diabetes criteria were defined according to the PROCEED study protocol [11]. EPD diagnosis was defined as the presence of one of the following criteria: 1) clinical history of steatorrhea; 2) faecal elastase of < 100 μg/g stool, or 3) quantitative faecal fat of > 7 g/d on a 100-g fat diet. Diabetes diagnosis was defined according to the criteria by the American Diabetes Association: abnormal values on 2 of the following tests or 2 abnormal values of the same test: 1) fasting blood sugar ≥ 126mg/dL; 2) HbA1c ≥6.5 %; 3) random blood glucose ≥ 200 mg/dL, or 4) use of anti-diabetic medications.

Luminex assay

The Stanford University Human Immune Monitoring Center performed the Luminex assay blinded to group assignments. Luminex -EMD Millipore Human 80 Plex kits were purchased from EMD Millipore Corporation, Burlington, MA. The human 80-plex Luminex kits includes 3 panels: Panel 1 includes the Milliplex HCYTA-60K-PX48, Panel 2 the Milliplex HCP2MAG-62K-PX23, and Panel 3 the Milliplex HSP1MAG-63K-06 and HADCYMAG-61K-03. A total of 150 μL of serum from each subject was processed. The detailed protocol description can be found at (https://iti.stanford.edu/himc/immunoassays.html). In brief, samples were diluted 3-fold (Panels 1 and 2) and 10-fold for Panel 3. Subsequently, 25 μL of the samples were mixed with antibody-linked polystyrene beads on 96-well filter-bottom plates and incubated at room temperature for 2 hours, followed by overnight incubation at 4 °C. Plates were pre-warmed for 20-30 minutes at room temperature and washed thrice. Detection antibodies were added to each well, followed by room temperature incubation on an orbital shaker for 2 hours at 500 rpm. The solution was removed by filtration and washed thrice. A biotinylated detection antibody, Streptavidin-PE, was added to the plates, incubated at room temperature for 40 minutes on an orbital shaker at 500 rpm, and washed thrice. Finally, a reading buffer was added, incubated at room temperature for 5 minutes on an orbital shaker at 500 rpm, and read in the Luminex FlexMap3D Instrument. As an estimate of analyte concentration, the median fluorescence intensity (MFI) average was used to compare the intensity in each clinical group instead of the absolute concentration values, as recommended by leading researchers in this field [14,15]. The Luminex assay included 80 cytokines/chemokines/adhesion molecules, as shown in Supplementary Table 1.

Statistical analysis

The purpose of the primary analysis of this study was to identify differences in circulating immune signature (proteins) levels between clinical stages of CP. As this is an explorative pilot study and no previous studies to our knowledge have investigated this, the sample size was not determined by statistical power calculation. The number of subjects chosen per group was based on sample availability, considerations of multiple testing with the Luminex assay, and general guidance for sample size construction for pilot studies in consultation with our biostatisticians at the CPDPC Coordination and Data Management Center [3,11].

Descriptive and inferential statistics were performed to summarise and differentiate the demographic and clinical characteristics of each CP subgroup, the total CP group, and controls. Data are reported as medians with interquartile range (IQR) or frequencies (%) unless otherwise indicated. Fisher's exact test, ANOVA, Kruskal-Wallis test, Student's t-test, and Wilcoxon rank sum test were used as appropriate. Ordinary least squares regression models were applied to investigate differences in MFI [16,17]. All group comparisons were adjusted for covariates of age, race, BMI, nonspecific binding, and plate effects. All three CP groups were equally weighted in comparison to the control group. Standard errors for ordinary least squares were adjusted for any heterogeneity in variance [18]. P-values were adjusted for multiple comparisons to control the false discovery rate at 5% [19,20]. The software packages R Studio version 1.3.1093 (RStudio PBC, Boston, Massachusetts, USA) and SAS version 9.4 (SAS Institute, Cary, North Carolina, USA) were used for statistical analyses.

Raincloud plots and a star plot were used to illustrate findings. Detrended logarithm MFI was used to illustrate Interleukin-6 (IL-6) and interleukin-8 (IL-8) due to skewness of data at high MFI levels. Star plots were constructed in R (www.r-project.org) with packages maptools [21], matrixcalc [22], plotrix [23], JPEN [24], and VCA [25]. Star plots are 2D visual classifiers of clustering results, and coordinates are two-dimensional embedding axes. Star plot locates vectors such that the separation is greatest among groups in 2D while trying to keep vector lengths ordered by biomarkers' importance values. Hence, the importance value is the ratio of the among-group to within-group sample variance [26].

Results

We selected 135 CP subjects and 50 controls for the final study population. Demographic and clinical characteristics are summarised in Table 1. Among CP subjects, 40 (30%) were classified as stage 1 CP, 46 (34%) as stage 2 CP, and 49 (36%) as stage 3 CP. CP subjects were older than controls (median age 55 vs. 39 years, $p<0.001$), but the age distributions across CP subgroups were not detectably different ($p=0.21$). Race varied among the groups ($p=0.02$) as the controls had a higher population of black people, and the stage 1 CP group had a higher population of Caucasians. Expectedly, CP duration increased with advancing clinical stage ($p=0.04$). A higher prevalence of RAP history was observed for stage 2 CP ($p=0.02$).

Immune signature expression in CP vs control group

Three of 80 candidate analytes' MFI was differentially expressed in CP subjects (stages 1-3 combined) vs. controls (Figure 1 and Table 2). Accordingly, the MFIs of IL-6 ($p<0.001$), IL-8 ($p<0.001$), and soluble intercellular adhesion molecule 1 (sICAM-1) ($p=0.016$) were all relatively increased in the CP group compared to the control group.

Immune signature expression across CP clinical stages

In a pairwise comparison between controls and CP subjects at different clinical stages, five analytes' MFI was significantly elevated in CP subjects at clinical stage 3 compared to controls (Figure 2 and Table 2); IL-6 ($p<0.001$), IL-8 ($p<0.001$), sICAM-1 ($p=0.002$),

hepatocyte growth factor (HGF) (p=0.007) and macrophage-derived chemokine (MDC) (p=0.018). There were no statistically significant differences in the expression of analytes across the individual clinical stages within the CP population (all p>0.05) (Table 2), although increasing levels of IL-6 and IL-8 were observed with advancing disease stages (Figure 2). Analytes did not separate clearly in clusters between the different CP clinical stages and controls (Figure 3).

Discussion

In this pilot study, we analysed samples from 135 subjects with various clinical stages of CP and 50 control subjects to identify immune signatures associated with CP clinical stages. This was performed via detailed immunophenotyping using a well-validated 80-multiplex assay. Three analytes, cytokine IL-6, chemokine IL-8, and adhesion molecule sICAM-1, were significantly elevated in the combined CP population. In addition, the levels of IL-6 and IL-8 increased with advancing disease stages, with the highest levels observed in advanced CP (clinical stage 3). Collectively, these findings indicate progressive elevation in pro-inflammatory cytokines and chemokines with advancing clinical stages of CP.

Immune signature expression in CP vs control group

In this study, pro-inflammatory mediators (IL-6, IL-8, and sICAM-1) were elevated in the combined CP population compared to the controls [27–29]. These findings differ from a previous observation, where five cytokines were differently expressed in CP subjects compared to controls (GM-CSF, IFN-β, Leptin, PDGFB, and Resistin) [3]. The inability to identify IL-6, IL-8, and sICAM-1 in the previous study may be due to a lack of power, as only 40 CP subjects were included. It is not entirely clear why the previously 5 identified markers were not seen in the current study, but it may have to do with sample acquisition and study design. Our data are consistent with parallel research using the PROCEED samples (unpublished).

Immune signatures across CP clinical stages

Immunological mechanisms govern the progression of CP through interaction between pancreatic non-immune and immune cells, as described in the introduction. These processes are controlled by immune signalling via cytokines, chemokines, and adhesion molecules, which are detectable in the systemic circulation [1,3,29]. Five analytes, IL-6, IL-8, sICAM-1, HGF, and MDC, were significantly elevated in CP subjects at an advanced disease stage (clinical stage 3) compared to controls. Furthermore, a tendency toward gradually elevating levels of IL-6 and IL-8 was observed with advancing disease stages, although we could not detect statistically significant differences between CP subgroups. The relatively small sample size and its associated lack of power may explain the lack of statistical significance in the groupwise comparisons of CP subgroups in this study.

IL-6 and IL-8 are pro-inflammatory proteins seen in multiple different inflammatory diseases [27,30]. Our observations suggest that IL-6 is involved in CP throughout the entire disease spectrum and may have a key role in accelerating the number of activated PSCs as the disease progresses. As IL-6 both activates PSCs and is produced by these cells, the

elevating levels of IL-6 in the systemic circulation with advancing CP stages may reflect an increasing number of activated PSCs as CP progresses [8]. Further studies of IL-6 signalling, including preclinical studies of anti-IL-6 treatment for CP, seem justified given the reproducibility of elevated IL-6 expression in several independent CP cohorts [31,32]. IL-8 is mainly observed in severely damaged areas of the pancreas, with a profound amount of fibrosis [28,33]. Collectively, our findings suggest that IL-6 and IL-8 are continuous promoters of inflammation in CP subjects and may have a key role in facilitating progression toward end-stage CP.

Study strengths and limitations

A key strength of the study relates to the rigorous methodology used across all sites for prospective collection, processing, and storage of biospecimens. The statistical analysis plan and adjustments were robust and carefully considered *pre-hoc* during the planning of the study. MFI was used as the unit of analyte levels in this paper, as using standard units may skew the statistical analysis, as some values may be above or below the limit of quantification, hence limiting assay sensitivity. This approach is in agreement with previous studies reporting higher statistical power using MFI compared to concentration-based units [15].

Some limitations pertain to our study. The cross-sectional study design precludes causal inference regarding the observed association, and future studies using longitudinal samples will be useful to further understand the temporal and dynamic nature of immune signalling in relation to CP progression. Further, the cytokine Transforming Growth Factor beta (TGF-β) is a key mediator of fibrosis in CP, but this was not investigated in this study as the Luminex panels do not include TGF-β [34]. Future studies of investigation of chemokines/cytokines/adhesion molecules in CP should include TGF-β.

Conclusion:

Our pilot study identifies a significant elevation in the pro-inflammatory mediators IL-6 and IL-8, as well as the adhesion molecule sICAM-1, in individuals with CP, with the highest levels observed in advanced disease stages. Our findings further highlight the potential of IL-6 and IL-8 as continuous drivers of inflammation in CP. Taken together with the existing literature, targeting IL-6 may be a promising avenue for therapeutic intervention in CP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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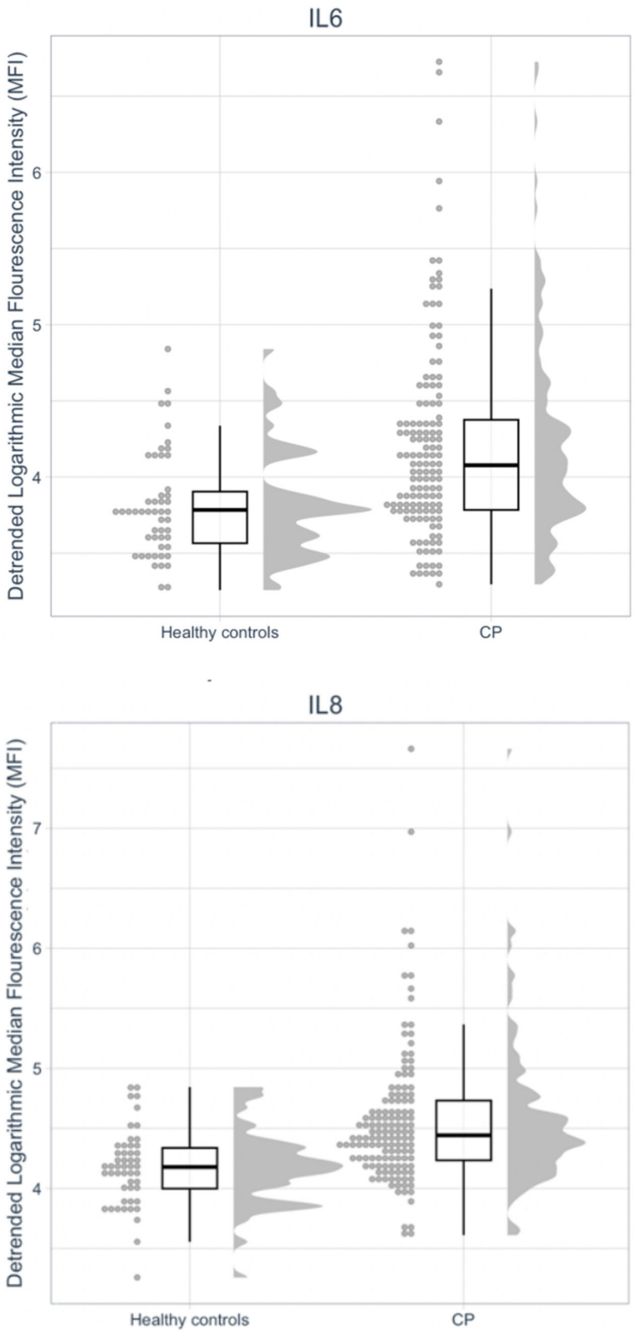

Figure 1:
Raincloud plots of the median fluorescence intensity (MFI) of biomarkers IL-6 and IL-8 in the total CP population.

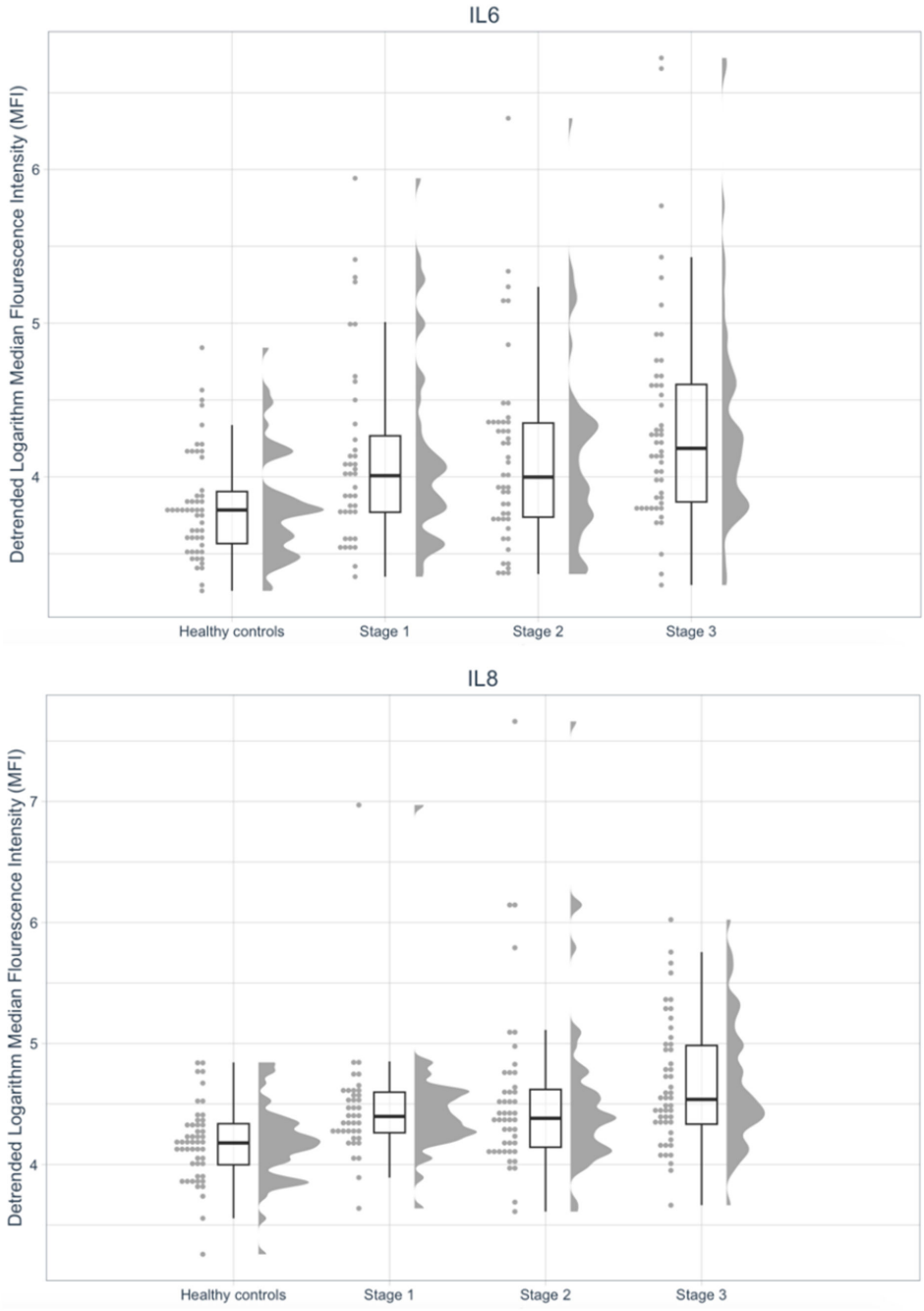

Figure 2:
Raincloud plots of the median fluorescence intensity (MFI) of biomarkers IL-6 and IL-8 in controls and CP patients across the different clinical stages.

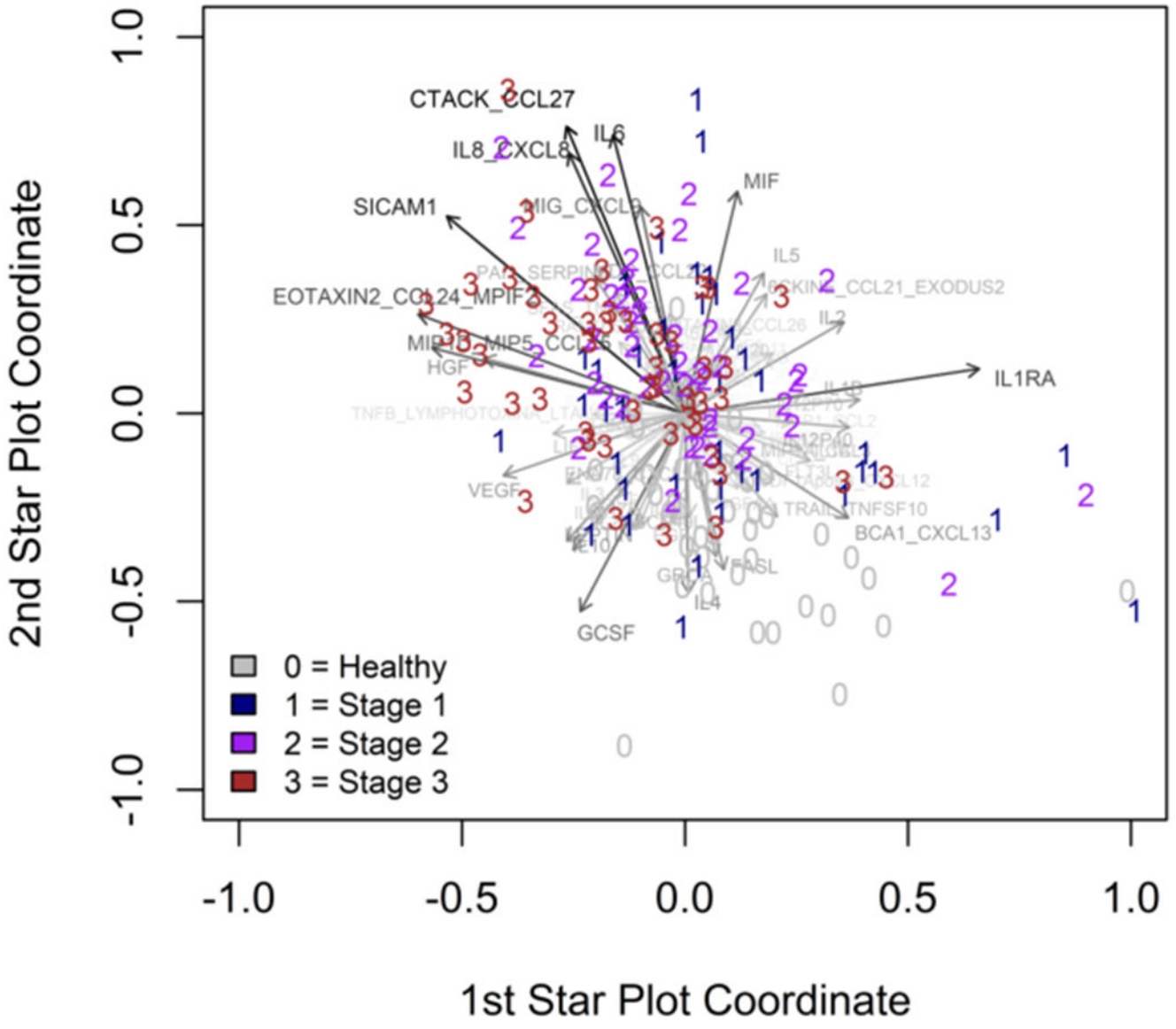

Figure 3:
Star plot of all 80 analytes within the subgroups of CP patients at different clinical stages.

Table 1.

Demographic and clinical characteristics of controls and chronic pancreatitis (CP) subgroups, by clinical stage.

	Control group (n=50)	Total CP population (n=135)	Clinical stage 1 (n=40)	Clinical stage 2 (n=46)	Clinical stage 3 (n=49)
Male sex, n (%)	20 (40.0)	74 (54.8)	21 (52.5)	24 (54.3)	28 (57.1)
Age, years (IQR)	39.0 (28-52)	55.0 (46-62) **	51.5 (44-65) ***	54.0 (45-61) ***	56.0 (51-62) ***
Age groups, n (%)					
<40	26 (52.0)	18 (13.3) ***	7 (17.5) ***	9 (19.6) ***	2 (4.1) ***
40-60	14 (28.0)	74 (54.8)	20 (50.0)	23 (50.0)	30 (61.2)
>60	10 (20.0)	43 (31.9)	13 (32.5)	14 (30.4)	17 (34.7)
BMI, category, n (%)					
Underweight	0	8 (5.9)	1 (2.5)	1 (2.2)	6 (12.2) *
Normal	20 (40)	73 (54.1)	24 (60.0)	25 (54.3)	24 (49.0)
Overweight	16 (32)	33 (24.4)	9 (22.5)	13 (28.3)	11 (22.4)
Obese	14 (28)	21 (15.6)	6 (15.0)	7 (15.2)	8 (16.3)
Race, n (%)					
Black or African	12 (24.0)	12 (8.9)	2 (5.0) **	6 (13.0)	4 (8.2)
White	34 (68.0)	112 (83.0)	37 (92.5)	36 (78.3)	39 (79.6)
Asian	3 (6.0)	6 (4.4)	0	1 (2.2)	5 (10.2)
More than one race	1 (2.0)	4 (3.0)	1 (2.5)	3 (6.5)	1 (2.0)
Other	0	1 (0.7)	0	0	0
Age at CP diagnosis, years (IQR)		50 (42-59)	51 (40-62)	51 (42-58)	50 (44-59)
Duration of CP, years (IQR)		2 (1-5)	1 (0-2)	2 (1-5)	4 (2-8) #
CP aetiology, n (%)					
Alcoholic Pancreatitis		58 (43.0)	16 (40.0)	19 (41.3)	23 (46.9) &
Idiopathic		53 (39.3)	18 (45.0)	16 (34.8)	19 (38.8)
Genetic		17 (12.6)	4 (10.0)	8 (17.4)	5 (10.2)
Obstructive		1 (0.8)	0	1 (2.2)	0
Post-necrotic		2 (1.5)	1 (2.5)	1 (2.2)	0
Hypertriglyceridemia		2 (1.5)	0	0	2 (4.1)
Miscellaneous		2 (1.5)	1 (2.5)	1 (2.2)	0
RAP, n (%)					
Yes		96 (71.1)	26 (65.0)	36 (78.3)	34 (69.4)
No		13 (9.6)	8 (20.0)	1 (2.2)	4 (8.2)
NA		26 (19.3)	6 (15.0)	9 (19.6)	11 (22.5)

IQR = Interquartile range, EPD = exocrine pancreatic dysfunction, BMI = Body mass index, RAP = Recurrent acute pancreatitis, NA = Not available.

Groupwise comparisons with False Discovery Rate corrections for multiple comparisons:

Significance of the difference between the control group and the CP subgroups:

* <0.05;

** <0.01;

*** <0.005

Significance of the difference between stage 1 compared to stage 2 and stage 3:

<0.01

Significance of the difference between stage 2 and stage 3:

& <0.05

Table 2.

Median Fluorescence intensity (MFI) of analytes differentially expressed in controls and across chronic pancreatitis (CP) clinical stages

	Control group	Total CP population	Clinical stage 1	Clinical stage 2	Clinical stage 3
IL6 (IQR)	44 (35-50)	59 (44-80) *	55 (43-71)	55 (42-78)	66 (46-100) ##
IL8 (IQR)	65 (55-77)	85 (69-114) *	81 (71-99)	80 (63-102)	94 (76-146) ##
HGF (SD)	75 (30)	87 (49) *	84 (35)	83 (47)	92 (59) ##
MDC (SD)	8094 (2118)	9258 (3597)	8448 (3197)	9457 (4068)	9717 (3383) #
sICAM1 (SD)	7984 (3204)	9004 (4280)	8901 (2727)	9003 (4828)	9088 (4797) #

Groupwise comparisons with False Discovery Rate corrections for multiple comparisons:

Significance of the difference between the control group and the total CP population:

*<0.01

Significance of the difference between the control group and the CP subgroups:

#<0.05;

##<0.01