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ON THE ROLE OF INTERLEUKIN 6 AND SOLUBLE INTERLEUKIN-6 RECEPTOR AS
POTENTIAL BIOMARKERS FOR CROHN'S DISEASE

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MONIKA ZIÓŁKIEWICZ
From Mikołów (Poland)

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1st Reviewer: Prof. Dr. Susanna Nikolaus, Klinik für Innere Medizin I mit den Schwerpunkten
Gastroenterologie, Hepatologie, Ernährungs- und Altersmedizin

2nd Reviewer: Priv.-Doz. Dr. Dirk Schmidt-Arras, Biochemisches Institut

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Prof. Dr. Dominik M. Schulte, Klinik für Innere Medizin I mit den Schwerpunkten
Gastroenterologie, Hepatologie, Ernährungs- und Altersmedizin

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1) **Introduction**

a) Crohn's disease

Crohn's disease (CD) and ulcerative colitis (UC) are the main subforms of chronic inflammatory bowel disease (IBD). In Germany, the current incidence of CD is 6.6 per 100,000 inhabitants [1] and its prevalence is 100-200 per 100,000 inhabitants [2]. CD can develop at any age and affects men and women at a similar rate [3], primarily young adults. Some risk factors which can contribute to the disease manifestation, are smoking, family disposition, appendectomy and oral contraceptives [3-5]. The clinical presentation varies depending on which site(s) in the gastrointestinal tract are affected. The relapses often end in incomplete remission [3].

Crohn's disease is characterized by transmural and intermittent inflammation. This means that it affects all the layers of the intestinal wall and that its lesions are discontinuous "skip lesions" [3]. In 30-40% of CD patients, only the small intestine is affected, both the small and large intestine are affected in 40-50% of cases, and in 15-25% of patients with CD, the lesions are only found in the large intestine. Around 90% of CD patients present with inflammation of the terminal ileum [4].

The most frequent symptoms are diffuse, spasmodic stomach-ache, diarrhoea (mostly without blood admixture), abdominal resistance, fever, fatigue and in severe cases, weight loss and malnutrition. Common macroscopic changes to the intestine are ulcerations, fistulas, strictures, perianal disease, stenosis due to the obstructed and fibrotic intestinal wall and abscesses. Ulcerations and crypt abscesses with conglomerates of macrophages can be found in the histological examination. Around 50 % of the CD patients present with non-caseating granulomas in all layers of the intestinal wall [4]. Moreover, CD shows extraintestinal manifestations which appear on the skin (erythema nodosum, pyoderma gangrenosum), in the joints (arthritis, ankylosing spondylitis), in the eyes (conjunctivitis, uveitis/iritis, episcleritis),

in the bones (osteoporosis), as well as affecting the hepatobiliary system (hepatomegaly, gallstones, fatty liver) [3, 4].

The aetiology of CD is multifactorial and is still not completely understood. Genetics, environmental factors, the immune system and the intestinal flora all play a role in the development of this medical condition [4, 6]. There are several hypotheses about its pathophysiology: One suggests that a chronic, deregulated mucosal immune response causes loss of tolerance for, and an inappropriate, innate immune response to the gut microbiota. Others claim that changes in the intestinal microbiota compound result in a pathological immune response. There is evidence to support both of these theories [6]. Studies show that patients with CD have reduced microbiome diversity [7] and that CD's development is also influenced by genetic predisposition, defects in the mucosal barrier function and environmental factors, such as nicotine [3, 4]. Modifications to the immune system result in a higher distribution of pro- than anti-inflammatory cytokines by the CD4 positive T cells which causes chronic inflammation of the intestine [4, 6]. Moreover, CD also increases the risk of developing colorectal cancer [4].

The clinical diagnostics include clinical examination, endoscopy with histopathology and laboratory results. During active disease, the colonoscopy shows signs of acute or chronic inflammation with ulcerations and abscesses. Although the serological parameters can be normal, patients frequently have high levels of C-reactive protein (CRP) and blood sedimentation rates (BSR), elevated leucocytes and signs of anaemia with low hemoglobin, serum iron and vitamin B12 levels [3, 4].

So far, there is no curative therapy for CD but several drugs have been approved for acute treatment and relapse prevention. The first-line treatments during an acute relapse are systemic and local corticosteroids (prednisolone, budesonide); remission can be achieved in 70% of the patients. However, corticosteroids are not suitable for relapse prevention as they have several side effects. Immunosuppressive drugs (azathioprine, 6-mercaptopurine, methotrexate) or

biologicals (e.g. anti-TNF antibodies like infliximab, adalimumab; monoclonal antibodies like vedolizumab) are indicated for long-term therapy. [3, 4]. There are also several new drugs in clinical trials. The operative resection of affected parts of the intestine is only indicated if there are complications.

The life expectancy of patients with Crohn's disease is hardly impaired, the quality of life might differ depending on the disease's clinical expression [3].

There are several ways of measuring CD activity by using laboratory assessments or activity scores. The most common clinical scores are the Crohn's Disease Activity Index (CDAI) and its simplified version, the Harvey-Bradshaw-Index (HBI). The CDAI contains 8 parameters: patients' abdominal pain, general well-being, total number of liquid stools, abdominal mass, complications of CD (i.e. fever, arthritis, uveitis, erythema nodosum, abscess etc.), use of anti-diarrhoea drugs, haematocrit level and changes in body weight. Definition of an active disease requires a CDAI > 150 points.

The Crohn's Disease Index of Severity (CDEIS) can be used to obtain endoscopic evidence of CD activity, this describes the lesions and ulcerations in the gastrointestinal tract. The CDEIS is not used as often because of the need for an invasive examination.

Despite having several subjective parameters, the CDAI is the score most commonly used to describe the disease activity in CD in clinical practice.

Another possible way of describing disease activity is to use laboratory parameters, for example CRP. Active disease is defined as CRP > 5mg/l and inactive disease as CRP <5mg/l.

b) Biomarkers

According to the World Health Organization (WHO), the United Nations and the International Labor Organization, a *biomarker* or *biological marker* is "any substance, structure, process or its products that can be measured in the body and influence or predict the incidence of outcome or disease" [8]. A reliable biomarker should be objective, quantifiable, cheap and easy to

determine. Ideally, its concentration should reflect the staging, monitoring and progress of the disease, effects of treatment and time to relapse; it should be specific for a given disease [9].

A differentiation is made between prognostic and predictive biomarkers. Prognostic biomarkers characterize the stage and the outcome of the disease, together with its progression and recurrence. They are independent of the therapy received by the patients. In contrast, predictive biomarkers show the possible response to the therapy and support treatment decisions [10].

In CD, the serum CRP levels approximately correlate with the disease's endoscopic activity [11]. However, it is unspecific, and is only elevated in two-thirds of the patients. Therefore, CRP cannot be used as a biomarker for CD [12].

A study shows that high-sensitivity CRP (hsCRP) is a good biomarker for CD but only in patients who had elevated hsCRP levels at the time of the diagnosis. It is not applicable for the patients who had normal hsCRP [12]. There are several other serological markers, such as anti-neutrophil cytoplasmic antibodies (pANCA), anti-Saccharomyces cerevisiae antibodies (ASCA) or anti-OmpC antibody but their sensitivity is only around 40-60% [4, 13]. The stool markers calprotectin and lactoferrin are also known to be CD markers, however they are not specific for CD and can be detected in all kind of chronically active inflammation, i.e. in patients with infectious colitis or diverticulitis [4].

Different studies have shown a decreased serum level of tryptophan in CD patients: Nikolaus et al. (2017) presented a study of more than 500 IBD patients which confirmed a negative correlation between tryptophan serum levels and disease activity. The decrease was greater in CD patients than in those with UC. Consequentially, these patients had increased levels of tryptophan metabolites, i.e. indoleamine 2,3 dioxygenase- 1 enzyme, which are responsible for the catabolism of tryptophan. Tryptophan deficiency could play a role in the pathogenesis of CD and reflect the disease's activity [14, 15].

The aim of the study is to determine whether interleukin 6 (IL-6), the soluble IL-6 receptor (sIL-6R) or the soluble gp130 protein (sgp130), could serve as a biomarker for CD.

c) IL-6 signalling

Interleukin- 6 (IL-6) is a pleiotropic cytokine, which was first cloned in 1986. It consists of 184 amino acids [16] and is produced by several cell types, such as antigen presenting cells (macrophages, dendritic cells), B- and T-cells, fibroblasts, epithelial cells, glial cells and keratinocytes [17], in response to different stimuli. It belongs to a cytokine receptor superfamily, which shares the same signal transducing subunit, gp130 protein. The currently known members are: IL-6 [18], IL-11 [19], ciliary neurotrophic factor (CNTF) [20], cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), leukemia inhibitory factor (LIF), oncostatin M (OSM) [21], neuropoietin (NPN) [22], IL-27 [23] and IL-31 [24]. A normal IL-6 serum concentration is 1-5 pg/ml [25].

IL-6 plays an important role in inflammation, immune response, metabolism, hematopoiesis and embryonic development [25]. It is responsible for the last step of the differentiation of B cells into antibody producing cells [16]. IL-6 is involved in T cells' growth and in their activation and proliferation [26]. Together with IL-2, it helps with cytotoxic T cell differentiation [27].

Furthermore, IL-6 acts as a growth factor for plasmacytoma and myeloma cells [26] and induces M1 murine myeloid leukemic cells' differentiation into macrophages [28].

IL-6 also participates in the acute phase response by indicating the secretion of acute phase proteins (e.g. CRP, alpha-1-antitrypsin, haptoglobin) in hepatocytes during inflammation and acting as a hepatocyte-stimulating factor. Together with IL-1, IL-6, is known to be the main inducer of CRP production [29, 30].

Moreover, IL-6 is produced in skeletal muscle cells after exercising. A study has shown that elevated plasma IL-6 levels can be detected after a 30-minute sprint. IL-6 later decreases again. This suggests that local systemic production might be due to the mechanical disruption of muscle fibres [31].

IL-6 plays an immense role in inflammation. It is produced by monocytes and macrophages during acute inflammation and by T cells during chronic states of inflammation. IL-6 expression is induced when the organism is under stress. The cytokine's release is stimulated by different inflammation triggers, such as $\text{TNF}\alpha$, IL-11, lipopolysaccharides, viruses and necrotic cells [32, 33]. Furthermore, IL-6 is responsible for the transition from the innate to the adaptive immune response and therefore, for the transition from acute to chronic inflammation. Neutrophils represent the biggest component of leukocytes recruited during acute inflammation. IL-6, acting through the sIL-6-R, suppresses neutrophil recruitment and makes way for the monocytes, which predominate in chronic inflammation [34, 35]. Inappropriate immune system control could lead to an imbalance in the IL-6 actions, causing the genesis of chronic inflammatory diseases.

IL-6 can exercise its effects through the membrane bound IL-6 receptor (IL-6-R) or the soluble IL-6 receptor (sIL-6-R). Both these complexes (IL-6/sIL-6R and IL-6/IL-6R) act as agonists and associate with glycoprotein 130 (gp130). Gp130, also known as CD130, is expressed on the surface of all human cells. The subunit is a 130kDa signal transducer which is shared among the cytokine receptor family [18].

The mRNA of sIL-6-R was first isolated in 1992. It lacks the membrane-spanning and cytoplasmic domain of the membrane-bound, IL-6R [36]. IL-6 binds to sIL-6-R and with similar affinity as to IL-6-R. The complex associates with sgp130 and activates intracellular signalling. This alternative pathway is called *trans-signalling*. sIL-6-R is found in concentrations of around 50 ng/ml in healthy human serum [37]. The IL-6/sIL-6-R complex does not bind to cells that express membrane-bound IL-6R.

Soluble IL-6R is generated by two independent mechanisms: limited proteolysis of the membrane protein (shedding) [38] and alternative mRNA splicing [39]. Most sIL-6-R is produced by shedding. The proteolytic cleavage is possible through the major proteases, known as *shedases*, ADAM10 and ADAM17 (A Disintegrin and Metalloproteinase family). These

are Zn^{2+} -dependent, membrane cell expressed, metalloproteases, which are crucial for embryonic development [40, 41].

IL-6 exercises its pro- inflammatory functions on the immune system through the soluble IL-6R. This includes stimulation of endothelial and smooth muscle cells [42], recruiting mononuclear cells and inhibition of T-cell apoptosis and T reg differentiation [43].

IL-6 also acts via the membrane bound IL-6-R; the ligand binds to the extracellular region of the receptor, thus triggering the association with the signal transducer gp130. This process is called classic signalling [18]. IL-6-R is only present on a few cells, such as hepatocytes, neutrophils, monocytes/macrophages and some lymphocytes [44, 45]. This would mean that only these cells could be activated by IL-6 however, due to the ubiquitous expression of gp130, IL-6 can potentially activate all human cells through sIL-6-R.

In chronic inflammation, the dying neutrophils are the main source of sIL-6R. Elevated CRP induces proteolytic shedding of the membrane-bound IL-6R in neutrophils so that they lose the ability to react to IL-6 directly and can only be activated by trans-signalling [38, 46].

A soluble form of the signal transducer gp130 (sgp130) can be detected which corresponds to the soluble form of IL-6R. It is found in human serum in concentrations of about 250-400 ng/mL. Sgp130 has been shown to inhibit the IL-6/sIL-6R complex. Alone, IL-6 does not associate with either gp130 or sgp130. The IL-6/IL-6-R complex only binds to intracellular gp130. In contrast, the IL-6/sIL-6 complex binds to both forms of gp130 with the same affinity. The IL-6/sIL-6-R is neutralized when it is bound to sgp130. Without such an inhibitor, IL-6 would essentially be able to stimulate all the body's cells [47]. An excess of sgp130 blocks all trans-signalling activity and therefore, sIL-6R and sgp130 can be seen as buffers for IL-6 in the blood. The level of sIL-6R determines the buffer's capacity because IL-6 binds to the sIL-6R in the first row [48].

Several studies have reported the detection of elevated IL-6, sIL-6R and sgp130 serum levels in IBD patients [49-55] and they appear to play an important role in the pathogenesis of the

disease. All of these molecules are also known to be elevated in rheumatoid arthritis [56, 57] and cancer [58].

All these findings could have a huge influence on the treatment of inflammatory bowel disease. A pilot randomised trial of an anti-IL-6R monoclonal antibody, Atlizumab, found clinical improvement in patients with Crohn's disease (demonstrated by a decreased Crohn's Disease Activity Index and normalization of acute- phase responses, but no endoscopic improvement in comparison with placebo [59]).

The aim of this study was to systematically evaluate IL-6, sIL-6R and sgp130 serum levels in a large cohort of outpatients suffering from Crohn's disease, as well as in healthy controls and identify whether these parameters correlate with disease activity, relapse and response to treatment. I was also interested in proving whether they could be used as biomarkers in CD. The sgp130 measurements for all the Crohn's patients and the healthy individuals were carried out during the exploratory phase of the study.

As has already been shown in several studies, I was expecting there to be strong modulations in IL-6 serum levels correlating with the disease's activity, inflammatory response and the clinical scores [50, 52-55] and slightly lower modulations in sIL-6R [49, 53-54].

To my knowledge, this is the largest cohort study on this topic; its characteristics allow optimal association analyses of several parameters and provide convincing data on the suitability and limitations of IL-6, sIL-6R and sgp130 as biomarkers in CD. It is a real-life, single-centre, cohort study with a duration of more than one-year, numerous blood samples and long-term patient follow up. The advantages of this study are that there were a large number of patients from a realistic environment, such as an outpatient clinic, and that they were accurately characterized, using 30 different parameters, including laboratory data, medication, past and current therapy, disease activity and medical history.

The purpose of this study was to review the partially contradictory data from previous studies by using the largest cohort of the, by far the best characterized, “all-comers”.

d) Ethics

Approval for this research was granted by the ethics committee of the medical faculty of Kiel University (AZ DA489/14) before the study.

2) **Methods**

a) Patients and clinical data collection

In my studies, I used serum samples which had been obtained from the Department of Internal Medicine outpatient clinic at the University Hospital Schleswig-Holstein (UKSH, Campus Kiel, Germany). The samples were left over from an epidemiological study on tryptophan metabolism. 212 Crohn’s disease patients’ data, with a total of 815 measurements, was obtained from the patients’ electronic hospital records and double-checked by at least two physicians. These were patients with a confirmed diagnosis and for whom there was sufficient data on their disease activity and medication, who were monitored using regular visits, clinical examinations, laboratory blood test results, activity scores (Crohn’s Disease Activity Index (CDAI) and Harvey Bradshaw Index (HBI)), and endoscopies. Moreover, serum samples collected from 100 age-matched healthy blood donors, were obtained from UKSH Department of Transfusion Medicine to serve as healthy controls. By agreement with the UKSH, the CONARIS Research Institute AG used the samples to measure IL-6, sIL-6R and sgp130. CONARIS is a research institute in Kiel, which concentrates on the development of pharmaceuticals for inflammatory diseases in their pre-clinical and early clinical stages.

The characteristics of the CD patients and the healthy controls are presented in Table 1.

Table 1 Characteristics of CD patients and healthy controls

	Crohn's disease	Controls
Number of individuals	212	100
Number of visits	2 (1–24)*	1
Males	34.7%	68.00%
Age at sampling [y]	37 (18–71)	44 (19–68)
Body mass index [kg/m ²]	23.7 (14.7–53.6)	n.d.
Weight [kg]	70 (35–168.8)	n.d.
Smokers	48.2%	n.d.
Time after diagnosis [years]	9 (0–40)	n/a
Crohn's Disease Activity Index (CDAI)	127.5 (0–583)	n/a
Harvey-Bradshaw Index (HBI)	3 (0–30)	n/a
Aminosalicylate therapy	22.5%	n/a
Immunosuppressive therapy	14.1%	n/a
Monoclonal antibody therapy, except for anti-tumour necrosis factor-alpha (TNF- α) antibodies	15.9%	n/a
Steroid therapy	41.2%	n/a
Tumour necrosis factor-alpha (TNF- α) inhibitor therapy	86.4%	n/a

*All values in this format represent median (range).

n/a, not applicable; n.d., not determined.

The samples for the study were collected between the 7th of January and the 6th of August 2015.

Only samples that contained at least 1 mL serum volume were used for the measurements, IL-6 and sIL-6R levels were detected, using ELISA.

After the blood sample had been taken from the IBD patients in the gastroenterological outpatient department of UKSH Campus Kiel, the serum was prepared in the UKSH Central Laboratory in Kiel and later sent to the Central Laboratory of the second UKSH campus in Lübeck for aliquotation and analysis, where the aliquots were stored at -20°C. After the analysis at the USKH, the remaining aliquots were transferred to the CONARIS laboratory and stored at below -70°C. The amount of serum sample required for all the analyses was 210 μ L (2 \times 100 μ L each for IL-6, and 5 μ L each for sIL-6R and spg130 for 500 μ L of a 1:100 dilution), plus a pipetting reserve. In 8 cases in part 1 of the study, only about 150-200 μ L was available, rather

than 250 μ L. In these cases, the sample volume for the IL-6 measurement was reduced from 100 to 90, 80 or 70 μ L, depending on the serum volume available (the missing volume was replaced by diluent from the kit). The results were then multiplied by a correction factor. This procedure was supported by assay linearity validation data provided by Ferring Pharmaceuticals in the context of the collaboration with CONARIS. The IL-6, sIL-6R and spg130 measurements were realized with enzyme-linked immunosorbent assay (ELISA).

b) Measurements of IL-6, sIL-6R and spg130

The IL-6 and sIL-6R serum levels of all patients (n= 212) and control samples (n=100) were measured and the spg130 serum levels for all control and in 81 CD patients' samples were measured during the exploratory phase of the study. All the measurements were performed using Quantikine[®] enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems/Bio-Tecne; Wiesbaden, Germany). Colorimetric data was acquired using an OPSYS MR microplate reader with Revelation Quicklink software (Dynex Technologies; Denkendorf, Germany).

The ELISA IL-6 measurements were carried out using D6050, Quantikine[®] Human IL-6 Immunoassay kits from R&D Systems (version 01.06, 749909.6, 11/13). The immunoassay works with E. coli-expressed recombinant IL-6. This system not only measures free IL-6 but also IL-6 bound to the soluble receptor.

The Quantikine Immunoassay uses the qualitative sandwich enzyme immunoassay technique. This means that a preformed monoclonal antibody, in this case one that was only specific for IL-6, was deposited on the plate. After the standards and the samples had been pipetted into the cavities of the micro titre plate, IL-6 could bind to the specific enzyme-linked polyclonal antibody. After incubation, all the unbound matter was washed away and a substrate solution was added. The intensity of the colour is proportional to the IL-6 concentration in the sample.

Following the measurements, a standard curve was established using a four-parameter logistic (4-PL) curve fit. The diluted samples' concentration was multiplied by the dilution factor.

With this method, the minimum detectable dose (MDD) of human IL-6 is $\geq 0,7$ pg/mL [60].

The ELISA measurements of sIL-6R were performed using DR600, Quantikine® Human sIL-6R Immunoassay kits from R&D Systems. These can measure human sIL-6R in serum, plasma, urine and cell culture supernates due to the recombinant sIL-6R produced in Sf21 cells, and its antibodies contained in the immunoassay.

The sIL-6R immunoassay measurements were performed in the same way as the D6050, Quantikine® Human sIL-6R Immunoassay (see above), with some small differences (see manufacturer's instructions, version 04.93, 750572.5, 10/14). The minimum detectable dose (MDD) of sIL-6R is 6,5 pg/mL with this method [61].

The ELISA measurements of sgp130 were performed with DGP00, Quantikine® Human soluble gp130 Immunoassay kits from R&D Systems. These can measure human sgp130 in serum, plasma and cell culture supernates because of the recombinant sgp130 produced in Sf21 cells, and its antibodies contained in the immunoassay.

The sgp130 immunoassay measurements were performed in the same way as the DGP00, Quantikine® Human IL-6 Immunoassay (see above), with some small differences (see: manufacturer's instructions, version 11.95, 750156.4, 4/14). The minimum detectable dose (MDD) of sgp130 is 0,08 ng/mL with this method [62].

c) Data analysis and statistics

More than 50% of all the IL-6 measurements were below the detection limit of 3.13 pg/mL. The entire IL-6 dataset was divided into three categories so that the statistical analysis could be performed: (1) below detection limit, (2) between detection limit and third quartile and (3) larger than third quartile. The comparison between the two extreme groups, 1 and 3, was made

for all the analyses in order to better differentiate between patients with high IL-6 levels and those with IL-6 concentrations just above the detection limit. All the parameters investigated in the study are listed in Table 2.

Table 2 General, clinical and laboratory parameters

Data group	Parameter
General data	Age at sampling [y]
	Allergy/asthma
	Appendectomy
	Arthritis as a comorbidity
	Body mass index (BMI), sorted into overlapping groups: group 1: high ($\text{BMI} \geq 25$) or normal ($18.5 \leq \text{BMI} < 25$) group 2: low (< 18.5) or normal ($18.5 \leq \text{BMI} < 25$)
	Food allergy
	Gender
	Positive family history of inflammatory bowel diseases
	Smoker (current)
	Smoker (former or current)
	Surgery on gastrointestinal tract (ever)
	Time after diagnosis [y]
Activity (CD)	Crohn's Disease Activity Index (CDAI)
	Harvey-Bradshaw Index (HBI)
Laboratory data	Alanine transaminase (ALT; formerly glutamic-pyruvic transaminase, GPT) [U/L]
	Aspartate transaminase (AST; formerly glutamic-oxaloacetic transaminase, GOT) [U/L]
	C-reactive protein (CRP) [mg/L]
	Creatinine [mg/dL]
	Hematocrit [vol%]
	Hemoglobin [g/dL]
	Interleukin-6 [pg/mL]
	Leukocytes [/nL]
	Soluble glycoprotein 130 [ng/ml]
	Soluble interleukin-6 receptor [ng/ml]
	Thrombocytes [/nL]
Medication	Aminosalicylate therapy
	Immunosuppressive therapy
	Monoclonal antibody therapy, except for anti-tumour necrosis factor-alpha (TNF- α) antibodies
	Steroid therapy
	TNF- α inhibitor therapy

Since more than one observation per patient was available in the majority of cases, univariate mixed models were used and all the available data was analysed to detect the associations with the existing parameters. I used the independent variable (*i.e.* clinical parameter) as the fixed effect and the patient identity code as the random effect. Linear mixed models were used for the continuous variables sIL-6R and sgp130, whereas logistic regression models were estimated for the ordinal variable IL-6. For all the mixed model analyses, all the independent continuous variables were dichotomized based on the median.

The measured concentrations of IL-6, sIL-6R and sgp130 were compared within patients, between patient groups and between patients and healthy controls. Moreover, an active and/or inactive time point was determined for each patient, based on either their disease activity scores (active: CDAI > 150 points; extremely active: CDAI > 250 points; inactive: CDAI ≤ 150 points) or their CRP levels (active: CRP > 5 mg/L; inactive: CRP ≤ 5 mg/L), with two different cohorts being used to make the comparison. First, all the active and inactive time points of all the patients were compared (full dataset/cohort). The second cohort consisted of the most active and the most inactive time points of those patients for whom both data were available (reduced dataset/cohort). Table 3 shows the number of patients and measurements for the cohorts and subcohorts.

Table 3 Numbers of patients and measurements per cohort and subcohort

Group	no. of patients	no. of measurements
All	212	815
All: activity by score	209	786
All: activity by CRP	212	814
Subset: activity by score	52	104
Subset: activity by CRP	56	112

CRP, C-reactive protein; no., number. Note that in a few cases, the Crohn's Disease Activity Index (CDAI) and the CRP level had not both been obtained at each visit.

A nonparametric bootstrap approach was used to present the data of active, inactive and healthy control samples for the median comparison. Sampling with replacement was performed at the proband level, and a suitable test statistic was calculated for each bootstrap sample. The confidence intervals were estimated using the 2.5% and 97.5% quantiles of the bootstrap test statistics' distribution. The P-values were determined by 2* the proportion of bootstrap test statistics larger or smaller than zero, depending on the sign of the test statistic based on the original sample. I used the difference in medians or the median of the differences for the continuous variables sIL-6R and sgp130 in the unpaired or paired comparisons, as test statistics. For IL-6, I applied the difference in proportions or the proportion of disagreement.

All the statistical tests were carried out two-sided, using a nominal significance level of 5%. The analyses were performed using the statistical software R (version 3.3.1). R packages nlme (version 3.1-128) and MuMIn (version 1.15.6), were used to estimate linear mixed models and the variance explained by the fixed effect (= marginal R²). The data was presented in boxplots by using the standard settings in R: the box corresponds to the interquartile range, the median is displayed as a bold line, the whiskers represent the most extreme values within the 1.5-fold of the interquartile range, and values outside this range are displayed as single dots.

3) **Results**

a) IL-6, sIL-6R and sgp130 in the complete cohort

Since more than 50% of the IL-6 levels were below the <3.13 pg/mL detection limit, I performed an ordinal analysis of the data and compared the two extreme groups: Those below the detection limit vs. those above the third quartile (figure 1A). The chart can be read as follows: below the 3.13 pg/mL detection limit (light grey), between the detection limit and the third quartile (grey) and above the third quartile (black). Figure 1A shows that the proportion

of IL-6 measurements above the detection limit is higher in CD patients than in the healthy controls. Because of the strong variation in the IL-6 measurements, a logarithmic transformation was performed for purely illustrative purposes (Figure 1B). Figures 1C and 1D represent sIL-6 and sgp130 levels on a logarithmic scale. A variation in comparison to the healthy controls, was also observed in the sIL-6 and sgp130 data, however, the increase of their median levels was less than 15% and therefore not significant when compared to the disease activity (Table 4).

Table 4 Circulating levels of IL-6, sIL-6R and sgp130

Groups	IL-6 (pg/mL)	sIL-6R (ng/mL)	sgp130 (ng/mL)
Controls	$\leq 3^{\ddagger}$ ($\leq 3-6$) (n = 100)	35 (20–53) (n = 100)	217 (136–432) (n = 100)
Inactive CD	≤ 3 ($\leq 3-6.872$) † (n =	40 (14–93) † (n =	227 (92–368) (n = 228)
Active CD	458) §	456)	232 (140–385) † (n =
	≤ 3 ($\leq 3-32.671$) *† (n =	36 (16–72) * (n =	135)
	328)	325)	

All values represent the median (range) and were rounded to the nearest integer.

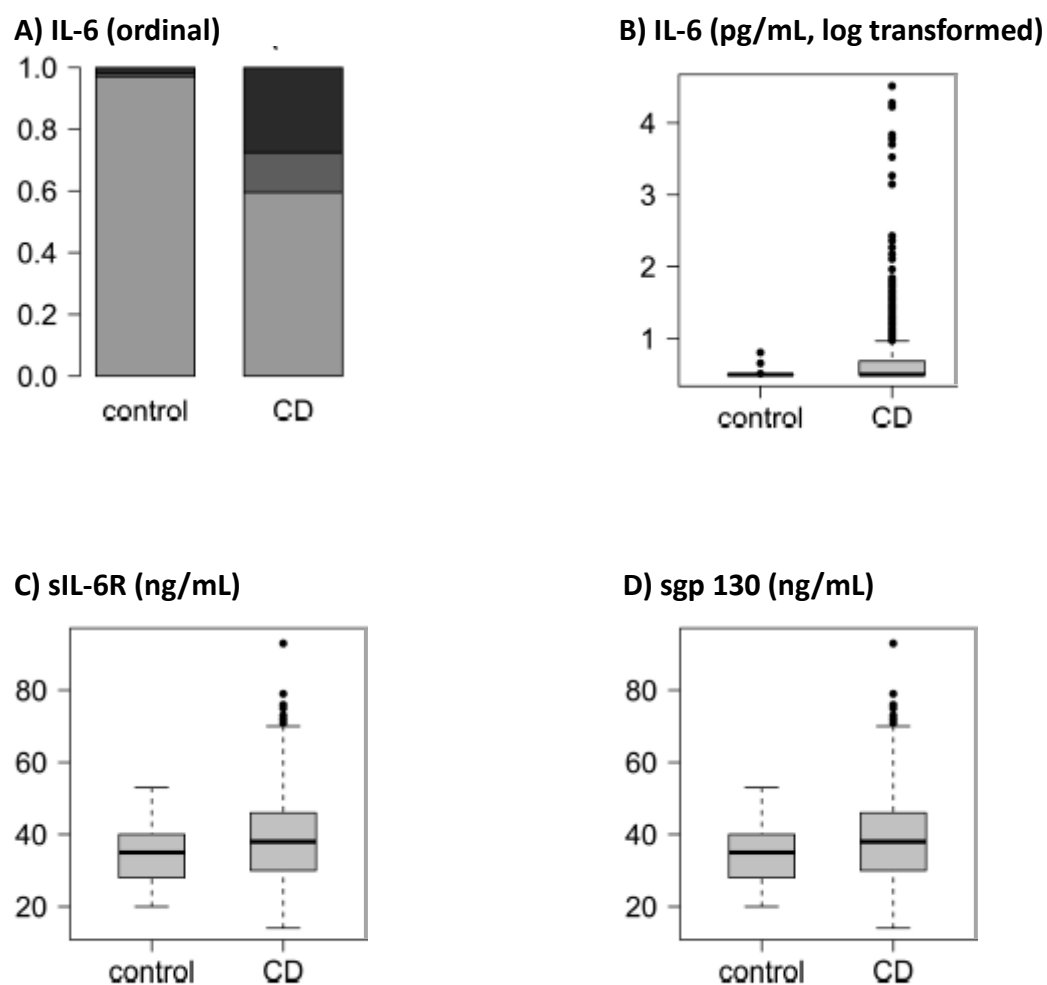
$^{*}p < 0.05$ or less for active (CDAI > 150 or CAI > 4) vs. inactive disease (CDAI \leq 150 or CAI \leq 4).

$^{\dagger}p < 0.05$ or less vs. healthy controls.

‡ Detection limit of the IL-6 ELISA: 3.13 pg/mL.

§ Number of samples from different visits. IL-6 and sIL-6R were measured in a total of 212 patients; sgp130 was measured in 81 patients.

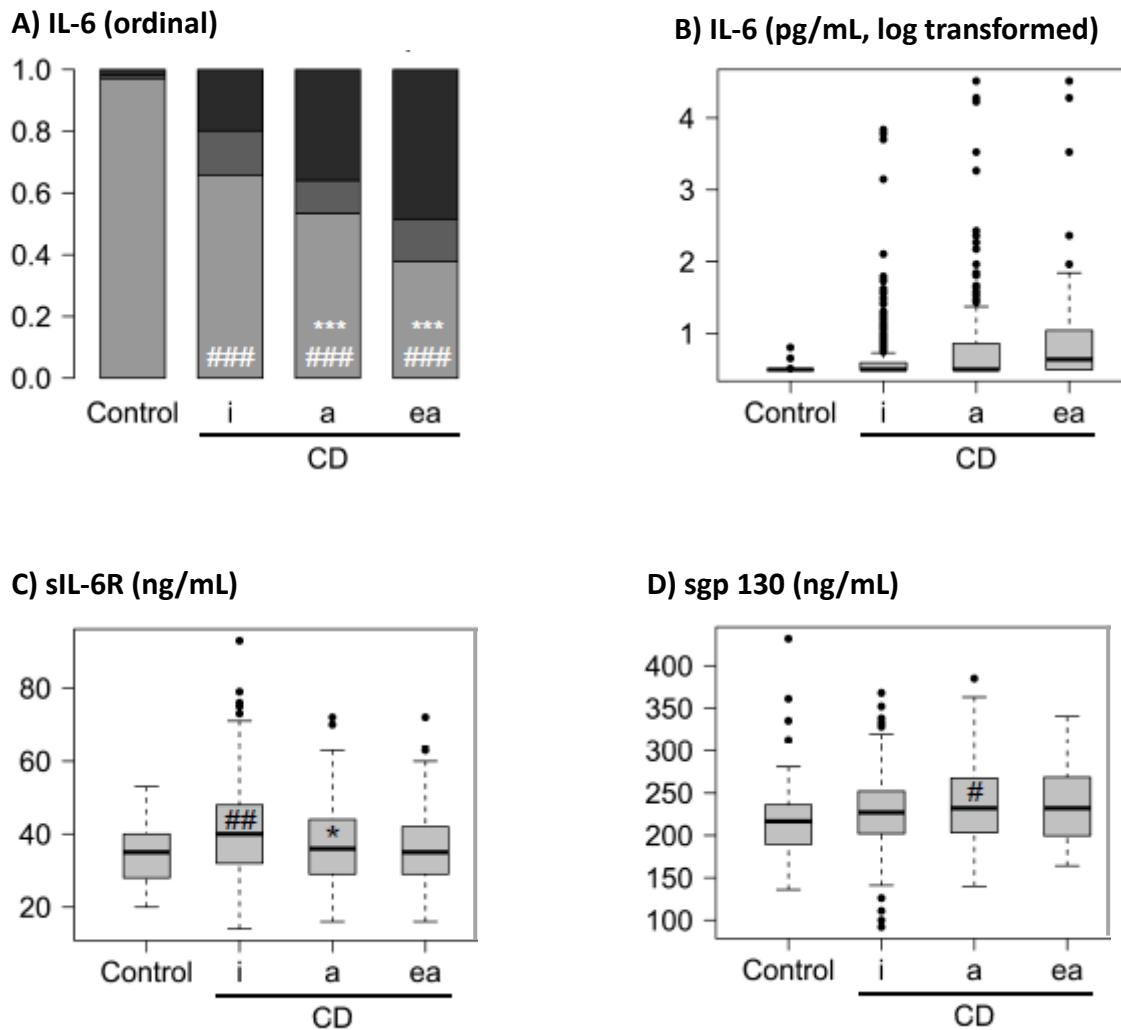
Figure 1



Overview of IL-6, sIL-6R and sgp130 serum levels in healthy controls and patients with Crohn's disease (CD). (A) Ordinal representation of IL-6 levels across all groups: below the detection limit of 3.13 pg/mL (light grey), between the detection limit and the third quartile (grey) and above the third quartile (black). (B) Logarithmic representation of IL-6 levels. (C) sIL-6R levels. (D) sgp130 levels.

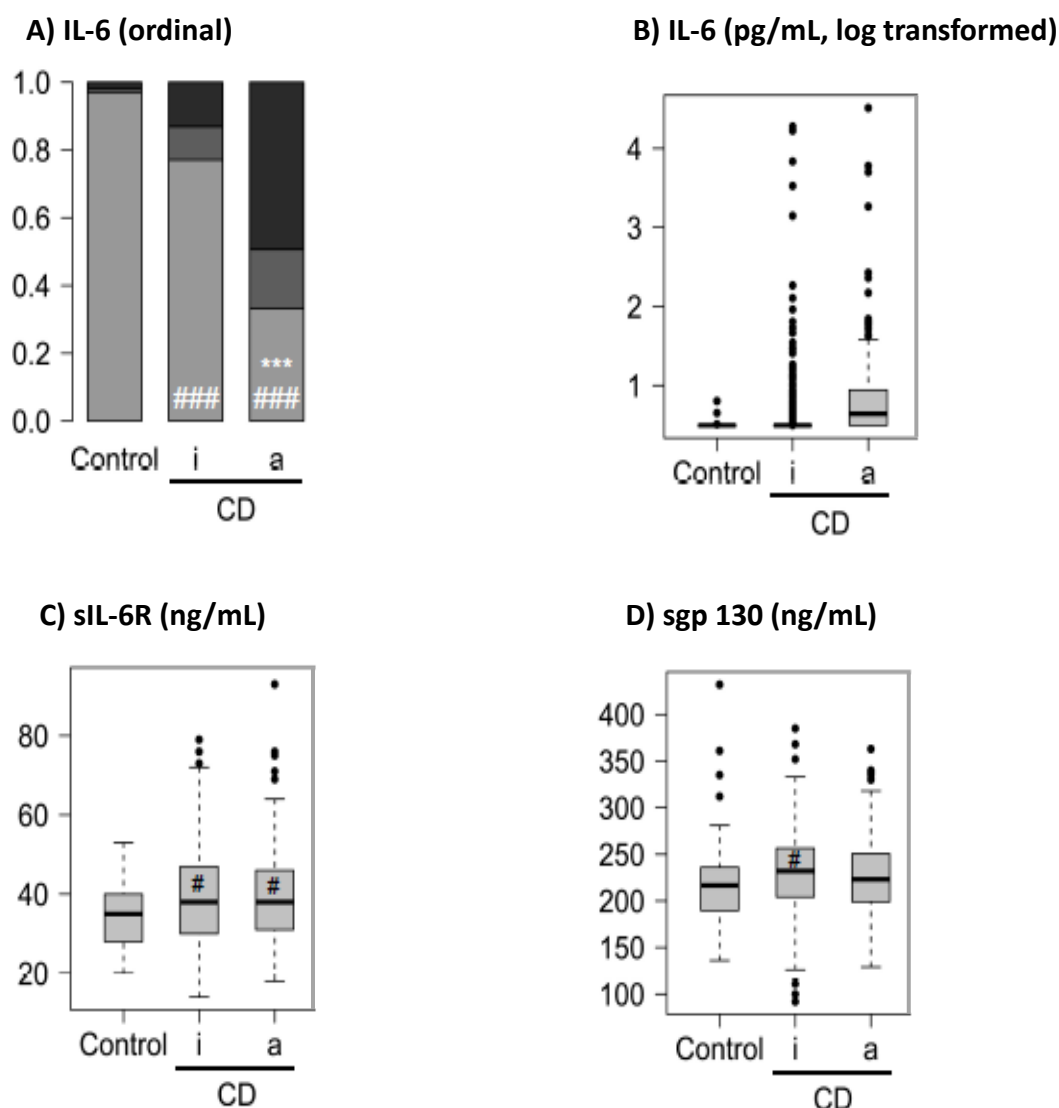
Moreover, the patients' cohort was investigated according to disease activity (Figure 2) and C-reactive protein (CRP) blood levels (Figure 3).

Figure 2



IL-6, sIL-6R and sgp130 serum levels in healthy controls and in all patients with Crohn's disease (CD), differentiated according to disease activity scores. (A) Ordinal representation of IL-6 levels across all groups: below the 3.13 pg/mL detection limit (light grey), between the detection limit and the third quartile (grey) and above the third quartile (black). The two extreme groups (below the detection limit and above the third quartile) were compared for significance analyses. (B) Logarithmic representation of IL-6 levels. (C) sIL-6R levels. (D) sgp130 levels. a, active disease [Crohn's Disease Activity Index (CDAI) > 150]; ea, elevated (moderate to severe) disease activity (CDAI > 250); i, inactive disease (CDAI ≤ 150). Significant differences between active and inactive disease: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Significant differences between active or inactive disease and controls: #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$.

Figure 3



IL-6, sIL-6R and sgp130 serum levels in healthy controls and in all patients with Crohn's disease (CD) differentiated according to C-reactive protein (CRP) levels. (A) Ordinal representation of IL-6 levels across all groups: below the detection limit of 3.13 pg/mL (light grey), between the detection limit and the third quartile (grey) and above the third quartile (black). The two extreme groups (below the detection limit and above the third quartile) were compared for significance analyses. (B) Logarithmic representation of IL-6 levels. (C) sIL-6R levels. (D) sgp130 levels. a, active disease (CRP > 5 mg/L); i, inactive disease (CRP ≤ 5 mg/L). Significant differences between active and inactive disease: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Significant differences between active or inactive disease and controls: #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$.

Figure 2 shows the IL-6, sIL-6R and sgp130 serum levels in healthy probands and in all the CD patients, differentiated according to disease activity score. Figure 2A is an ordinal (below the 3.13 pg/mL detection limit (light grey), between the detection limit and the third quartile (grey) and above the third quartile (black)), Figure 2B is a logarithmic representation of all IL-6 data

(in pg/mL), 2C shows a logarithmic representation of the sIL-6R (in ng/mL) and 2D of sgp130 (in ng/mL). All figures show the healthy controls compared to the CD patients, who were divided into three groups depending on their activity score: a, active disease [Crohn's Disease Activity Index (CDAI) > 150]; ea, extremely active disease (CDAI > 250) and i, inactive disease (CDAI ≤ 150). In addition, significant differences between active and inactive disease (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), as well as between active or inactive disease and controls (#, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$), can be seen. Figure 2A shows a significant difference of $p < 0.001$ between the healthy controls and inactive, active and extremely active disease. Furthermore, there is a significant difference of $p < 0.001$ between active/extremely active and inactive disease, all according to disease activity.

In Figures 2C and 2D, it is possible to observe a minor increase in sIL-6R and sgp130 levels however, both are independent of disease activity.

The investigation of all patients according to their CRP levels, is shown in Figure 3. In this case only the two groups with extreme IL-6 levels (Figure 3A; ordinal representation) were compared: Those below the detection limit and those above the third quartile; the disease activity was divided into: a, active disease (CRP > 5 mg/L) and i, inactive disease (CRP ≤ 5 mg/L). The remaining descriptions are the same as in Figure 2.

When the ordinal IL-6 levels and the disease activity measured by CRP were compared, a significant difference of $p < 0.001$ between the patients with active or inactive disease and the controls, as well as between those with active and inactive disease, was noted. Furthermore, there was a significant difference of $p < 0.05$ between the active/inactive disease group and the controls in terms of sIL-6R levels (Figure 3C) and the same difference between the inactive disease group and healthy controls in terms of sgp130 levels (Figure 3D).

The analysis of the disease activity score (Figure 2) and CRP (Figure 3) showed an association between elevated IL-6 levels and disease activity which was more noticeable with CRP than with CDAI (Figures 2A and 3A). The IL-6 levels were higher in patients with extremely active

disease (“ea” in Figure 2; CDAI> 250) than for all active patients taken together (“a” in Figure 2; CDAI>150). However, it should be mentioned that more than 50% of all patients (with inactive and active disease) did not show any IL-6 elevation at all (Table 4; Figure 2A).

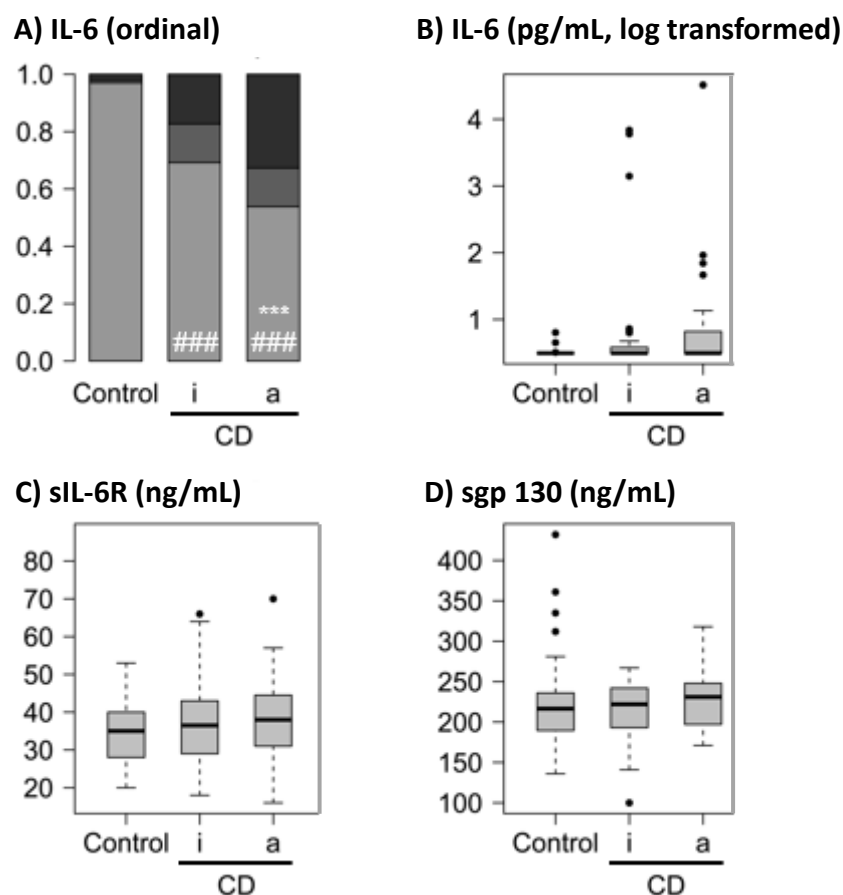
There was a slight, apparent elevation of sIL-6R levels in line with CDAI (Figure 2C) and a more obvious one in comparison to CRP levels (Figure 3C), but this was not statistically significant and did not correlate with the disease activity.

b) IL-6, sIL-6R, sgp130 levels in subcohorts with matched measurements

Due to the variable number of measurements per patient in the complete dataset, a reduced dataset was created to investigate whether this factor and the large number of patients with inactive disease, contributed to the drawing of possibly false conclusions from the complete dataset (Table 4).

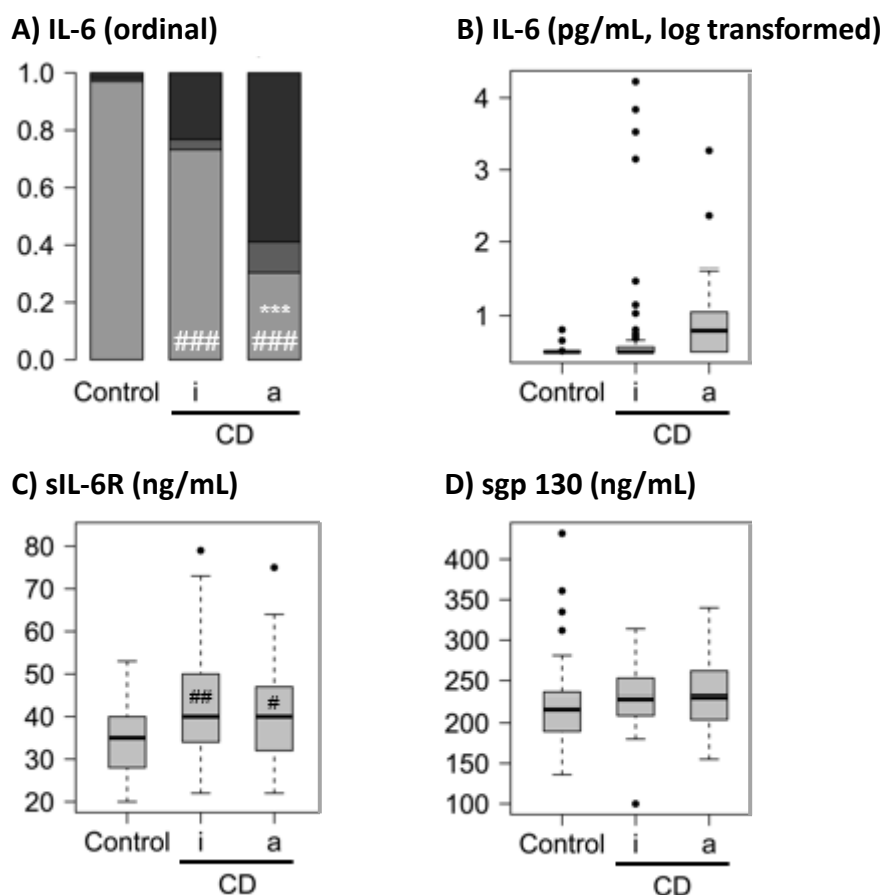
Only patients with at least one time point for an active and one time point for an inactive disease were included in the reduced data set. In cases which had more than one time point, only the two most extreme time points were included (the highest and the lowest CDAI or CRP levels) and analysed (Figures 4 and 5).

Figure 4



IL-6, sIL-6R and sgp130 serum levels in healthy controls and in selected patients with Crohn's disease (CD), differentiated according to disease activity scores. Only patients with at least one time point with active and one with inactive disease were included. If more than one time point per condition was available, only data from the two most extreme time points (highest and lowest disease activity score) were included. (A) Ordinal representation of IL-6 levels across all groups: below the 3.13 pg/mL detection limit (light grey), between the detection limit and the third quartile (grey) and above the third quartile (black). For significance analyses, the two extreme groups (those below the detection limit and those above the third quartile) were compared. (B) Logarithmic representation of IL-6 levels. (C) sIL-6R levels. (D) sgp130 levels. a, active disease [Crohn's Disease Activity Index (CDAI) > 150]; i, inactive disease (CDAI ≤ 150). Significant differences between active and inactive disease: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Significant differences between active or inactive disease and controls: #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$.

Figure 5



IL-6, sIL-6R and sgp130 serum levels in healthy controls and in selected patients with Crohn's disease (CD) differentiated according to C-reactive protein (CRP) levels. Only patients with at least one time point with CRP > 5 mg/L and one time point with CRP ≤ 5 mg/L were included. If more than one time point per condition was available, only data from the two most extreme time points (highest and lowest CRP levels) were included. (A) Ordinal representation of IL-6 levels across all groups: below the 3.13 pg/mL detection limit (light grey), between the detection limit and the third quartile (grey) and above the third quartile (black). For significance analyses, the two extreme groups (those below the detection limit and those above the third quartile) were compared. (B) Logarithmic representation of IL-6 levels. (C) sIL-6R levels. (D) sgp130 levels. a, active disease (CRP > 5 mg/L); i, inactive disease (CRP ≤ 5 mg/L). Significant differences between active and inactive disease: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Significant differences between active or inactive disease and controls: #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$.

Figures 4 and 5 represent the comparison between the reduced dataset and the disease activity score (CDAI), and with the CRP levels. Significant differences of $p < 0.001$ are shown in IL-6

levels, not only between active or inactive disease and the controls, but also between active and inactive disease. Moreover Figure 5 shows a significant difference of $p < 0.01$ between inactive disease and the controls, and of $p < 0.05$ between active disease and the controls in the comparison between sIL-6R levels and CRP levels.

There was no significant difference between the results obtained with the full data set and those from the reduced data set (Figures 4 and 5; cf. Figures 2 and 3), proving the accuracy of the results.

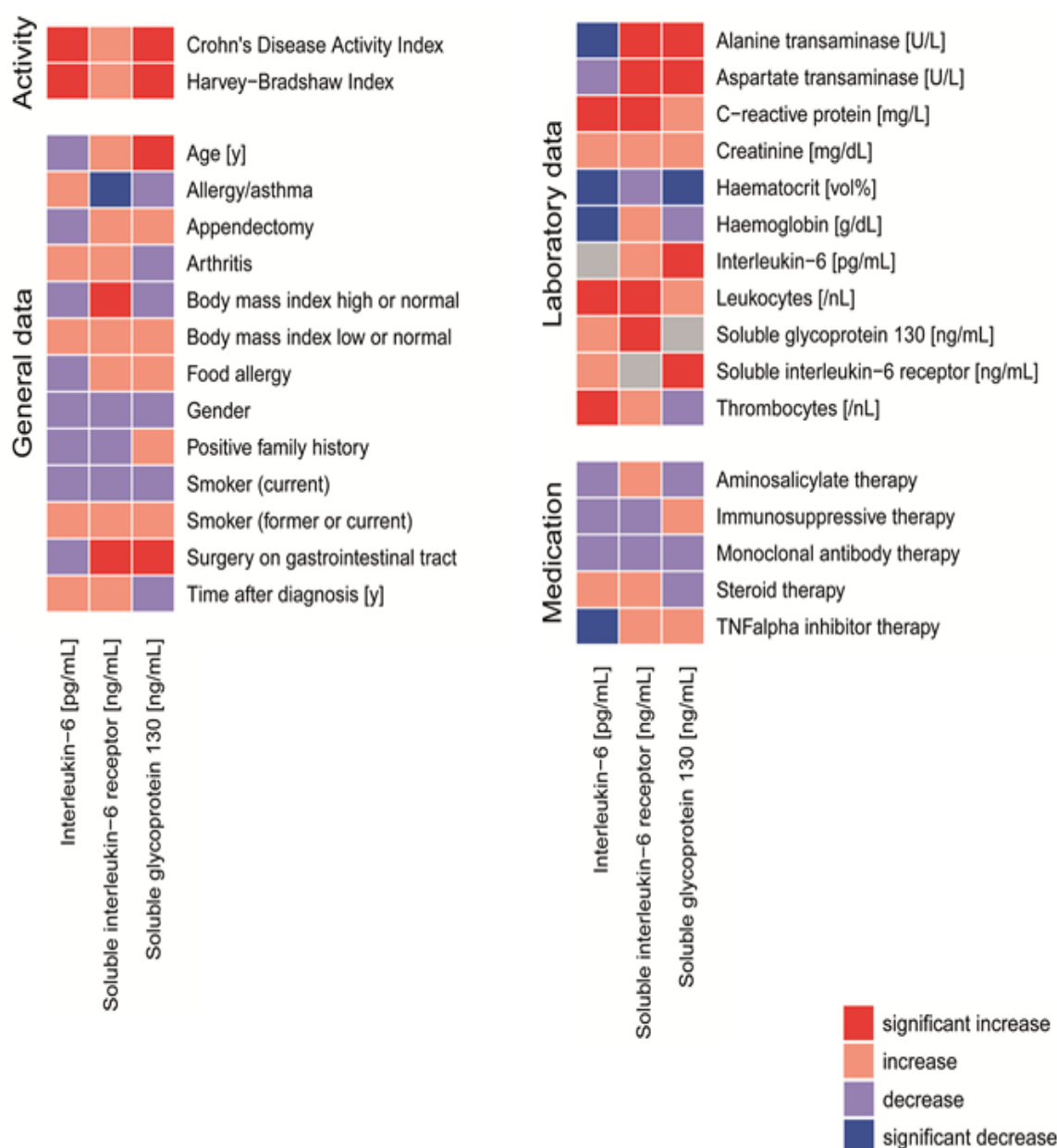
To summarize, a biologically and statistically significant increase in IL-6 levels was observed in association to the CDAI (Figures 2A and 4A) and CRP (Figures 3A and 5A). The association with the CDAI, the increase was only observed in a minority of patients with active CD (Figures 2A and 4A). Regarding the CRP, the majority of patients with active disease showed significant elevation of IL-6 blood levels. No significant increase in sIL-6R and gp130 was observed in this study.

c) Association of IL-6, sIL-6R and sgp130 levels with general, clinical and laboratory parameters

The parameters which were investigated for association with IL-6, sIL-6R and sgp130 are all listed in Table 2 and the results are presented in Figure 6.

Figure 6 is a heat map in which the associations are visualized in colour: dark red indicates a significant increase, red an increase, blue a decrease and dark blue a significant decrease in the parameters indicating a positive or negative association. Grey fields show a parameter with only one characteristic value. The complete data and the p-values for the significance analyses are presented in the supplementary tables: for ordinal IL-6 levels in Supplementary Table 1, sIL-6R levels in Supplementary Table 2 and sgp130 in Supplementary Table 3.

Figure 6



Association heat maps for Crohn's disease (CD; A). Tendentially or significantly positive (increase) or negative (decrease) associations between ordinal interleukin-6, soluble interleukin-6 receptor or soluble glycoprotein 130 serum levels and other parameters are visualised by colour code. For the complete data and p-values, see Supplementary Tables 1–3. Grey fields indicate parameters with only one characteristic value or self-association.

Supplementary Table 1: Correlation of ordinal interleukin-6 levels with general, clinical and laboratory parameters

Data group	Parameter	Crohn's disease (CD)		
		OR	95% CI	P-value
General data	Age at sampling [y]	0.51	0.20 - 1.31	0.15
	Allergy/asthma	1.72	0.45 - 6.49	0.43
	Appendectomy	0.28	0.06 - 1.41	0.11
	Arthritis as a comorbidity	1.02	0.48 - 2.17	0.96
	Body mass index (BMI) high ($BMI \geq 25$) or normal ($18.5 \leq BMI < 25$)	0.88	0.35 - 2.19	0.78
	Body mass index low (< 18.5) or normal ($18.5 \leq BMI < 25$)	1.63	0.38 - 7.08	0.51
	Food allergy	0.88	0.25 - 3.11	0.84
	Gender	0.76	0.28 - 2.07	0.59
	Positive family history of inflammatory bowel diseases	0.41	0.13 - 1.30	0.13
	Smoker (current)	0.70	0.27 - 1.82	0.46
	Smoker (former or current)	1.43	0.58 - 3.53	0.44
	Surgery on gastrointestinal tract	0.56	0.23 - 1.39	0.2
	Time after diagnosis [y]	1.00	0.40 - 2.52	1
Activity (CD)	Crohn's disease activity index (CDAI)	4.18	2.07 - 8.43	2.2e-05
	Harvey-Bradshaw Index (HBI)	4.28	2.13 - 8.59	1.2e-05
Laboratory data	Alanine transaminase (ALT) [U/L]	0.28	0.14 - 0.54	7.7e-05
	Aspartate transaminase (AST) [U/L]	0.60	0.33 - 1.09	0.092
	C-reactive protein (CRP) [mg/L]	22.53	10.39 - 48.85	7e-21
	Creatinine [mg/dL]	1.20	0.63 - 2.29	0.58
	Haematocrit [vol%]	0.28	0.14 - 0.54	0.00014
	Haemoglobin [g/dL]	0.27	0.14 - 0.53	0.00012
	Leukocytes [/nL]	5.34	2.74 - 10.38	1.1e-07
	Soluble glycoprotein 130 [ng/mL]	1.46	0.57 - 3.73	0.43
	Soluble interleukin-6 receptor [ng/mL]	1.05	0.54 - 2.05	0.87
	Thrombocytes [/nL]	4.72	2.43 - 9.15	3.1e-06
Medication	Aminosalicylate therapy	0.56	0.13 - 2.36	0.42
	Immunosuppressive therapy	0.68	0.16 - 2.86	0.59
	Monoclonal antibody therapy, except for tumour necrosis factor-alpha (TNF- α) inhibitors	0.57	0.19 - 1.77	0.33
	Steroid therapy	1.31	0.46 - 3.75	0.62
	TNF-α inhibitor therapy	0.21	0.21 - 0.21	0.014

Bold font: significantly correlated ($p < 0.05$).

N/a, not applicable.

Supplementary Table 2: Correlation of soluble interleukin-6 receptor levels with general, clinical and laboratory parameters

Data group	Parameter	Crohn's disease (CD)			
		Beta	95% CI	R ²	P-value
General data	Age at sampling [y]	1.09	-1.38 - 3.56	0.0021	0.39
	Allergy/asthma	-3.83	-6.53 - 1.14	0.0083	0.0054
	Appendectomy	1.65	-1.83 - 5.13	0.0018	0.35
	Arthritis as a comorbidity	0.10	-1.30 - 1.50	1.7e-05	0.89
	Body mass index (BMI) high (BMI ≥ 25) or normal (18.5 ≤ BMI < 25)	3.75	1.56 - 5.95	0.025	0.00084
	Body mass index low (< 18.5) or normal (18.5 ≤ BMI < 25)	1.90	-0.72 - 4.51	0.0051	0.15
	Food allergy	1.75	-0.71 - 4.21	0.0024	0.16
	Gender	-0.92	-3.98 - 2.15	0.0014	0.56
	Positive family history of inflammatory bowel diseases	-0.34	-3.94 - 3.26	0.00017	0.85
	Smoker (current)	-0.55	-3.22 - 2.12	0.00049	0.69
	Smoker (former or current)	0.58	-1.77 - 2.94	0.00061	0.63
	Surgery on gastrointestinal tract	2.98	0.77 - 5.20	0.015	0.0084
	Time after diagnosis [y]	1.33	-1.08 - 3.74	0.0031	0.28
Activity (CD)	Crohn's disease activity index (CDAI)	0.93	-0.27 - 2.14	0.0015	0.13
	Harvey-Bradshaw Index (HBI)	0.43	-0.74 - 1.60	0.00033	0.47
Laboratory data	Alanine transaminase (ALT) [U/L]	2.55	1.43 - 3.67	0.012	9.2e-06
	Aspartate transaminase (AST) [U/L]	1.23	0.18 - 2.29	0.0027	0.022
	C-reactive protein (CRP) [mg/L]	1.66	0.45 - 2.87	0.005	0.0072
	Creatinine [mg/dL]	0.76	-0.46 - 1.98	0.001	0.22
	Haematocrit [vol%]	-0.53	-1.87 - 0.81	0.00051	0.43
	Haemoglobin [g/dL]	0.27	-1.06 - 1.61	0.00013	0.69
	Interleukin-6 [pg/mL]	0.36	-0.13 - 0.85	0.0011	0.15
	Leukocytes [/nL]	1.20	0.11 - 2.29	0.0026	0.031
	Soluble glycoprotein 130 [ng/mL]	3.95	2.57 - 5.32	0.032	3.6e-08
	Thrombocytes [/nL]	0.89	-0.40 - 2.17	0.0014	0.18
Medication	Aminosalicylate therapy	0.99	-3.04 - 5.03	0.0011	0.63
	Immunosuppressive therapy	-2.94	-6.82 - 0.93	0.0072	0.14
	Monoclonal antibody therapy, except for tumour necrosis factor-alpha (TNF-α) inhibitors	-0.68	-3.15 - 1.80	0.00043	0.59
	Steroid therapy	0.93	-1.64 - 3.50	0.0017	0.47
	TNF-α inhibitor therapy	1.11	-1.24 - 3.46	0.0011	0.35

Bold font: significantly correlated ($p < 0.05$).
CI, confidence interval; n/a, not applicable.

Supplementary Table 3: Correlation of soluble glycoprotein 130 levels with general, clinical and laboratory parameters

Data group	Parameter	Crohn's disease (CD)			
		Beta	95% CI	R ²	P-value
General data	Age at sampling [y]	17.61	2.89 - 32.33	0.038	0.019
	Allergy/asthma	-10.95	-33.63 - 11.73	0.0056	0.34
	Appendectomy	22.07	-4.99 - 49.13	0.021	0.11
	Arthritis as a comorbidity	-3.73	-13.36 - 5.90	0.0017	0.45
	Body mass index (BMI) high (BMI ≥ 25) or normal (18.5 ≤ BMI < 25)	-4.93	-19.75 - 9.88	0.0031	0.51
	Body mass index low (< 18.5) or normal (18.5 ≤ BMI < 25)	1.84	-17.21 - 20.89	0.00022	0.85
	Food allergy	11.98	-5.23 - 29.19	0.0082	0.17
	Gender	-4.47	-22.24 - 13.30	0.0023	0.62
	Positive family history of inflammatory bowel diseases	2.92	-17.61 - 23.45	0.00084	0.78
	Smoker (current)	-7.41	-23.80 - 8.97	0.0061	0.37
	Smoker (former or current)	12.41	-3.88 - 28.69	0.019	0.13
	Surgery on gastrointestinal tract	24.75	10.50 - 39.00	0.071	0.00071
	Time after diagnosis [y]	-8.62	-24.85 - 7.62	0.0092	0.3
Activity (CD)	Crohn's disease activity index (CDAI)	9.96	1.28 - 18.64	0.013	0.025
	Harvey-Bradshaw Index (HBI)	10.69	2.13 - 19.25	0.015	0.015
Laboratory data	Alanine transaminase (ALT) [U/L]	11.71	3.85 - 19.58	0.017	0.0036
	Aspartate transaminase (AST) [U/L]	9.07	1.82 - 16.32	0.011	0.014
	C-reactive protein (CRP) [mg/L]	2.28	-5.88 - 10.45	0.00065	0.58
	Creatinine [mg/dL]	3.96	-4.17 - 12.10	0.002	0.34
	Haematocrit [vol%]	-11.70	-20.60 - -2.80	0.017	0.01
	Haemoglobin [g/dL]	-4.82	-14.16 - 4.53	0.0029	0.31
	Interleukin-6 [pg/mL]	4.08	0.74 - 7.42	0.011	0.017
	Leukocytes [/nL]	1.95	-5.56 - 9.47	0.00048	0.61
	Soluble interleukin-6 receptor [ng/mL]	27.20	18.92 - 35.47	0.085	4e-10
	Thrombocytes [/nL]	-2.82	-11.65 - 6.01	0.001	0.53
Medication	Aminosalicylate therapy	-0.49	-22.23 - 21.25	2.4e-05	0.96
	Immunosuppressive therapy	8.39	-17.28 - 34.07	0.005	0.52
	Monoclonal antibody therapy, except for tumour necrosis factor-alpha (TNF-α) inhibitors	-5.25	-28.16 - 17.65	0.0018	0.65
	Steroid therapy	-1.69	-16.90 - 13.51	0.00043	0.83
	TNF-α inhibitor therapy	0.33	-15.83 - 16.48	7.6e-06	0.97

Bold font: significantly correlated ($p < 0.05$).
CI, confidence interval; n/a, not applicable

For ordinal IL-6 levels, a significant positive association was detected with the disease activity scores: CDAI (OR 4.18; 95%CI 2.07-8.43; p 2.2e-05) and HBI (OR 4.28; 95%CI 2.13-8.59; p 1.2e-05). The laboratory parameters representing inflammation and disease activity consistently showed significant association with the IL-6 levels. Specifically, CRP (OR 22.53; 95%CI 10.39-48.85; p 7e-21), leucocytes (OR 5.34; 95%CI 2.74-10.38; p 1.1e-07) and thrombocytes (OR 4.72; 95%CI 2.43-9.15; p 3.1e-06) were significantly increased while haematocrit (OR 0.28; 95%CI 0.14-0.54; p 0.00014) and hemoglobin (OR 0.27; 95%CI 0.14-0.53; p 0.00012) were significantly decreased. Moreover, significantly reduced IL-6 levels were found in patients who were being treated with anti-inflammatory therapy, in this case tumour necrosis factor-alpha (TNF- α) inhibitors (OR 0.21; 95%CI 0.21-0.21; p 0.014); (Figure 6, Supplementary Table 1).

A significant positive association was found between sIL-6R and the patient's body mass index, BMI (Beta 3.75; 95%CI 1.56-5.95; R^2 0.025; p 0.00084). Moreover, sIL-6 levels were significantly increased in patients with a history of gastrointestinal surgeries (Beta 2.98, 95%CI 0.77-5.20; R^2 0.015; p 0.0084) and the raised levels were also positively associated with inflammatory markers CPR (Beta 1.66; 95%CI 0.45-2.87; R^2 0.005; p 0.0072) and leucocytes (Beta 1.2; 95%CI 0.11-2.19; R^2 0.0026; p 0.031); (Figure 6, Supplementary Table 2).

The CDAI and HBI disease activity scores were significantly positively associated not only with the IL-6 levels, but also with the sgp130 levels (CDAI: Beta 9.96; 95%CI 1.28-18.64; R^2 0.013; p 0.025; HBI: Beta 10.69; 95%CI 2.13-19.25; R^2 0.015; p 0.015). In addition, there was an association between a significant increase in sgp130 levels and the age of the patients (Beta 17.61; 95%CI 2.89-32.33; R^2 0.038; p 0.019) and also with the previous history of gastrointestinal tract surgeries (Beta 24.75; 95%CI 10.5-39; R^2 0.071; p 0.00071). A significant negative association was noted with the haematocrit level (Beta -11.7; 95%CI -20.6- -2.8; R^2 0.017; p 0.01); (Figure 6, Supplementary Tables 1 and 3).

Furthermore, a significantly positive association was observed between the sIL-6R serum levels and sgp130 (Figure 6, Supplementary Tables 2 and 3), representing the slightly increased IL-6 buffer (Figures 1-5, Table 5). In addition, the IL-6 levels were significantly associated with the sgp130 serum levels (Beta 4.08; 95%CI 0.74-7.42; R^2 0.011, p 0.017); (Figure 6, Supplementary Table 3).

The final association was seen between serum levels of the liver enzymes AST and particularly ALT, and the parameters measured in this study: ALT was negatively associated with IL-6 levels (OR 0.28; 95%CI 0.14-0.54; p 7.7e-05) and positively with both sIL-6R (Beta 2.55; 95%CI 1.43-3.67; R^2 0.012; p 9.2e-06) and sgp130 levels (Beta 11.71; 95%CI 3.85- 19.58; R^2 0.017; p 0.0036). The AST levels showed similar changes but were of lower significance (Figure 6, Supplementary Table 1-3).

To summarize, IL-6, sIL-6R and sgp130 levels are mainly associated with clinical and laboratory parameters and the therapy for CD, and therefore represent the different stages of disease activity. The significantly negative association of IL-6 and the significantly positive associations of sIL-6R and sgp130 with the liver enzymes, is a new finding, which to my knowledge has not yet been described in the literature.

4) Discussion

The main purpose of this study was to determine whether IL-6, sIL-6R and sgp130 were suitable as biomarkers in Crohn's disease. The aim was to explore whether or not these laboratory parameters play a role in the disease's clinical diagnostics, especially because of the contradictory findings in the literature which show either a significant increase or decrease in the single parameters, or no association with CD at all [49-55]. My research has confirmed the previous findings of overall elevated IL-6 levels in patients with CD, particularly in those with active disease [49-50, 52-55].

Table 5 Circulating levels of IL-6, sIL-6R and sgp130 in patients with Crohn's disease (selected publications)

Publications	Groups	IL-6 (pg/mL)	sIL-6 (ng/mL)	sgp130 (ng/mL)	Selected associations (p < 0.05) ^a
Mahida et al. 1990 (52)	Healthy controls (n=16) Active CD (n= 21)	< 20 (<20-30) [median (range)] 47 (<20-250)	n.d. n.d.	n.d. n.d.	IL-6 & albumin, no association with CRP
Hyams et al. (50)	Healthy controls (n=29) Pediatric CD (n=49)	0.4 ± 0.2 [mean ± SEM] 4 ± 1b	n.d. n.d.	n.d. n.d.	Significant association between IL-6 and disease activity
Mitsuyama et al. 1995 (49)	Healthy controls (n=69) Inactive CD (n= 9) Active CD (n= 15)	n.d. [mean ± SEM] 5 ± 4 16 ± 12c	107 ± 2 114 ± 7 142 ± 9b, c	n.d. n.d. n.d.	(+) IL-6 & CPR; (+) IL-6 & platelet counts; sIL-6R & CRP
Reinisch et al. 1999 (63)	Healthy controls (n=32) Inactive CD (n= 44) Mod. active CD (n=44) ^d Highly active CD (n=48)	< 1.3 (<1.3) [median (range)] 1.3 (1.3-8) b 3 (1.3- 35) b, c 6 (1.3- 35) b, c	n.d. n.d. n.d. n.d.	n.d. n.d. n.d. n.d.	(+) IL-6 & disease activity (in primarily inflammatory CD patients); (+) IL-6 & CRP; (+) IL-6 & oromucosoid
Gustot et al. 2005 (53)	Healthy controls (n=15) Inactive CD (n= 20) Active CD (n= 30)	2 (0-4) [median (range)] 3 (0-7) 15 (0-69) b, c	33 (21-50) 29 (20-50) 38 (31-56) c	273 (213-375) 278 (229-449) 220 (138-310) b, c	(+) IL-6 & CRP; (+) sIL-6 & CRP; (-) sgp130 & CRP
Mitsuyama et al. 2006 (51)	Healthy controls (n=17) Inactive CD (n= 10) Active CD (n= 13)	n.d. (only paired analyses with selected samples) n.d. n.d.	n.d. (see IL-6) n.d. n.d.	43 (27-59) [median (IQR)] 63 (44-83) 66 (44-88)	n.d.
Nancey et al. 2008 (54)	Healthy controls (n=20) Inactive CD (n= 50) Active CD (n= 44)	3 ± 1 [mean ± SEM] 50 ± 4b 80 ± 9 b, c	58 ± 6 82 ± 5b 77 ± 5b	n.d. n.d. c	(+)IL-6 & CRP, but not sIL-6R & CRP
Mitsuyama et al. 1991 (55)	Healthy controls (n=61) Inactive CD (n= 20) Active CD (n= 9)	n.d. 4-84 7-10	n.d. n.d. n.d.	n.d. n.d. n.d.	(+) CRP & disease activity

All numbers above 1 were rounded to the nearest integer.

^aPositive (+) or negative (–) associations between the indicated parameter combinations. ^bp < 0.05 or less vs healthy controls.

^cp < 0.05 or less vs inactive disease.

^dModerately active: Crohn's Disease Activity Index (CDAI) 150–250; highly active: CDAI >250.

CD, Crohn's disease; IL-6, interleukin-6; IQR, interquartile range; n, number of individuals per group; n.d., not determined; SEM, standard error of the mean; sgp130, soluble gp130; sIL-6R, soluble interleukin-6 receptor.

However, in this study, only 50% of the patients with active disease showed elevated IL-6 levels in serum. The median serum IL-6 levels were not elevated in patients with either active or inactive disease, compared to the healthy controls (based on disease score). These results were contradictory to some small previous studies' findings, in which the IL-6 levels were elevated in active and inactive disease. Moreover, the elevation ranges in those studies were narrower than those in my study [52-55].

A positive correlation was found between the activity scores (CDAI and HBI) and the IL-6 serum levels, confirming the results of previous studies [53, 55, 65]. However, it is difficult to use the CDAI score as a parameter for inflammatory activity, since it measures the degree of illness in CD and includes subjective characteristics, such as pain and general well-being.

The IL-6 serum levels might be different to those in the inflamed intestinal mucosa, since the blood taken from patients has always passed through the liver on the way from the intestine to the sampling site. As we know, the liver is one of the largest sources and consumers of IL-6 in the body. This is different for patients with, for example, rheumatoid arthritis, for whom the samples were taken directly from the inflamed joints where the local IL-6 levels might be higher than in the blood circulation. Therefore, one can speculate that high levels of IL-6 found in biopsies taken from an inflamed intestine [53-55, 64-66] may not lead to IL-6 levels in blood serum being increased to the same extent.

Similar results are found in CD patients' TNF- α serum levels; depending on the study, the TNF- α either seems to be elevated in patients with active disease [67] or there are no differences between CD patients' serum levels and those of controls [68]. Nevertheless, there is a strong, proven increase in mucosal TNF- α -producing cells and TNF- α levels in the stool of patients with active disease [69-70]. Furthermore, there is a well-established and highly effective therapy in CD using several different anti-TNF- α antibodies, which shows very good symptom

control and an improvement in well-being and disease activity in more than 60% of the patients treated with it [71].

With regard to sIL-6R, only patients with active CD showed an increase in serum levels, confirming the findings of the small pilot study by Gustot et al. (2005) [53] and the general knowledge of sIL-6R levels in the healthy population and in patients suffering from chronic inflammation [25]. In contrast to my results, Nancey et al. (2008) [54] and particularly Mitsuyama et al. (1995), [49] measured higher overall levels.

The negative association between sIL-6R levels and allergies/asthma found in my study, contradicted the findings in the literature. Ullah et al. (2015) discovered that blocking trans-signaling in the IL-6 cascade leads to the suppression of certain asthma types, in particular, the mixed granulocytic type. Moreover, sputum obtained from asthma patients contained high IL-6 and sIL-6R levels which only correlated weakly with these parameters' levels in the serum. [72]. These findings are in accordance with those of previous studies which show higher IL-6 and sIL-6R levels at the location of the inflammation, for example, inflamed intestinal mucosa in CED and inflamed joints in rheumatoid arthritis [53-55, 64-66].

A positive association between sIL-6R levels and the body mass index (BMI), confirmed the study findings reported by Kraakman et al. (2015). They show that trans-signaling is the main pathway responsible for recruiting macrophages to adipose tissue and inducing obesity [73].

Overall, higher spg130 levels were observed for active CD in the current study, however no significant difference between active and inactive disease was apparent. Once again, these findings confirmed the results reported by Gustot et al. (2005) [53] and the general consensus on spg130 levels [25], but showed overall higher spg130 levels than those detected by Nancy et al. (2008) [54].

It is hard to determine the reason for the deviations in sIL-6R and sgp130 levels in all these studies, however, they do not appear to play a significant role in the pathogenesis of Crohn's disease.

This study confirmed the majority of the findings reported in the literature concerning general, clinical and laboratory parameters and showed positive associations between IL-6 levels and disease activity, CRP, leukocytes and platelet levels [49-50, 53, 55, 63]. Regarding the therapy, significantly reduced IL-6 levels were only seen in those patients who were treated with TNF- α inhibitors, no other medication influenced the IL-6 serum levels. These findings were already known from the literature [52-53].

This study's novel finding was the negative and significant association between the levels of the liver enzymes AST, and particularly ALT, and the components of the IL-6 system. High ALT levels were simultaneously associated with reduced IL-6 levels and high sgp130 and sIL-6R levels. Similar but less significant, associations were shown for AST serum levels. The majority of the studies carried out previously, have shown elevated ALT or AST levels in CD, which are mainly transient and related to inflammatory bowel disease-associated liver diseases [74-79]. However, there are also contradictory results in the literature; Vadstrup (2004) detected decreased ALT levels in CD patients in his study [80]. These findings might contradict the popular belief that IL-6 trans-signaling plays a pro-inflammatory role in the inflammation of intestinal mucosa, but they are consistent with research from animal studies on IL-6 and liver injuries. The studies by Klein et al. (2005), Lam et al. (2010) and Malchow et al. (2011), showed that IL-6 has a protective function following liver injury, the cytokine seems to trigger hepatic differentiation, regeneration and wound healing [81-83]. The results of this large cohort study imply that there is more to the interaction between the IL-6 system and the liver enzyme levels, than is currently known. Further research is needed to determine the role of IL-6 in CD patients' livers.

To summarize, the present study surprisingly shows that only a minority of the CD patients have elevated IL-6 serum levels, however, IL-6 is a reliable biomarker of disease activity in these patients. At present, measuring IL-6 in serum for CD patients is not to be recommended in clinical practice. However, IL-6 serum level measurements might be useful for identifying patients who could profit from drugs targeting IL-6 signaling pathways. SIL-6R and sgp130 are not useful as biomarkers in CD.

5) Supplementary material

Supplementary Table 4: Statistical data for Figure 1a

Ordinal groups for interleukin-6	Control	Crohn's disease
Values < detection limit (3.13 pg/mL)	0.97	0.59
detection limit \leq values \leq 3 rd quartile	0.01	0.13
3 rd quartile < values	0.02	0.28

Supplementary Table 5: Statistical data for Figure 1b–d

Variables	Control	Crohn's disease
Interleukin-6 (logarithmic; pg/mL)	0.5 (0.5–0.8)	0.5 (0.5–4.51)
Soluble interleukin-6 receptor (ng/mL)	35 (20–53)	38 (14–93)
Soluble glycoprotein 130 (ng/mL)	217 (136–432)	228 (92–385)

All values represent median (range) and were rounded to the nearest integer.

Supplementary Table 6: Statistical data for Figure 2

Active vs controls					Inactive vs controls			Inactive vs active			Enhanced activity vs controls			Inactive vs enhanced activity		
Var.	Dis.	Est.	95% CI	<i>p</i>	Est.	95% CI	<i>p</i>	Est.	95% CI	<i>p</i>	Est.	95% CI	<i>p</i>	Est.	95% CI	<i>p</i>
IL-6 o.	CD	0.38	0.28 - 0.5	< 0.001	0.21	0.14 - 0.28	< 0.001	0.29	0.13 - 0.54	< 0.001	0.54	0.41 - 0.66	< 0.001	0.50	0.23 - 0.77	< 0.001
sIL-6R	CD	1.00	-3 - 7	0.37	5.00	2.5 - 9	0.002	3.00	0 - 5	0.044	0.00	-4 - 6	1	2.00	-3 - 6	0.36
sgp130	CD	15.50	1 - 33	0.024	10.50	-5.5 - 23	0.16	2.00	-11 - 19	0.77	15.50	-12 - 46	0.19	8.00	-14 - 40	0.57

All data except for small *p* values were rounded to the second decimal place.

Bold font, *p* value < 0.05.

95% CI, 95% confidence interval; CD, Crohn's disease; Dis., disease; Est., estimate; IL-6 o., ordinal data for interleukin-6; sgp130, soluble glycoprotein 130; sIL-6R, soluble IL-6 receptor.

Supplementary Table 7: Statistical data for Figure 3

Active vs. controls					Inactive vs. controls			Inactive vs. active		
Variable	Disease	Estimate	95% CI	<i>p</i> value	Estimate	95% CI	<i>p</i> value	Estimate	95% CI	<i>p</i> value
IL-6 ordinal	CD	0.58	0.46 - 0.67	< 0.001	0.13	0.07 - 0.19	< 0.001	0.42	0.26 - 0.59	< 0.001
sIL-6R	CD	3.00	0 - 8	0.016	3.00	0 - 8	0.03	0.00	-2 - 2	0.96
sgp130	CD	6.50	-6 - 19	0.32	15.50	2 - 28	0.022	3.00	-11 - 21	0.68

All data except for small *p* values were rounded to the second decimal place.

Bold font, *p* value < 0.05.

95% CI, 95% confidence interval; CD, Crohn's disease; IL-6, interleukin-6; sgp130, soluble glycoprotein 130; sIL-6R, soluble IL-6 recepto

Supplementary Table 8: Statistical data for Figure 4

Variable	Disease	Active vs. controls			Inactive vs. controls			Inactive vs. active		
		Estimate	95% CI	<i>p</i> value	Estimate	95% CI	<i>p</i> value	Estimate	95% CI	<i>p</i> value
IL-6 ordinal	CD	0.36	0.22 - 0.5	< 0.001	0.18	0.06 - 0.31	< 0.001	0.32	0.12 - 0.57	< 0.001
sIL-6R	CD	3.00	-1.5 - 8	0.15	1.50	-3 - 7	0.51	2.00	-2 - 4	0.22
sgp130	CD	14.50	-20 - 26	0.33	5.50	-19 - 20	0.82	12.00	-30 - 50	0.56

All data except for small *p* values were rounded to the second decimal place.

Bold font, *p* value < 0.05.

95% CI, 95% confidence interval; CD, Crohn's disease; IL-6, interleukin-6; n/a, not applicable; sgp130, soluble glycoprotein 130; sIL-6R, soluble IL-6 receptor.

Supplementary Table 9: Statistical data for Figure 5

Variable	Disease	Active vs. controls			Inactive vs. controls			Inactive vs. active		
		Estimate	95% CI	<i>p</i> value	Estimate	95% CI	<i>p</i> value	Estimate	95% CI	<i>p</i> value
IL-6 ordinal	CD	0.64	0.51 - 0.77	< 0.001	0.22	0.1 - 0.34	< 0.001	0.44	0.24 - 0.66	< 0.001
sIL-6R	CD	5.00	1 - 8.5	0.012	5.00	1 - 12	0.006	-1.00	-5 - 2	0.33
sgp130	CD	14.50	-2 - 37	0.068	12.50	-2 - 34	0.074	2.50	-22 - 26	0.65

All data except for small *p* values were rounded to the second decimal place.

Bold font, *p* value < 0.05.

95% CI, 95% confidence interval; CD, Crohn's disease; IL-6, interleukin-6; sgp130, soluble glycoprotein 130; sIL-6R, soluble IL-6 receptor.

Supplementary Table 10: Associations of interleukin-6, soluble interleukin-6 receptor and soluble glycoprotein 130 levels with disease location

Disease	Location	IL-6 (ordinal data)		sIL-6R		sgp130	
		OR	<i>p</i> value	Effect est.	<i>p</i> value	Effect est.	<i>p</i> value
CD	Stomach to proximal ileum affected	1.65	0.36	1.11	0.002	0.18	< 0.001
CD	Terminal ileum affected	3.45	0.016	-1.34	0.003	-2.67	< 0.001
CD	Colon affected	1.86	0.13	-1.32	0.002	-16.63	0.019

All data except for small *p* values were rounded to the second decimal place.

Bold font, *p* value < 0.05.

CD, Crohn's disease; Effect est., estimated effect size; IL-6, interleukin-6; OR, odds ratio; sgp130, soluble glycoprotein 130; sIL-6R, soluble IL-6 receptor.

6) Summary

Background: The aim of the study was to determine whether IL-6, sIL-6R and sgp130 were suitable as biomarkers in Crohn's disease. To date, the published data on this topic originates from studies with small cohorts and the results are contradictory. I evaluated the levels of IL-6, sIL-6R and sgp130 as markers for disease activity in a large cohort of Crohn's disease patients recruited from an outpatient clinic.

Methods: A total of 815 measurements from 212 patients with confirmed Crohn's disease were included. General, clinical and laboratory parameters were collected and monitored, including the patients' disease activity status. The data used as controls were obtained from 100 age-matched, healthy blood donors.

Results: The majority of the measurements showed IL-6 serum levels below the detection limit. However, a small cohort had IL-6 levels which were significantly elevated in active versus inactive disease, and also in comparison to the healthy controls. The positive associations with thrombocytes, leukocytes and C-reactive protein serum levels known from previous research, were confirmed in this study.

The serum concentrations of sIL-6R and sgp130 were only slightly elevated and showed no statistical significance.

Moreover, a negative association was found between IL-6 levels and serum alanine transaminase (ALT) and aspartate transaminase (AST) while sIL-6 and sgp130 were positively associated with ALT and AST.

Conclusions: Only a small proportion of patients with Crohn's disease show elevated IL-6 levels in their blood samples. However, in these patients, the IL-6 is strongly associated with the disease activity and can be used as a biomarker. According to these results, sIL-6R and sgp130 do not appear to play a role in clinical practice.

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