Usefulness and analytical performances of complement multiplex assay for measuring complement biomarkers in plasma

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Abstract

Introduction. The complement system is involved in numerous diseases, through diverse mechanisms and degree of activation. With the emergence of complement targeting therapeutic, simple and accessible tools to evaluate the extent of complement activation are strongly needed.

Methods. We evaluated two multiplex panels, measuring complement activation fragments (C4a, C3a, C5a, Bb, Ba, sC5b9) and intact components or regulators (C1q, C2, C3, C4, C5, FD, FP, FH, FI). The specificity of each measurement was assessed by using complement proteins depleted sera and plasma collected from patients with complement deficiencies. Normal values distribution was estimated using 124 plasma samples from healthy donors and complement activation profile was assessed in plasma collected from 31 patients with various complement-mediated disorders.

Results. We observed good inter-assay variation. All tested protein deficiencies were accurately detected. We established assay-specific reference values for each analyte. Except for C3, C4 and C4a, the majority of the measurements were in good agreement with references methods or published data.

Conclusion. Our study substantiates the utility of the Complement Multiplex assay as a tool for measuring complement activation and deficiencies. Quantifying complement cleavage fragments in patients exhibiting classical or alternative pathway activation allowed evaluating the activation state of the whole cascade.

Key words: Complement, biomarkers, therapeutic, ELISA

Highlights

- The complement system is implicated in 50+ diseases with varied mechanisms of activation of deficits.
- Accurately assessing the level of complement activation remains a challenge.
- Quantifying complement activation fragments and protein could provide an instantaneous snapshot of the cascade's activation state.
- We presented the use of complement multiplex ELISA for simultaneously measuring 14 complement proteins.
- The data presented here pave the way for the utilization of the multiplex ELISA technique for quantifying complement proteins and fragments.

Revised manuscript (clean version) Click here to view linked References

Usefulness and analytical performances of complement multiplex assay for measuring complement biomarkers in plasma

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Methods. We evaluated two multiplex panels, measuring complement activation fragments (C4a, C3a, C5a, Bb, Ba, sC5b9) and intact components or regulators (C1q, C2, C3, C4, C5, FD, FP, FH, FI). The specificity of each measurement was assessed by using complement proteins depleted sera and plasma collected from patients with complement deficiencies. Normal values distribution was estimated using 124 plasma samples from healthy donors and complement activation profile was assessed in plasma collected from 31 patients with various complement-mediated disorders.

Results. We observed good inter-assay variation. All tested protein deficiencies were accurately detected. We established assay-specific reference values for each analyte. Except for C3, C4 and C4a, the majority of the measurements were in good agreement with references methods or published data.

Conclusion. Our study substantiates the utility of the Complement Multiplex assay as a tool for measuring complement activation and deficiencies. Quantifying complement cleavage fragments in patients exhibiting classical or alternative pathway activation allowed evaluating the activation state of the whole cascade.

Key words: Complement, biomarkers, therapeutic, ELISA

Abbreviations:
C3G: C3 glomerulopathy
EIA: Enzyme Immunoassay
FD: Factor D
FH: Factor H
FI: Factor I
FP: Factor P or Properdin
LP: complement lectin pathway
SLE: systemic lupus erythematosus
sC5b-9: soluble C5b-9

1. Introduction

In 2023, the complement system is recognized to be involved in over fifty pathologies, through diverse mechanisms and degrees of activation [1–10]. Nevertheless, accurately assessing the level of complement activation in each pathology, remains a challenge.

The complement system is organized into a complex enzymatic cascade with numerous protein interactions [11]. Throughout the activation process of this cascade, inactive proteins, circulating in the bloodstream, are sequentially cleaved and activated, leading to the assembly of enzymatic complexes (convertases). These convertases then generate complement cleavage fragments that either deposit on tissues or are released into the circulation. Consequently, the complement activation results in the consumption of specific proteins (i.e., C3 and/or C4), the deposition of complement on tissues (i.e. C3b, C4d, C5b-9), and the release of C3 and C5 activation fragments (C3a, C5a) that carry biological activity. Notably, these activation fragments not only promote inflammation at the site of complement activation by participating in immune cells activation and recruitment but also in opsonization, phagocytosis and lysis, thus contributing to the destruction of pathogens and abnormal cells [11].

Both an excess and a deficiency in complement activation can be pathological. Therefore, the complement exploration in plasma aims to disclose acquired or inherited protein deficiencies, to detect autoantibodies to complement proteins or to identify indirect evidences of complement activation (such as the consumption of C3 and C4 or an elevation in plasma soluble C5b9 (sC5b-9) levels)[12].

Currently, the routine search for complement activation relies solely on the identification of consumption of the two main proteins, C3 and C4, by their quantification. Nevertheless, evaluating the extent of complement activation only based on decreased C3 and/or C4 plasma levels poses several challenges. Indeed, each complement protein undergoes independent synthesis and catabolic processes, which can also considerably vary under pathological conditions (i.e. inflammation) [13] and across individuals. In addition, each complement protein, along with its activation fragments are rapidly metabolized *in vivo* and have their own half-life. For example, in systemic lupus erythematosus (SLE), variation in C3 or C4 levels can be the result of inflammation, consumption or deficiency, making them very difficult to interpret[14]. In the context of research work, other measurements of complement

proteins or cleavage fragments can be carried out, but never exhaustive enough to fully understand the activation of the entire cascade.

Hemolytic assays have been proposed to assess complement activity or to study the function of specific steps in the cascade [15–17]. However, these experiments, that necessitate a high technical expertise, are tricky to establish and to standardize and some reagents are difficult to obtain. More recently, the emergence of transcriptomic and proteomic tools has yielded valuable insights into the involvement of complement in various disease conditions [18]. However, while these methods offer crucial information about changes in gene transcription and protein expression, they do not assess protein functions and are still primarily confined to the domain of research. The unique dynamic activation process within the complement cascade underscores that measuring complement activation at the protein level is the only reliable way to accurately evaluate the extent of complement cascade activity in a particular pathology.

Quantifying complement activation fragments will provide a more accurate picture of the true state of cascade activation. Monoclonal antibodies designed for specific neoepitopes on these fragments have the potential to expand our ability to map the complement system more comprehensively and to detect and characterize an ongoing or recent complement activation.

Over the past decade, there has been a surge in the development of numerous complement inhibitors, highlighting the necessity for new biomarkers to guide their utilization and ensure their effectiveness in biological contexts[19–24]. It has now become imperative to validate a dependable and robust method for quantifying complement proteins and cleavage fragments, suitable for both research and routine applications.

Complement multiplex ELISA now provides the possibility of simultaneously measure intact complement proteins and their activation fragments at different levels of the cascade.

This study aimed to evaluate the performance of a multiplex ELISA in identifying complement protein deficiencies or cascade activation. Additionally, we established assay-specific reference values for each analyte and discussed the relevance and interpretability of complement assessment in plasma using this multiplex approach.

2. Methods

2.1. Specimen collection

EDTA plasma samples were collected from healthy donors and patients, sent to the laboratory on ice in less than 3 hours, centrifuged for 5 minutes at 4000 rpm, +4°C. Plasma was distributed in at least 4 polypropylene tubes and aliquots were immediately frozen at -80°C until assayed. All samples selected for the validation of the MicroVue Complement Multiplex assays were thawed extemporaneously for the experiment, without any additional 'freeze-thaw' cycle.

Plasma EDTA samples from healthy donors were tested for the validation and the calculation of reference values (n=124, from COVID-HOP clinical trial, NCT: 04418375). Plasma EDTA samples from patients with complement-mediated diseases (n=31) were tested to evaluate the suitability of this assay in pathological conditions. The 31 patients with a complement-mediated disease were patients with an inherited or acquired complement disorder predominantly affecting the classical pathway (n=7), the alternative pathway (n=23) or the terminal pathway (n=1) (detailed in **Table 1**). For comparison analysis to routinely used methods, two additional collections of plasma EDTA samples from 17 patients with a clear cell renal cell carcinoma and 54 patients with C3 glomerulopathy (C3G) were used (see below).

2.2. Use of complement depleted sera

Commercial sera (ComptTech®) depleted of complement proteins C1q, C2, C3, C4, C5, FB, factor D (FD), factor H (FH), factor I (FI) and properdin/factor P (FP) were tested to validate the sensitivity of detection for complement protein deficiencies.

2.3. MicroVue Complement Multiplex assays

The MicroVue Complement Multiplex assays (Quidel Ortho®, A900) are designed to measure multiple complement proteins within the same biological sample (up to 8 analytes per well) based on the ELISA technique. We tested two panels evaluating multiple complement analytes in two independent 96-well plates. Panel 1 measured the complement activation fragments and regulators: C3a, C5a, C4a, Ba, Bb, sC5b-9, FH and FI. Panel 2 included the intact complement proteins: C1q, C2, C3, C4, C5, FD and FP (the contribution of each component in the cascade is detailed in **Figure 1**).

For the assay, samples were thawed in batch and stored on ice until their deposition in the microplate. Plasma samples were diluted 1:100 in specimen diluent (1X) for the measurement of panel 1 and 1:1000 for the measurement of panel 2 and added onto the plate (50 µL/well), according to the manufacturer's instructions. Calibrators (n=6) and high/low controls were diluted in specimen diluent and deposited extemporaneously onto the plates (50 µL/well). The negative control for background subtraction consisted of 50 µL of specimen diluent (supplemented by a FH/FI competitor for the Panel 1 kit). Each sample was deposited in one well and each experimental condition was tested once. After 120 ± 10 minutes incubation, plates were washed three times with wash buffer (1X) (300 µL/well). Each well was then incubated with 50 µl of the detection mix for 60 ± 10 minutes. After 3 additional washes, the bound analyte was revealed by an enzymatic reaction using 50 µl of streptavidin horseradish peroxidase incubated for 20 ± 1 minutes and washed six times before the addition of 50 µl of substrate. The chemiluminescence was then read extemporaneously on a dedicated automated system (Q-View™ Imager LS). The intensity of the spots corresponding to each analyte was measured and the concentrations were evaluated according to the calibrator lot number of each kit using the Qview software®. Negative subtraction was used to correct any background intensity.

2.3. Inter-assay variability

Aliquots of the same plasma sample from healthy donors (N=20 for panel 1, N= 8 for panel 2) were tested in two independent experiments to assess the inter-assay variability for each analyte. This included also calibrators from the same lot (n=2 lots of 6 calibrators for each panel).

2.4. Comparison to reference methods

We conducted a comparative analysis using aliquots of the same plasma sample from individuals from diverse groups. These samples were subjected to testing using the Microvue Multiplex Complement assays and also evaluated using ELISA or immunonephelometry assays, which are routinely used for complement assessments in our laboratory.

Comparison of plasma C3 and C4 levels were carried out using 54 samples, including 16 healthy donors, 17 patients presenting with clear cell renal cell carcinoma and 21 patients

with complement-mediated diseases. The measurements were performed by the routinely used immunonephelometric assay (Atellica™ NEPH 630, Siemens).

Comparison of FH, FI and sC5b-9 measurements were conducted using 74 samples, comprising 54 samples from patients diagnosed with C3G for all 3 measurments and 15, 14 and 9 samples from patients with complement-mediated diseases for FH, FI and sC5b-9 measurement respectively. The Microvue Multiplex Complement assay was compared to routinely used in-house ELISA assays for FH and FI (as previously described in[25,26]) and the Microvue™ sC5b-9 Plus Enzyme Immunoassay (EIA) (Quidel®, A029) for sC5b-9 measurement (performed according to the manufacturer's procedure). FH and FI measurements were expressed as percentage relative to measurements obtained from a pool of healthy donors. To standardize the comparison with the reference method, FH and FI measurements obtained with Microvue Multiplex Complement assay were normalized with the same strategy (expressed as percentage of measurements from 41 healthy donors). FH or FI deficiency was defined for percentages below 65% of concentrations observed in healthy donors. The threshold of 300 ng/ml was used to define elevated sC5b-9 levels in the EIA sC5b-9 Plus Microvue™ assay (Quidel®, A029) (threshold based on measurement in 100 healthy donors).

2.5. Assay-specific references values in healthy donors and comparison to patients with complement classical or alternative pathway mediated diseases

The reference values for complement proteins measurements using the Microvue Multiplex Complement assay were established based on measurements obtained from 124 and 70 healthy donors (for panel 1 and panel 2, respectively). The reference values were determined as the mean $\pm$ 2 standard deviations of the respective complement proteins levels in healthy donors.

Subsequently, a comparison was conducted between complement protein measurements in healthy donors and those in patients with complement-mediated diseases (**Table 1**).

2.6. Statistical analysis

The data are expressed as either the median with interquartile range (IQR) or as the mean $\pm$ standard deviation (SD) for continuous variables, and as percentages for categorical variables.

The agreement between the measurement of FH, FI, sC5b-9, C3 and C4 by the reference technique and the Multiplex Complement assay was assessed using a Bland-Altman analysis, with the calculation of the limits of agreement corresponding to the 95% confidence intervals (IC95%). The agreement between the reference method and the Microvue Multiplex Complement assay for the assessment of FH and FI deficiency and sC5b-9 elevation was evaluated through kappa coefficient calculation. Spearman's correlation or Pearson were used as appropriate to assess the correlation between i) the measurement of each analyte using different techniques and ii) replicate measurements of each analyte on aliquots of the same sample. Variation coefficients were calculated to assess interassay variability. High and low controls from the same lot were tested in independent experiments and values obtain used to calculate variation coefficients to assess reproducibility. Reference values of complement analytes in healthy donors were defined as the mean $\pm$ 2SD of the EDTA plasma concentration of all the 124 healthy donors tested (n=70 for panel 2). All statistical analyses were performed using R (version 4.2.1) with the ggplot and pheatmap packages or GraphPad Prism software (v8).

3. Results

3.1. Assay evaluation: inter assay variability, comparison to references methods and use of complement depleted sera

3.1.1 Inter-assay variability

Reproducibility was assessed by calculating coefficient of variation of high and low controls from the same lot. Six individual experiments were performed with the same lot for measurement of panel 1 (**Supplemental Table 1**), and coefficient of variation were all between 7 and 25%. Three individual experiments were performed with the same lot for measurement of panel 2 (**Supplemental Table 2**), and coefficient of variation were comprized between 4 and 26%. To assess the variability of the MicroVue Complement Multiplex assays, we performed measurements on two separate aliquots of the same plasma sample from healthy donors. These measurements were carried out in two independent experiments for each analyte, with a sample size of n=20 for panel 2 and n=8 for panel 1. Additionally, measurements were taken using two calibrators lots, each including calibrators 1 to 6. The spearman correlation

coefficients obtained for each analyte were all above 0.70 (ranges: 0.70-0.95). The coefficients of variation were comprised between 10 and 26%. The mean of coefficient of variation, correlation coefficient, and p-value for each analyte can be found in **Figure 2.**

3.1.2. Comparison to reference methods

The measurement of 5 analytes included in the MicroVue Complement Multiplex assay is routinely performed in our laboratory by other methods. Therefore, we were able to compare the results obtained using the multiplex method with those obtained using routine methods for specific samples.

FH, FI and sC5b-9 measurements are routinely performed by ELISA. Correlation coefficient for FH, FI and sC5b-9 levels were 0.84, 0.55 and 0.68, respectively. Kappa coefficient for the assessment of FH and FI deficiencies and sC5b-9 elevation were 0.77, 0.21 and 0.55 for FH, FI and sC5b-9, respectively (**Figure 3**).

C3 and C4 quantification are routinely performed by immunonephelometry. When considering all samples with both measurements available, we found no correlation between the two techniques (**Figure 4 A**). When considering separately a first group combining healthy donors and cancer patients without complement-mediated diseases in the correlation, the correlation coefficients were 0.61 and 0.47 for C3 and C4 measurements (**Figure 4 B**). When considering only patients with complement-mediated diseases, correlation coefficients were 0.46 and 0.43 for C3 and C4 measurements (**Figure 4 C**). Bland & Altman analysis showed a systematic bias in higher values. (**Figure 4D)**.

3.1.3. Complement depleted sera and plasma from deficient patients

To assess the capacity of MicroVue Complement Multiplex assay to detect complement protein deficiencies, we tested commercially available sera that had been depleted of C1q, C2, C3, C4, C5, FB, FD, FH, FI and FP, as well as 4 sera samples from healthy donors. In all 10 depleted sera, we found very low or undetectable measurements of the corresponding depleted complement protein (**Figure 5**).

We further examined 25 samples from patients previously known to carry complement protein deficiencies (mainly linked to heterozygous or homozygous documented deficiencies, except for 2 CFH deficiencies without documented genetic background and one CFH-CFHR1 hybrid without CFH deficiency) (**Figure 6**). In all patients with homozygous deficiency, the

respective protein was systematically found at the lower limit of detection (C1q, C2, C5, FP, FH and FI). Samples from patients carrying heterozygous deficiency (C1q, C2, FH, FI) also exhibited low levels of the deficient protein. As excepted, FH measurement in sample carrying CFH-CFHR1 hybrid did not demonstrated any decreased FH measurement.

3.2. Assay-specific reference values in healthy donors

A total of 124 samples from healthy donors were tested on panel 1 and 70 on panel 2 to calculate the assay-specific reference values for each complement protein or activation fragment. The distribution of plasma concentrations measured for each analyte in healthy donors and the detection range is shown in **Figure 7.**

We studied the distribution of the reference values, according to the standard curves for each assay, to analyze their capacity to detect an increase and/or decrease concentration for each protein/fragment. The data is summarized in **Table 2** and showed that all standard curves covered largely the distribution of the normal values allowing valuable use for detecting decreased or increased levels of the complete proteins, except for C3 and C4. For the activation fragments, the normal values being low, the standard curves were adapted for the measurement of increase levels except for C4a which normal values covered all the measuring range.

3.3. Complement proteins and fragments concentrations in patients with alternative or classical pathways activation

We focused on assessing the levels of intact proteins susceptible to be decreased in case of classical pathway activation (C1q, C2, C3, C4 and C5 consumption) or alternative pathway activation (C3 and C5 consumption) and the dosage of the cleavage fragments generated along the cascade. In most patients with alternative pathway activation, we found an elevation of fragments Ba, Bb, C3a, C5a and sC5b-9 quantification (**Figure 8A**) whereas, in most patients with classical pathway activation, we found a combined elevation of only C3a and C5a fragments. However, we detect only 4 samples with intact C5 consumption in alternative pathway activation group (**Figure 8B**). Intact C3 and C4 measurements were even increased in both alternative pathway and classical pathway activation groups. Visual representation of fragments dosage (Ba, Bb, C3a, C5a and sC5b-9) demonstrating an elevation in samples with complement activation compared to controls are presented in **Figure 9**.

4. Discussion

We hereby report the first study focusing on the performance of multiplex ELISA allowing the simultaneous quantification of complement proteins and cleavage fragments, conducted using samples from both healthy donors and patients presenting with diseases involving complement activation at various levels. The aim of the present work was not to perform a complete performance validation procedure as requested by the ISO 15189, but to address the main points useful when deciding the use of a new assay. Therefore, we have focused on the evaluation of the assay's specificity (thanks to the use of depleted serum and plasma from patients with a known deficiency), inter-assay variation, reliability (by comparison with assay methods used in routine) and ability to identify the activated pathway in a pathological context.

The specificity of the different measurements has been validated by using both depleted sera (for C1q, C2, C3, C4, C5, FB, FD, FH, FI and FP) and plasma samples collected from patients with complement protein deficiencies (FH, FI, FP, C1q, C2, C5). Thus, these assays can be employed for detecting complete deficiencies for these specific proteins.

Even if the inter-assay variation evaluation on human samples has been performed in only two experiments, the test exhibited good performance. The correlation coefficients were all above 0.7, and the coefficients of variation ranged from 10 to 26%. Nevertheless, it is important to contextualize this latter coefficient of 26% which was obtained for the measurement of sC5b-9. This coefficient was calculated from values that were uniformly very low, all originating from healthy donors. Overall, these metrics fall within the performance ranges obtained with ELISA technology [27]. The reproducibility, here evaluated only on internal quality controls (IQC) provided by the kit on 6 experiments for panel 1 and 3 experiments for panel 2, has to be more precisely determined on larger series, eventually with the use of any appropriate sample serving as IQC.

When a reference method was available within our laboratory, we conducted a comparison between the results obtained using the multiplex ELISA and those obtained with the reference method. The comparison was highly satisfactory for FH and sC5b-9, with high kappa coefficients. The kappa coefficient was lower for the categorisation of lower *vs* normal FI, mainly due to false low FI with multiplex ELISA, but with a good categorisation of the only

deficient sample tested with the two methods. Importantly, the multiplex ELISA accurately identified both homozygous and heterozygous deficiencies of FH and FI.

When available in the literature, the ranges of values obtained from samples collected from healthy donors were found to be comparable to those previously reported for C1q, FH and FI [28]. For C2 and FD, the assay allowed detection of deficiency, however reference values appeared to differ from available commercial assays (ELISA, radial immunodiffusion or turbidimetric assays), indicating the absence of standardization among assays [29,30]. Concerning properdin measurement, we observed significant heterogeneity in levels obtained in healthy donors. We speculated that this variability might be attributed to gender differences and the phenomenon of X chromosome inactivation[31,32] but the gender of the healthy donors was not available.

The relationship between C3 and C4 measurements obtained using the multiplex ELISA and those obtained by immunonephelometry was different in samples taken from patients with complement-mediated pathologies and those taken from healthy donors or patients without complement mediated disease. Notably, there was no C3 consumption (i.e., decrease levels) despite 10 samples with low C3 identified on nephelometry measurement. We have even identified an increase in samples from patients with classical pathway activation (active SLE, cryoglobulinemia, C1 Inhibitor deficiency). These discrepancies might be explained by epitope specificity of the antibodies used for protein capture and detection. Regarding C3 measurement, the monoclonal antibodies used in the design of the multiplex assay were directed against C3a and C3c whereas in immunonephelometry, the antibodies were polyclonal and directed against C3c. Further in-depth analysis is required to understand these unexpected results, notably by studying more precisely the epitopes recognized by the different antibodies.

The simultaneous combination of activation fragment measurements for C3a, C4a, Ba, Bb, C5a, and sC5b-9 in a single test is particularly advantageous from a technical standpoint.

This may avoid the need to store multiple aliquots per sample to avoid freeze-thaw cycles which significantly affect the accuracy of complement fragment measurements. The time cost is thereby reduced, also due to the decrease in the number of experiments to be conducted. Indeed, the complement exploration and notably the measurement of activation fragments encounters different difficulties or pitfalls primarily related to the pre-analytic conditions. These conditions include the sample matrix (e.g., for blood: plasma EDTA, plasma citrate,

serum), the conditions of sample transportation (time and temperature) and of sample storage (time, temperature).

The values obtained for Ba, Bb, C3a, C5a and sC5b-9 from samples collected from healthy donors were similar to those previously reported [29,31].

There is a growing number of pathologies for which the assessment of precise complement activation becomes essential, in order to choose the appropriate therapeutic strategy. Yet the available analyses remain limited in routine practice as well as in research laboratories[33,34]. The simultaneous measurement of activation fragments provides an instantaneous snapshot of the cascade's activation state, thereby helping to address this challenge. In our study, the values obtained with samples collected from patients with complement-mediated diseases allowed a good classification of the activated pathway (classical or lectin *vs* alternative pathway). Indeed, we observed higher levels of Ba and Bb fragments in patient samples with alternative pathway-mediated diseases as compared to samples from healthy donors and from patients with classical pathway-mediated diseases. We also noticed higher values for C5a and sC5b-9 in this group of patients, whereas C3a values were elevated in both patient groups as compared to healthy donors. These observations in patients' samples illustrate the importance of the alternative pathway amplification loop for the generation of C5 convertases and the activation of the terminal complement pathway. This emphasizes the importance of simultaneously measuring various activation fragments at different levels of the cascade.

However, interpreting the C4a levels among the different donor groups appears to be challenging. In fact, the values observed in the samples of healthy donors were widely dispersed, covering all the measuring ranges. These C4a values are comparable to those previously reported in plasma from kidney transplanted recipients without comparison with healthy donors [6] but differ from another older study that used a radioimmunoassay[35]. The physiopathological significance of this marker still remains to be demonstrated, for now its effector functions have mainly been studied *in vitro* [36]. The availability of different monoclonal antibodies specific to complement activation fragment neoepitopes has opened up new avenues of research in the complement exploration field. Notably it is now possible to assess the presence of fragments complement deposition or generation *in situ* in different experimental and clinical situation such as in kidney diseases[37], infections[38] cancer[39] and organ or hematopoietic stem cell transplantation[40,41]. While these new tools have

improved our understanding of the pathophysiology of various disease conditions, they also highlight the necessity for the development of non-invasive biomarkers.

Quantifying complement fragments in blood, urine, or other bodily fluids can serve as one of such non-invasive biomarkers which has the potential to change the diagnosis and the management of numerous complement-mediated diseases. In routine clinical practice, the use of the multiplex ELISA may allow grouping the relevant analytes to be measured according to the clinical context. For example, simultaneous dosages of FH, FI, along with the fragments sC5b-9, Bb, would be very effective for the diagnosis and the monitoring of patients suspected from atypical haemolytic uremic syndrome or C3G [42,43]. In addition, with the emergence of new drugs targeting the complement system, there is an increasing need for soluble biomarkers as companion tests to select and monitor patients who would benefit from such new treatments [44].

Our study has several limitations. First, our access to donors demographic data was very restricted, preventing any interpretation of the measurements in relation to their age or gender. Additionally, we only analyzed 31 patient samples with complement-mediated disorders. Therefore, the represented pathology panel is limited. For a routine use, a complete validation following ISO 15189 is needed.

5. Conclusion

In conclusion, the data presented here pave the way for the utilization of the multiplex ELISA technique for quantifying complement proteins and fragments. We have observed good test inter assay variation. Measurements of FH, FI, and sC5b-9 were in good agreement with references methods. However, measurements of C3 and C4 should be interpreted with better knowledge of their biological significance. Protein deficiencies were accurately detected by this assay. Lastly, quantifying complement cleavage fragments in patients exhibiting classical pathway or alternative pathway activation allowed capturing the activation state of the cascade as a whole.

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Declaration of interest

The authors declare to have no conflict of interest relative to this study.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used OpenAI- chatGPT in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Author contributions

Conceptualization: MADD; Experiments: AG, MRA, MD, JP, MR, PVM ; Data curation: MSM ; Formal analysis: MSM; Resources: MC, BP, DL, GF ; Supervision: MADD; Writing- original draft: MSM AD MADD ; and Writing - review & editing: AG, MRA,, LR, VFB, SC

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Figure legends

Figure 1: **The MicroVue Complement Multiplex assays used to measure multiple complement proteins in the same biological sample.** Panel 1 measured the complement activation fragments: C3a, C5a, C4a, Ba, Bb, soluble C5b-9 (sC5b-9) and the regulators factor H (FH) and factor I (FI) highlighted in orange. Panel 2 included the intact complement proteins: C1q, C2, C3, C4, C5, factor D (FD) and properdin/factor P (FP), highlighted in purple.
The complement system comprises more than 30 soluble and membrane-bound proteins. Three different pathways can lead to its activation: the classical (CP), lectin (LP), and alternative (AP) pathways. When activated, these serine protease cascades converge to the formation of enzymatic complexes, the C3 and C5 convertases, that cleave and activate C3 and C5, respectively. This allows the generation of the main effectors of this system. The anaphylatoxins C3a and C5a promote inflammation, the opsonins C3b/iC3b participate to pathogens opsonization, and C5b initiates the formation of the membrane attack complex (MAC) (C5b-9) that induces the lysis of pathogens or damaged cells. CP and LP are initiated by the recognition of pathogen-associated molecular patterns or damage-associated molecular patterns by pattern-recognition molecules (C1q and mannose-binding lectin, MBL, respectively). CP is mostly triggered by immune complexes containing IgG and IgM, recognized by C1q molecules. Further recruitment of C1r and C1s leads in C2 and C4 cleavage and classical C3 convertase formation (C4b2a). LP is initiated by mannose containing glycan, recognized by MBL or ficolins then activating mannose associated binding lectin associated serine proteases (MASPs), also leading to classical C3 convertase (C4b2a) formation. The formation of classical C3 convertases (C4b2a) accompanies with the release of C4a.
Conversely, AP is constantly activated at a low level in the fluid phase, by spontaneous hydrolysis of C3 in bioactive C3(H_20), allowing generation of small quantity of C3b. In the presence of an activating surface (such as apoptotic/necrotic cells and bacteria), C3b covalently binds to the surface. Thus, Factor B (FB) binds to C3b and is then cleaved by factor D (FD), with the release of Ba fragment resulting in the formation of an alternative C3 convertase (C3bBb) on the cell surface. Alternative C3 convertase (C3bBb) is stabilized by properdin. When the AP C3 convertase dissociates there is a release of Bb fragment. Alternative and classical C3 convertases cleave C3 in C3a and C3b. C3b can be used to form new C3 convertases, creating an amplification loop. Finally, the terminal pathway (TP) is common to all 3 complement pathways: C3 convertases are converted to C5 convertases by the association of several molecules of C3b and cleave C5 into C5a and C5b. C5b then associates with C6, C7, C8 and few molecules of C9 to form the membrane attack complex (MAC, or C5b-9). Soluble form of C5b-9 (sC5b-9) is generated on activation of complement, comprising basic MAC associated with regulatory protein clustering and or vitronectin.
To avoid self-aggression under physiological conditions, the complement system is tightly regulated in the circulation and on the host cell surface by a network of soluble (C4 binding protein C4BP, factor H (FH), factor I (FI) vitronectin and clusterin) and membrane-bound regulators (membrane cofactor protein (MCP) or CD46, complement receptor 1 (CR1) or CD35, decay accelerating factor (DAF) or CD55 and CD59).

Figure 2: **Multiplex Complement inter-assay variability assessment: inter-assay correlation of complement analytes measurements in replicates of EDTA plasma samples from healthy donors and calibrators.** Correlation coefficient was calculated with Spearman method.

Figure 3: **Comparison of the ELISA multiplex measurement of FH, FI and sC5b-9 with the reference ELISA used in routine.** (a) Correlation coefficient. Dashed lines show limits for normal values. (b) Bland-Altman plots. Mean measurement of both methods is plotted on the

X-axis. Difference in measurement (current-ELISA multiplex) is plotted on the Y-axis. Solid blue line shows the mean difference or bias in measurements of the assay. Green and red dashed lines show the upper and lower limits of agreement (LOA). Numerical values of bias and LOA (95% IC) are given in the table. (c) Kappa coefficient evaluating agreement of reference method and ELISA multiplex to assess FH and FI deficiency and sC5b-9 elevation.

Figure 4: **Comparison of intact C3 and C4 complement proteins measurement using the ELISA Multiplex Complement Assay and the nephelometric assay used in routine.** (a) Correlation coefficient in all tested samples (b) Correlation coefficient in non-complement mediated disease: healthy donors and patients with clear cell renal cell carcinoma (ccRCC). (c) Correlation coefficient in complement-mediated diseases. (d) Bland-Altman plots. Mean measurement of both methods is plotted on the X-axis. Difference in measurement (current-ELISA multiplex) is plotted on the Y-axis. Solid blue line shows the mean difference or bias in measurements of the assay. Green and red dashed lines show the upper and lower limits of agreement (LOA). Numerical values of bias and LOA (95% IC) are given in the table. Complement-mediated disease: "Alternative P activators " = 3 anti-FH Ab, 1 C3 Nephritic factor, 11 FH deficiency (2 without genetic background, 6 homozygote, 3 heterozygote), "Alternative P deficit" = 1 FP deficiency; "Classical P activators" = 1 C1 inhibitor deficiency; "Classical P deficit " = 2 C1q deficiency (1 homozygote, 1 heterozygote), 1 C2 deficiency; "Final P deficit" = 1 C5 deficiency . *Abbreviations*: P: pathway.

Figure 5: **Validation of the MicroVue Complement Multiplex assay for the detection of quantitative deficiencies in complement proteins with depleted sera.** Ten commercially available complement protein depleted sera and 4 sera from healthy donors were tested. Abbrevation: dpl : depleted

Figure 6: **Distribution of complement proteins plasma levels in patients with complement-deficiencies using the Microvue Complement Multiplex Assay**. Dashed lines show lower limit of range deducted from the 124 healthy controls tested (mean-2DS). *Abbreviations*: DefC1qHo: homozygote C1q deficiency, DefC1qHe: heterozygote C1q deficiency, DefC2Ho: homozygote C2 deficiency, DefC2He: heterozygote C2 deficiency, DefC5Ho: homozygote C5 deficiency, DefFPHo: homozygote FP deficiency, DefFHHo: homozygote FH deficiency, DefFHHe: heterozygote FH deficiency, DefFHWoGenet: FH deficiency without genetic FH background, DefFIHe: heterozygote FI deficiency, DefFIHo: homozygote FI deficiency, HD: healthy donor, HybrCFHCFHR1: hybrid CFH-CFHR1.

Figure 7: **Distribution of each complement analyte measured by the MicroVue Complement Multiplex assays in healthy donors.** Dashed lines show normal ranges deducted from the n= 70 healthy donors tested for C1q, C2, C3, C4, C5, FD and FP and from n=124 donors for C3a, Ba, Bb, C4a, C5a, sC5b-9, FH and FI measurements. Distributions values are given in Table 2. For each measurement, X axis limit corresponds to ELISA multiplex highest limit of detection.

Figure 8: **Distribution of complement proteins and fragments plasma levels in patients with diseases associated with a complement activation using the Microvue Complement Multiplex Assay**. Dashed lines show normal range deducted from the 124 healthy controls tested.
Abbreviations: AcDefC1inh: acquired C1 inhibitor deficiency, AFH: auto-antibody anti-FH, AP: alternative pathway, CP: classical pathway, Cryo: cryoglobulinemia, DefFHHe: heterezygote FH deficiency, DefFHWoGenet: FH deficiency without genetic FH background, DefFIHe: heterezygote FI deficiency, DefFIHo: homozygote FI deficiency, HD: healthy donor, HybrCFHCFHR1: hybrid CFH-CFHR1, SLE: systemic lupus erythematosus.

Figure 9: **Visual representation of complement fragment quantification in samples from healthy donors and patients with diseases associated with a complement activation**
Each row represents a sample and each column a measured analyte. Analytes concentrations were centered and scaled (by subtracting the mean and then dividing by the standard deviation) in the column direction for Z score visualization.

Tables

Table 1: Pathological samples from patients with complement-mediated diseases **(n=31)** tested for measurement of intact complement proteins and fragments by the MicroVue Complement Multiplex assay.

	Main complement		
expected effect on complement activation	**Alternative pathway (n=23)**	**Classical pathway (n=7)**	**Terminal pathway (n=1)**
Increased activation (n=25)	FH deficiency n= 16 (He/Ho/without genetic FH background n=6/8/2) Hybrid CFH-CFHR1 n=1 FI deficiency n= 2 (He/Ho, n=1/1) Anti-FH Aab n=3	Acquired C1 inhibitor deficiency n=1 Systemic Lupus Erythematosus n=1 Cryoglobulinemia n=1	
Defect in complement activation (n=6)	Properdin deficiency (Ho) n=1	C1q deficiency (Ho) n=2 C2 deficiency n=2 (He/Ho, n=1/1)	C5 deficiency (Ho) n=1

Aab: autoantibody; FH: Factor H, FI: Factor I; He: heterozygous; Ho: homozygous.

Table 2: Distribution of complement proteins and fragments concentrations in healthy individuals with MicroVue Complement Multiplex assays

	N samples	Median [IQR]	Extreme values Min - Max	Mean (SD)	mean+/ 2SD	ELISA multiplex upper and lower values of standard curve	Proposal interpretation of potential use: detection of …
C1q (ug/ml)	70	91.5 [58.6, 109.3]	34.5 - 226.9	93.8 (41.3)	11.1 - 176.5	2.1 - 1380	Increase & decrease
Intact C2 (ng/ml)	70	21142.9 [16443.9, 29157.6]	8078 - 48151	23027.5 (9502.9)	4021.7 - 42033.2	580 - 519660	Increase & decrease
Intact C3 (ug/ml)	70	395.5 [326.7, 542.5]	173.4 - 3080	493.5 (388.6)	0 - 1270.7	85 - 27470	Needs further investigations
Intact C4 (ug/ml)	70	53.9 [35.4, 78.8]	30.0 - 1053	86.6 (133.1)	0 - 352.8	23 - 13770	Needs further investigations
Intact C5 (ug/ml)	70	201.2 [151.5, 253.4]	107.3 - 370.0	205.8 (67.2)	71.5 - 340.1	1.6 - 790	Increase & decrease
Factor D (ng/ml)	70	831.1 [711.9, 984.6]	379.8 - 1586	859.9 (225.9)	408.1 - 1311.6	12 - 7490	Increase & decrease
Factor P (ng/ml)	70	30856.1 [23287.4, 50647.6]	17632 - 108672	39590.1 (21394.0)	0 - 82378.1	400 - 321800	Increase & decrease*
Factor H (ug/ml)	124	351.1 [298.6, 409.7]	174.4 - 654.0	359.5 (92.5)	174.6 - 544.5	13 – 1322	Increase & decrease
Factor I (ng/ml)	124	20971.5 [16665.7, 26144.5]	5218 - 59968	22767.2 (9344.9)	4077.4 - 41456.9	1928 - 88518	Increase & decrease
Ba (ng/ml)	124	451.2 [394.7, 550.7]	245.7 - 970.1	474.2 (126.4)	221.3 - 727.0	26 - 3039	Increase & decrease
Bb (ug/ml)	124	1.2 [0.9, 1.5]	0.4 - 3.1	1.3 (0.5)	0.2 - 2.4	0.19 - 26	Increase
C3a (ng/ml)	124	53.0 [37.7, 71.3]	21.0 - 270.2	66.5 (49.5)	0 - 165.5	19 - 10208	Increase
C4a (ng/ml)	124	1515.9 [763.4, 2337.2]	73.00 - 5807	1838.4 (1442.5)	0 - 4723.5	55 – 6832	Needs further investigations
C5a (ng/ml)	124	6.1 [4.8, 8.9]	1.0 - 35.8	7.1 (4.4)	0 - 15.9	0.74 - 348	Increase
sC5b-9 (ng/ml)	124	245.5 [159.2, 314.7]	127.0 - 685.8	253.79 (105.4)	43.0 - 464.5	127 - 62256	Increase

*Due to a significant standard deviation, the range of values reported as mean +/- 2SD includes 0. Nevertheless, it is possible to identify values lower than the normal values, since the minimum normal value was 17632 ng/ml, with a detection limit of 450 ng/ml.

Supplemental Material

Figure(s)
Click here to access/download;Figure(s);Fig1-R1-Meuleman et al.pdf

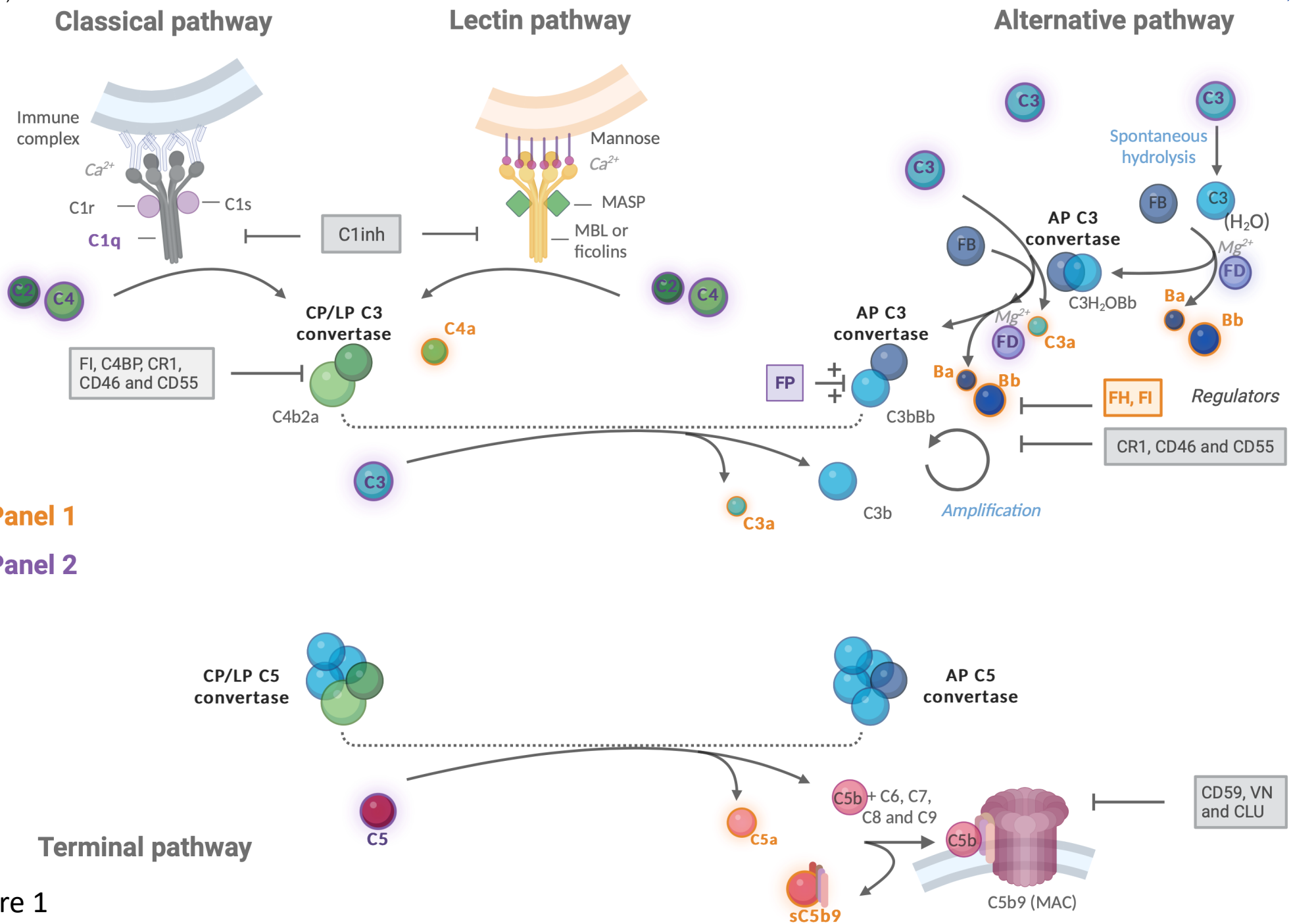

Figure 1

Figure(s) Click here to access/download;Figure(s);Fig2-R1-Meuleman et al.pdf

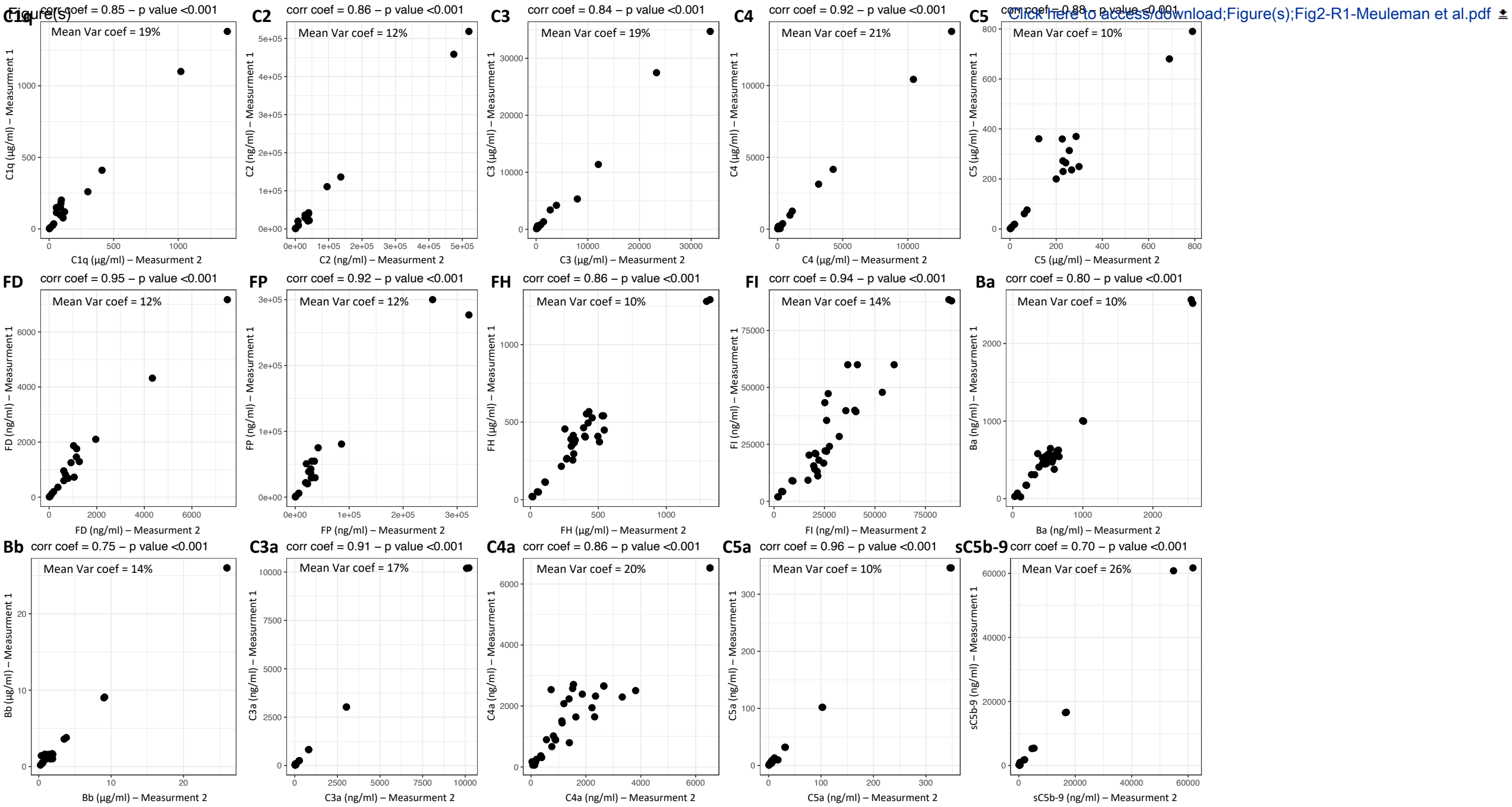

Figure 2

Figure(s)

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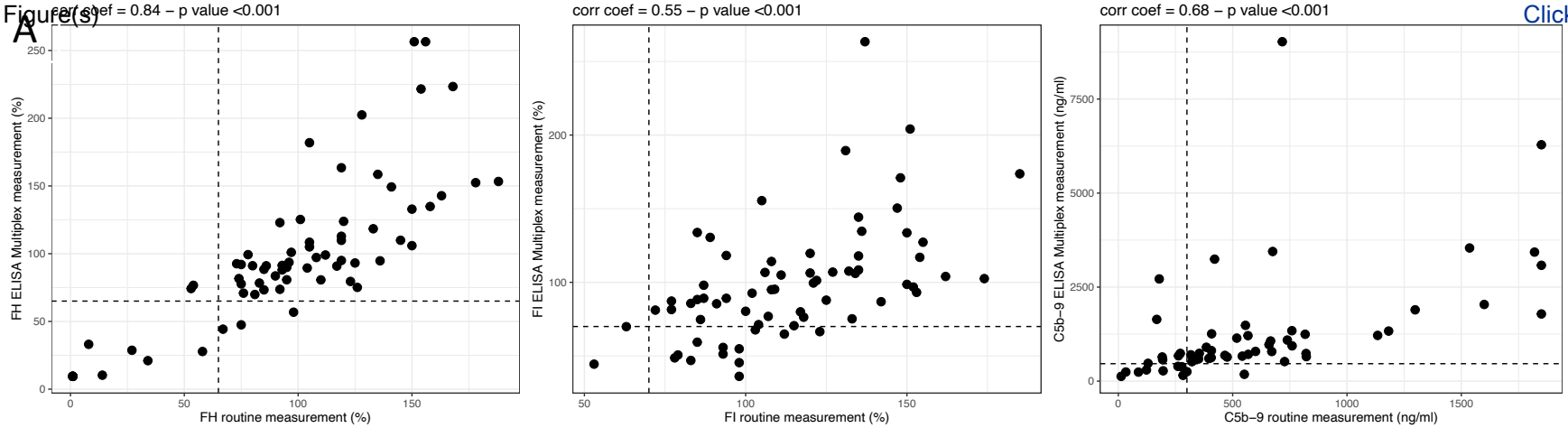
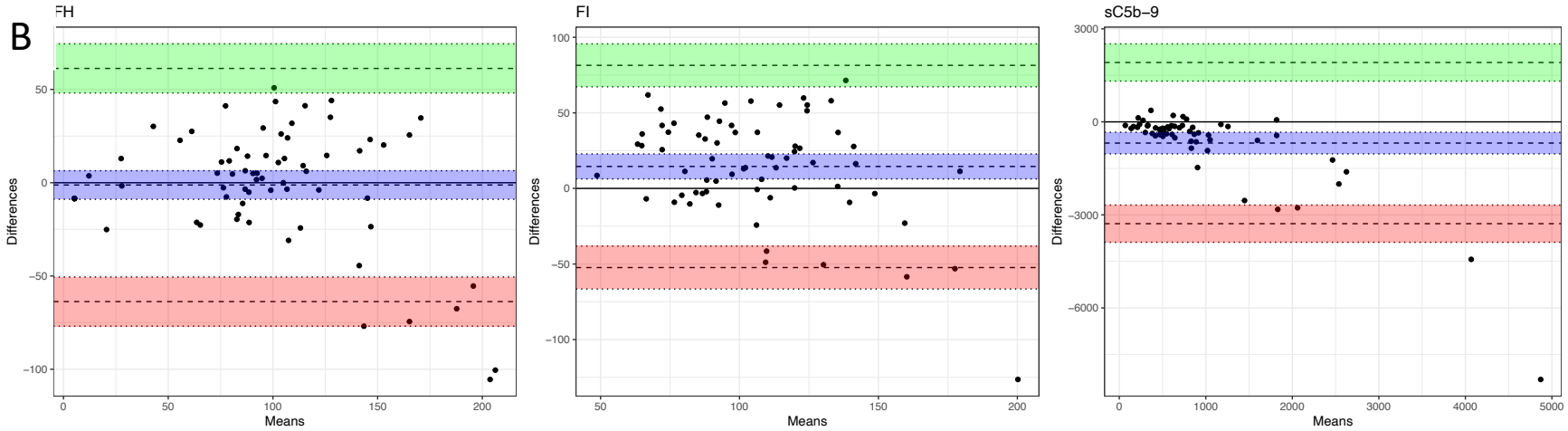
A



B



Measurement	FH	FI	sC5b-9
Bias (95% CI)	-1.2 (-8.9 to 6.4)	14.5 (6.3 to 22.8)	-684.2 (-1032.9 to -335.5)
Upper limit of agreement (95% CI)	61.3 (48.1 to 74.4)	81.4 (67.3 to 95.6)	1915.0 (1315.7 to 2514.4)
Lower limit of agreement (95% CI)	-63.7 (-76.9 to -50.6)	-52.4 (-66.5 to -38.2)	-3283.4 (-3882.8 to -2684.1)

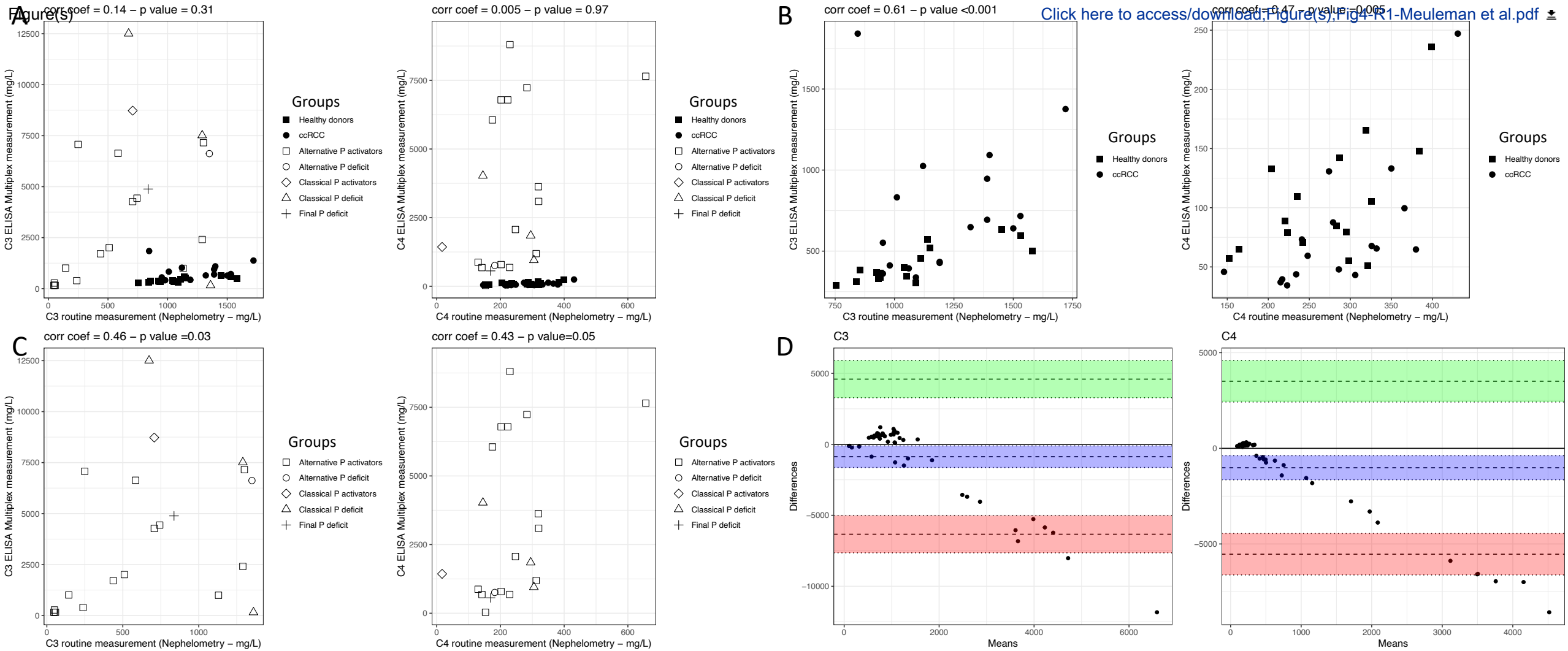
C

	FH	FI	sC5b-9
Kappa coefficient	0.77	0.21	0.55
p value	<0.001	<0.001	<0.001

Figure 3

Figure(s)

Click here to access/download;Figure(s);Fig4-R1-Meuleman et al.pdf



Measurement	C3	C4
Bias (95% CI)	-870.3 (-1631.1 to -109.4)	-1014.5 (-1644.1 to -384.9)
Upper limit of agreement (95% CI)	4593.1 (3284.8 to 5901.3)	3506.7 (2423.9 to 4589.4)
Lower limit of agreement (95% CI)	-6333.6 (-7641.9 to -5025.3)	-5535.7 (-6618.4 to -4453.0)

Figure 4

Figure(s)

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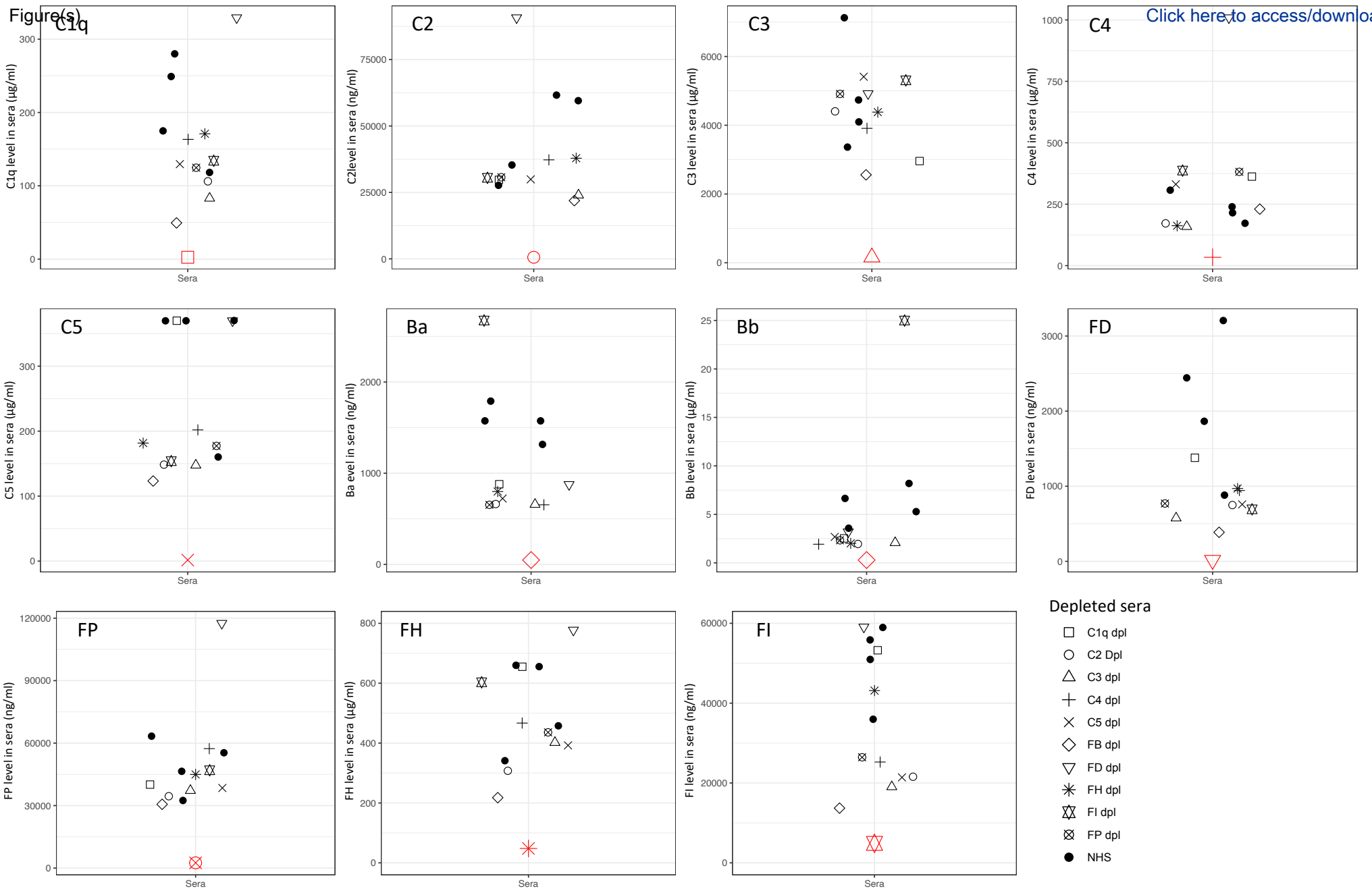

Figure 5

Figure(s)
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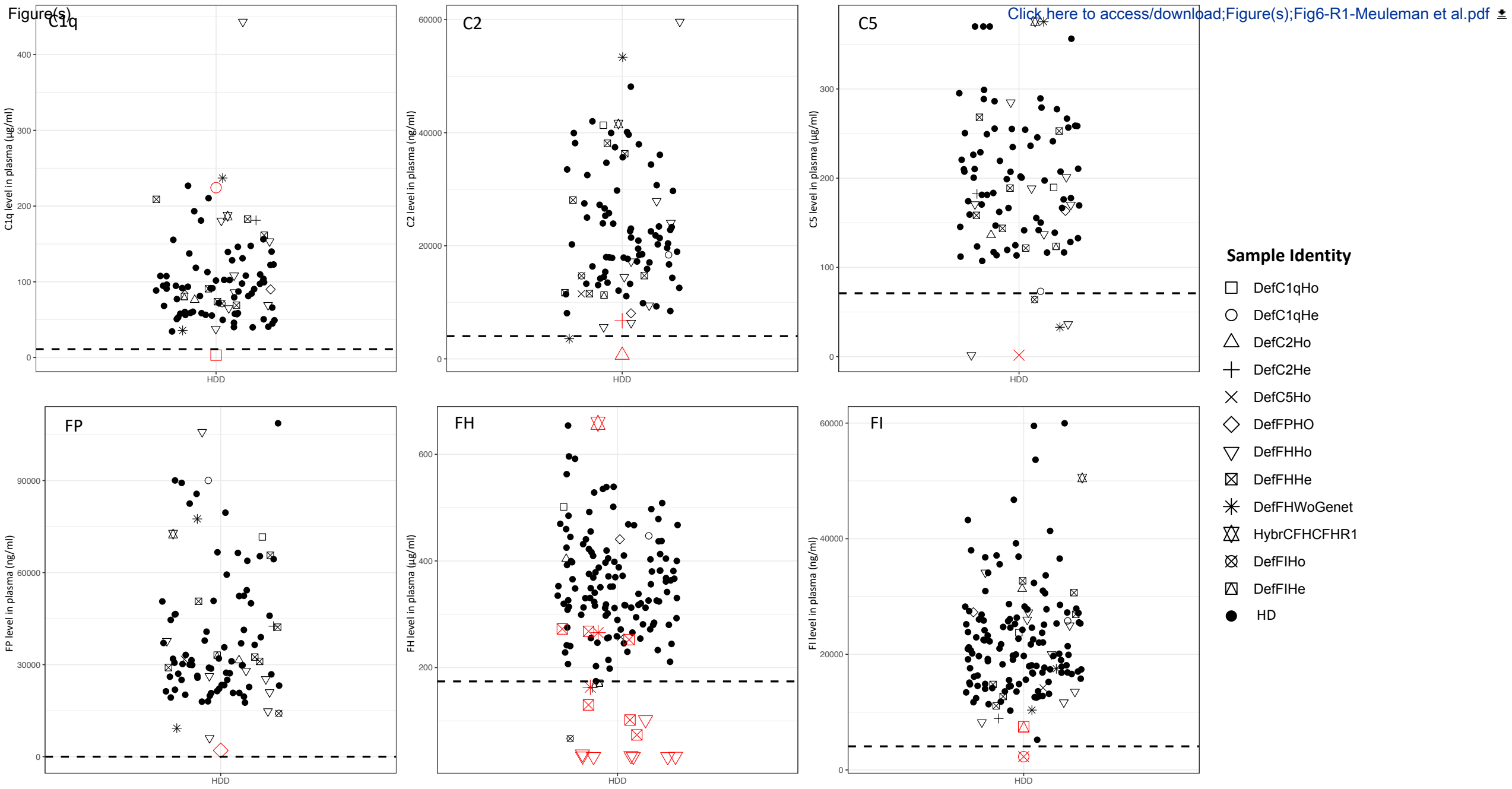

Figure 6

Figure(s)
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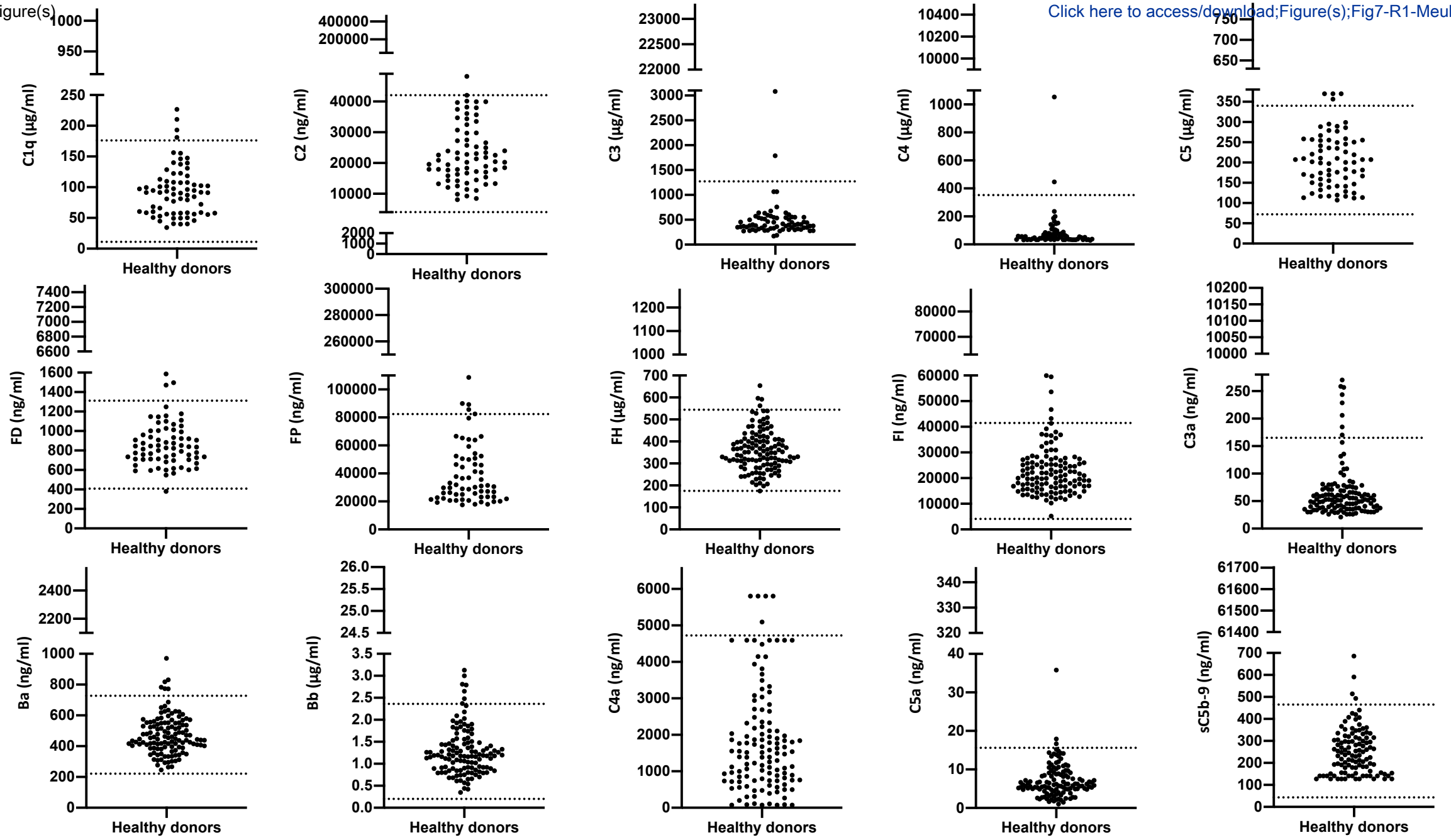

Figure 7

Figure(s)
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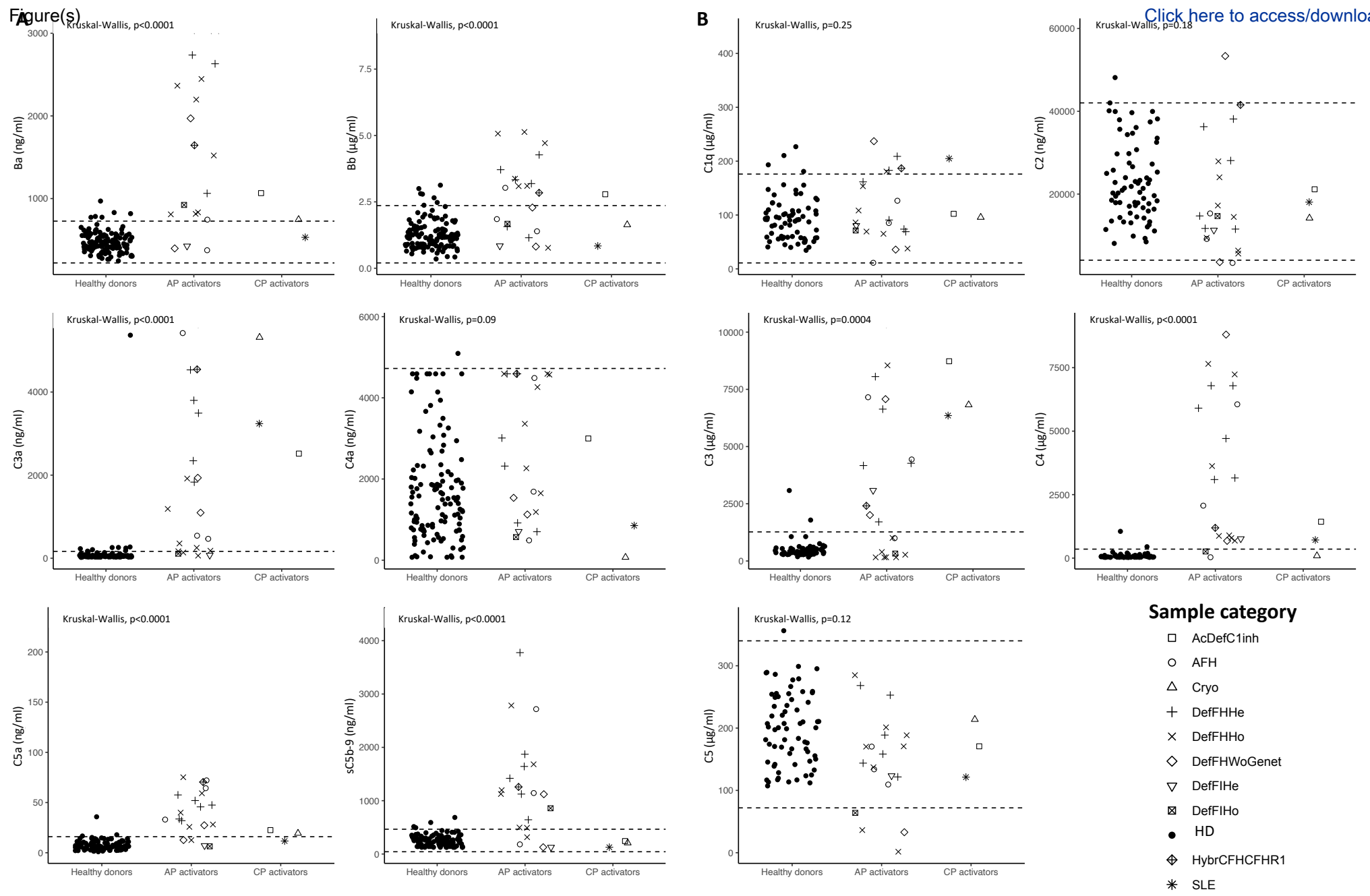

Figure 8

Figure(s)

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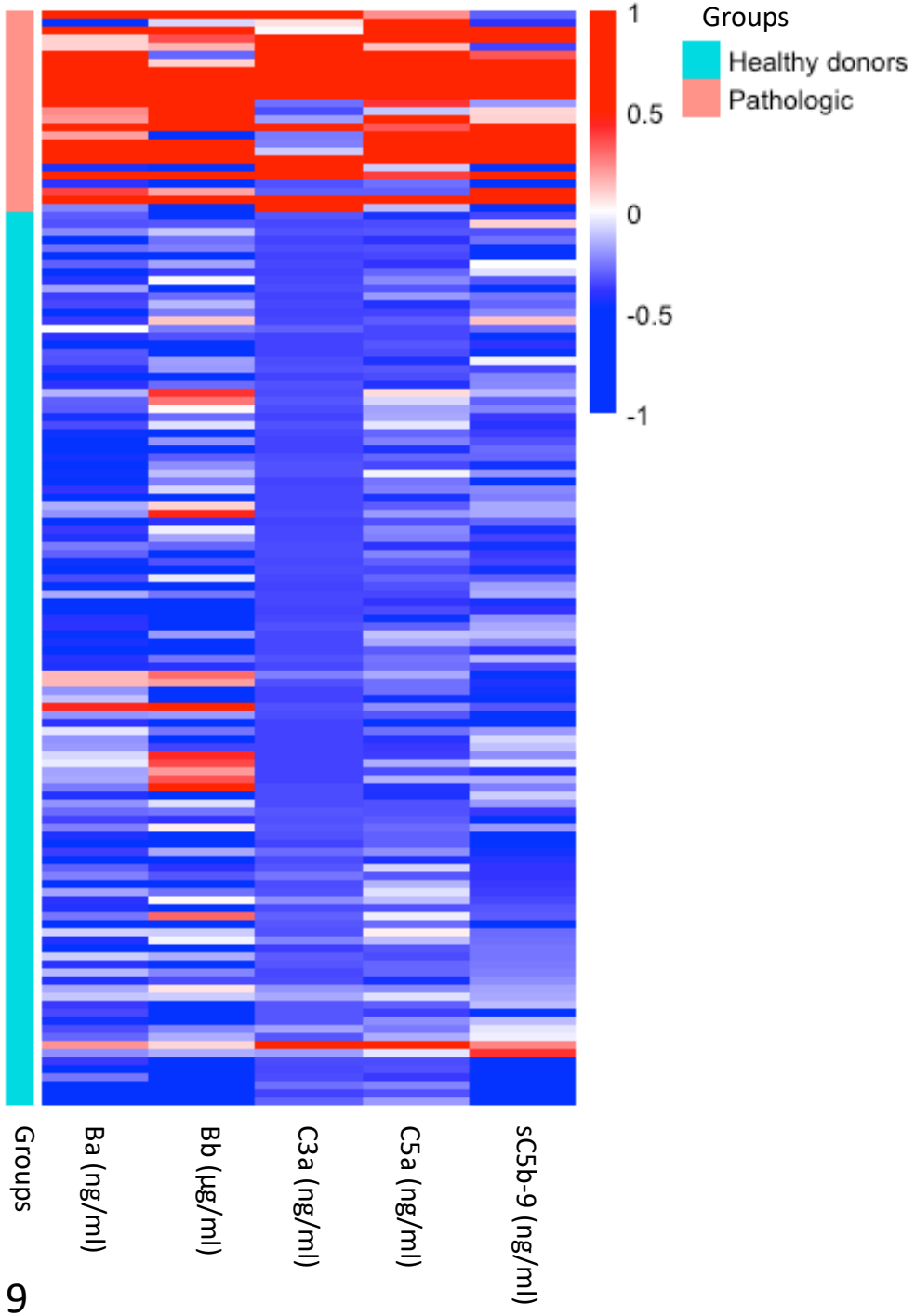

Figure 9