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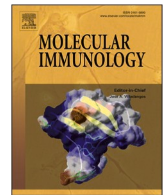

Complement ratios C3bc/C3 and sC5b-9/C5 do not increase the sensitivity of detecting acute complement activation systemically

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A R T I C L E I N F O

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A B S T R A C T

Background: Complement activation plays an important pathogenic role in numerous diseases. The ratio between an activation product and its parent protein is suggested to be more sensitive to detect complement activation than the activation product itself. In the present study we explored whether the ratio between the activation product and the parent protein for C3 (C3bc/C3) and for C5 (sC5b-9/C5) increased the sensitivity to detect complement activation in acute clinical settings compared to the activation product alone.
Materials and methods: Samples from patients with acute heart failure following ST-elevated myocardial infarction (STEMI) and from patients with out-of-hospital cardiac arrest (OHCA) were used. C3, C3bc and C5, sC5b-9 were analysed in 629 and 672 patient samples, respectively. Healthy controls (n = 20) served to determine reference cut-off values for activation products and ratios, defined as two SD above the mean.
Results: Increased C3bc/C3- and sC5b-9/C5 ratios were vastly dependent on C3bc and sC5b-9. Thus, 99.5 % and 98.1 % of the increased C3bc/C3- and sC5b-9/C5 ratios were solely dependent on increased C3bc and sC5b-9, respectively. Significantly decreased C3 and C5 caused increased ratios in only 3/600 (0.5 %) and 4/319 (1.3 %) samples, respectively. Strong correlations between C3bc and C3bc/C3-ratio and between sC5b-9 and sC5b-9/C5-ratio were found in the STEMI- ($r = 0.926$ and r = 0.786, respectively) and the OHCA-population ($r = 0.908$ and r = 0.843, respectively; $p < 0.0001$ for all). Importantly, sC5b-9 identified worse outcome groups better than sC5b-9/C5-ratio.
Conclusion: C3bc and sC5b-9 were sensitive markers of complement activation. The ratios of C3bc/C3 and sC5b-9/C5 did not improve detection of complement activation systemically.

Abbreviations: C3, Complement protein 3; C5, Complement protein 5; PRP, Pattern recognition protein; sC5b-9/TCC, Terminal complement complex.

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1. Introduction

The human complement system is a rapid and efficient immune surveillance system that protects the host and preserves homeostasis. Through pattern recognition proteins (PRP), the complement system provides an instant protection against exogenous threats, such as foreign intruders and organisms, or endogenous danger evoked by altered host cells and damaged self (Ricklin et al., 2010).

Complement is a cascade system built up of an elaborate network of soluble and cell-surface bound components, PRP, proteases, receptors and regulators (Bajic et al., 2015). Activation occurs via three routes, the classical, the lectin and the alternative pathway, all converging and leading to cleavage of the central complement component C3. The terminal pathway, on the other hand, leads to formation of the terminal C5b-9 complement complex (TCC), which either is formed in the soluble phase (sC5b-9) or inserted into a membrane as the membrane attach complex.

The cascade system is under strict control and a non-tuned balance between activation and regulation can trigger complement-driven pathological processes and diseases. Eculizumab, a monoclonal antibody targeting and preventing cleavage of C5, is FDA approved for only four rare conditions (Hillmen et al., 2006; Legendre et al., 2013; Paul, 2013; Dhillon, 2018). However, complement is suggested to play a key role in numerous other disease entities, where off-label use of eculizumab has been provided (Ricklin et al., 2017). This is an interesting development but also challenging, requiring solid complement-diagnostic tools and methods. Thus, a number of new complement inhibitors are under development to target different parts of the complement system, and some have already passed clinical trials and are and in clinical use. The development and use of complement inhibitors for several diseases have recently been extensively reviewed (Garred et al., 2021)

In the clinical setting, it may be difficult to ascertain the presence of complement activation. Low levels of C3 and C4 could be interpreted as an increased consumption and thus increased activation, but could also be explained by reduced synthesis due to liver failure, or dilution of the sample. Increased values can be seen during an acute phase reaction and in chronic inflammatory conditions (Ward, 2010; Jalal et al., 2018). The measurement of complement activation products is required for documenting *in vivo* complement activation. However, activation of complement occurs primarily locally, and a slightly increased formation of an activation product can easily be interpreted as insignificant. Thus, different reports have suggested that the ratio between an activation product and its parent protein (e.g. C3dg/C3, C3bc/C3 or C4bc/C4) is a more sensitive indicator of activation compared to the measurement of the activation product alone (Nurnberger and Bhakdi, 1984; Nielsen et al., 1996; Kim et al., 2019). This is an interesting approach that specifically takes into account borderline elevated samples. However, the evidence from the literature on this issue is limited.

The aim of the present study was to explore whether ratios between the activation products C3bc and sC5b-9 and their respective parent proteins C3 and C5 are more sensitive markers of complement activation compared to the activation products alone. Samples from two different prospective clinical studies including patients with acute heart failure following ST-elevation myocardial infarction (STEMI) (Husebye et al., 2014) and comatose out-of-hospital cardiac arrest (OHCA) patients were included (Nakstad et al., 2020).

2. Material and methods

2.1. Clinical trial samples

2.1.1. Levosimendan in Acute Heart Failure Following Myocardial Infarction (LEAF)

EDTA plasma samples from 60 patients were obtained from the previously published LEAF trial (Husebye et al., 2013). All patients had large STEMI, (the STEMI population), complicated by clinical symptoms of acute heart failure. The patients were further subdivided into patients with cardiogenic shock (shock-group), or heart failure without cardiogenic shock (non-shock group). Blood samples were drawn from inclusion to day 5. Complement activation products, including C3bc and sC5b-9 were measured and these results have already been published (Orrem et al., 2018). A total of 207 samples from 25 patients, previously analysed for C3bc, were available and analysed for C3 in the current study and enabled us to calculate the C3bc/C3 ratio. A total of 281 samples from 60 patients, previously analysed for sC5b-9, were available and analysed for C5 in the current study and enabled us to calculate the sC5b-9/C5 ratio.

2.1.2. Norwegian Cardio-Respiratory Arrest Study (NORCAST)

EDTA plasma samples were obtained from the Norwegian Cardio-Respiratory Arrest Study (NORCAST), a prospective study on blinded prognostication assessment in comatose patients with OHCA (the OHCA population) from both cardiac and non-cardiac causes (Nielsen et al., 1996). All patients were treated with standardized operating procedures (SOP) including targeted temperature management (TTM) to 33 °C for 24 h (Nielsen et al., 1996). Blood samples were obtained on admission and at day three and classified according to the patient's clinical outcome at six months post arrest defined as cerebral performance category (CPC) score 1–2 (good outcome) or CPC score 3–5 (poor outcome) (Mak et al., 2016). The complement activation products, including C3bc and sC5b-9, were measured and presented in a subsequent study (Chaban et al., 2021). In total, 422 samples from 246 patients, quantified for C3bc, and 391 samples from 230 patients, quantified for sC5b-9, were available for C3 and C5 quantification, enabling us to calculate the C3bc/C3- and sC5b-9/C5 ratio, respectively.

2.2. Controls

Twenty healthy controls (age 30–58 years) were analysed for C3bc, C3, sC5b-9 and C5 to define positive cut-off values of the ratios. A positive value was defined as two standard deviations above the mean and a normal value as equal or lower than the mean.

2.3. Detection of the complement components C3 and C5 and the complement activation products C3bc and sC5b-9

C3 was measured using nephelometry, BN II System from Siemens, (Erlangen, Gemany) at the Routine Immunological Laboratory at Oslo University Hospital. A commercially available enzyme-linked immunosorbent assay (Abcam, Cambridge, UK) was used to detect complement component C5. C3 and C5 were measured in both studied clinical cohorts, whereas C3bc and sC5b-9 already had been measured as mentioned above. The control samples were measured for C3 and C5 as described above and C3bc and sC5b-9 were quantified as described in detail previously (Bergseth et al., 2013)

2.4. Statistics

Graphpad prism version 8 (San Diego, CA) was used for statistical analyses. Data were analysed with one-way ANOVA followed by Bonferroni's multiple comparison test, and correlation analyses were measured in logarithmic scale using the non-parametric spearman correlation test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Control samples, cut-off values and C3 and C5 concentrations

Cut-off values were based on measurement of the control samples and defined as two standard deviations below/above the mean (Fig. 1, grey bars). C3bc positive value was defined as > 4.9 AU/mL, C3bc/C3

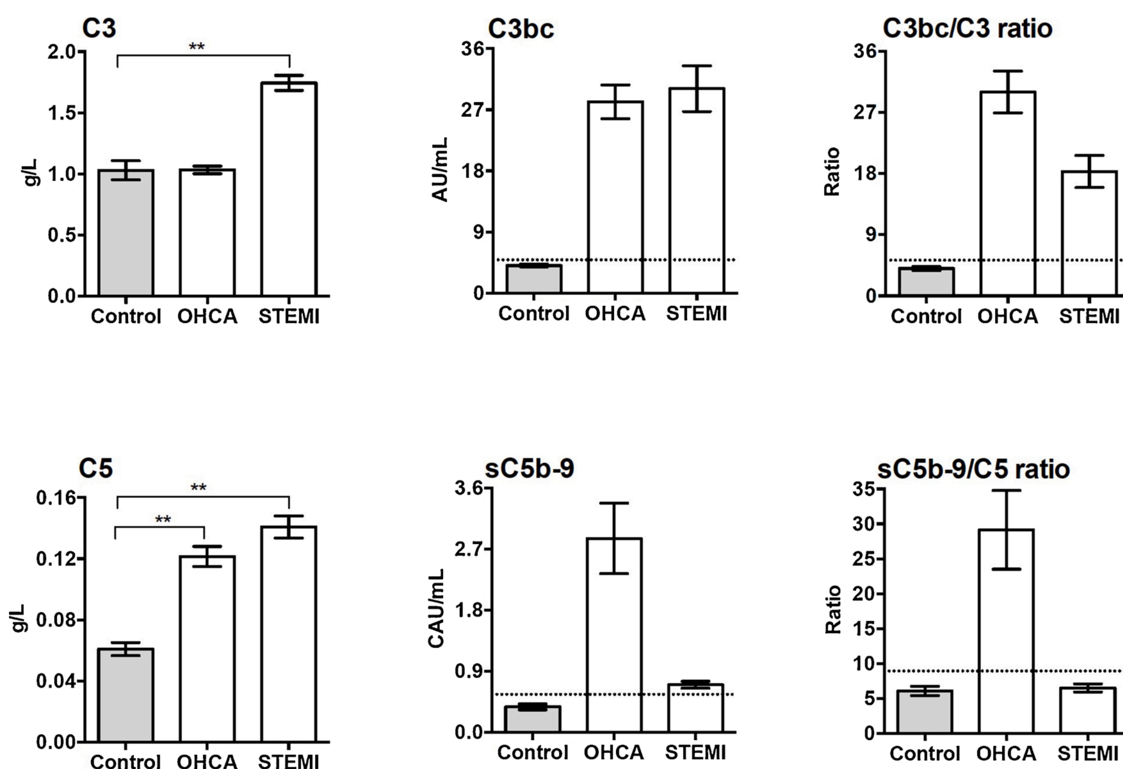


Fig. 1. C3, C3bc, C5 and sC5b-9 were analysed in samples from 20 healthy individuals (controls; grey bars) and in samples from two critically ill patients populations; a) patients with acute heart failure following ST-elevation myocardial infarction, the STEMI-population, b) patients with out-of hospital cardiac arrest, the OHCA-population (white bars). The ratios between the activation products and the native components were thereafter calculated. Positive cut-off values were defined as two standard deviations above the mean of the control, indicated by the dotted lines giving the following positive values; C3bc > 4.9 AU/mL, C3bc/C3 > 5.24 (upper panel), sC5b-9 > 0.56 AU/mL and sC5b-9/C5 ratio > 8.94 (lower panel). Statistical comparisons of C3- and C5-levels were performed between the controls and the MI- and CA-population. Data are presented as means ± 95 % CI. $^{**}p < 0.001$.

positive ratio as > 5.24, sC5b-9 positive value as > 0.56 AU/mL and sC5b-9/C5 positive ratio as > 8.94 (indicated by dotted lines in Fig. 1). For comparison, the mean values with 95 % CI for C3, C3bc, C5, sC5b9 and the calculated ratios C3bc/C3 and sC5b-9/C5 are shown for the STEMI and OHCA patients, respectively (Fig. 1). Notably, C3 concentration was significantly higher in the STEMI patients and C5 concentration was significantly higher in both STEMI and OHCA patients, compared to the control group ($p < 0.001$ for both) (Fig. 1, left panel.)

3.2. C3-activation as evaluated by levels of C3bc and the ratio of C3bc/C3

Increased C3bc/C3-ratio was vastly dependent on increased formation of C3bc. Altogether 597 out of 600 ratio-positive values (99.5 %) from both patient populations had increased levels of C3bc alone, whereas only three samples with increased ratios were caused by low or normal C3-values (Table 1). Notably, 15 out of 612 C3bc-positive samples (2.5 %) were not associated with increased ratios, underscoring the high sensitivity of C3bc alone. In the STEMI population, 100 % of the samples from the shock group and 91 % from the non-shock group were both C3bc and C3bc/C3 ratio positive (Table 1). In the OHCA population, altogether 96 % of the samples were both C3bc and C3bc/C3 ratio positive.

3.3. Terminal pathway activation evaluated by levels of sC5b-9 and the ratio sC5b-9/C5

Increased sC5b-9/C5-ratio was vastly dependent on increased formation of sC5b-9. From both patient populations, altogether 313 out of 319 ratio-positive samples (99.1 %) had increased levels of sC5b-9 alone, whereas only six samples with increased ratios were caused by normal or low sC5b-9-values (Table 2). Out of 510 sC5b-9 positive samples, 197 were not associated with increased ratios. In the STEMI population, 79 % (30/38) of the samples from the shock group were sC5b-9-positive, whereas only 42 % (16/38) of the samples were sC5b-9/C5-ratio positive. In the OHCA population, 92 % (149/162) of the samples from the poor outcome group were sC5b-9-positive, whereas only 71 % (115/162) of the samples were sC5b-9/C5-ratio positive.

Table 1
C3-activation measured by increased formation of the activation product C3bc, versus increased formation of the C3bc/C3 ratio in patients with acute heart failure following ST-elevation myocardial infarction (**STEMI**) and patients with out-of-hospital cardiac arrest (**OHCA**).[a]

Patient population	C3bc: **positive** C3bc/C3: normal	C3bc: normal C3bc/C3: **positive**	C3bc: **positive** C3bc/C3: **positive**	C3bc: normal C3bc/C3: normal	Number of samples
STEMI	9 (4)[b]	0	192 (93)	6 (3)	207
Shock	0	0	37 (100)	0	37
Non-shock	9 (5)	0	155 (91)	6 (4)	170
OHCA[c]	6 (1.5)	3 (0.5)	405 (96)	8 (2)	422
Poor outcome	1 (0.5)	1 (0.5)	163 (97)	3 (1.5)	168
Good outcome	4 (1.5)	2 (1.0)	198 (96)	3 (1.5)	207

[a] C3bc positive defined as >4.9 AU/mL and normal as ≤ 4.9 AU/mL. C3bc/C3 ratio positive defined as >5.24 and normal as ≤ 5.24.
[b] Number of samples with percentages in parenthesis.
[c] In the cardiac arrest study clinical data were available for 375 out of 422 samples.

Table 2
C5-activation measured by increased formation of sC5b-9 (TCC) versus increased formation of the sC5b-9/C5 ratio in patients with acute heart failure following ST-elevation myocardial infarction (**STEMI**) and patients with out-of-hospital cardiac arrest (**OHCA**).[a]

Patient population	sC5b-9: **positive** sC5b-9/C5: normal	sC5b-9: normal sC5b-9/C5: **positive**	sC5b-9: **positive** sC5b-9/C5: **positive**	sC5b-9: normal sC5b-9/C5: normal	Number of samples
STEMI	109 (39)[b]	4 (1)	44 (16)	124 (44)	281
Shock	15 (39)	1 (3)	15 (39)	7 (18)	38
Non-shock	94 (39)	3 (1)	29 (12)	117 (48)	243
OHCA[c]	88 (22.5)	2 (0.5)	269 (69)	32 (8)	391
Poor outcome	35 (21)	1 (1)	114 (71)	12 (7)	162
Good outcome	46 (23)	1 (0.5)	135 (67.5)	18 (10)	200

[a] sC5b-9 positive defined as >0.56 AU/mL and normal as ≤ 0.56 AU/mL. sC5b-9/C5 ratio positive defined as >8.94 and normal as ≤ 8.94.
[b] Number of samples with percentages in parenthesis.
[c] In the cardiac arrest study clinical data were available for 362 out of 391 samples.

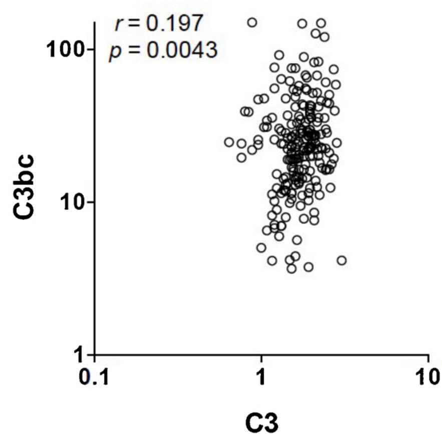
3.4. Correlations between the activation product C3bc, C3, and the ratio of C3bc/C3

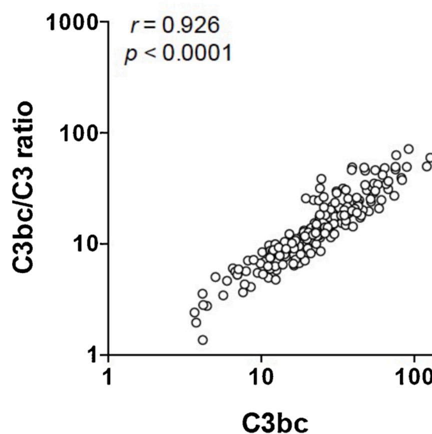
Correlation between C3bc and the ratio of C3bc/C3 measured in both the STEMI- and OHCA populations were highly significant ($p < 0.0001$), with high correlation coefficients ($r = 0.926$ and $r = 0.908$, respectively, Fig. 2, right panel). In contrast, only weak correlations were found between C3bc and C3 with $r = 0.197$ ($p = 0.0043$) and $r = 0.117$ ($p = 0.016$), respectively, (Fig. 2, left panel). In controls, the correlation between C3bc and C3 was moderate ($r = 0.474$, $p = 0.034$), whereas no correlation was found between C3bc and C3bc/C3-ratio ($r = 0.035$, $p = 0.88$) (data not shown).

3.5. Correlations between the activation product sC5b-9, C5 and the ratio of sC5b-9/C5

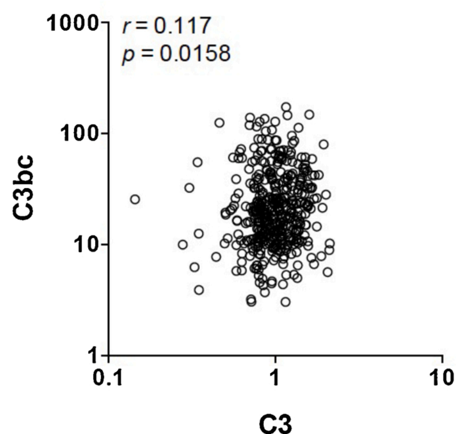
The correlation between sC5b-9 and the ratio of sC5b-9/C5 in the STEMI and OHCA populations were highly significant ($p < 0.0001$) with high correlation coefficients, $r = 0.786$ and $r = 0.843$, respectively (Fig. 3, right panel). No correlation between sC5b-9 and C5 were found, $r = 0.048$ (p = 0.42) and $r = 0.027$ ($p = 0.59$), respectively (Fig. 3, left panel). In controls, we did not find any correlation between sC5b-9 and

STEMI





OHCA



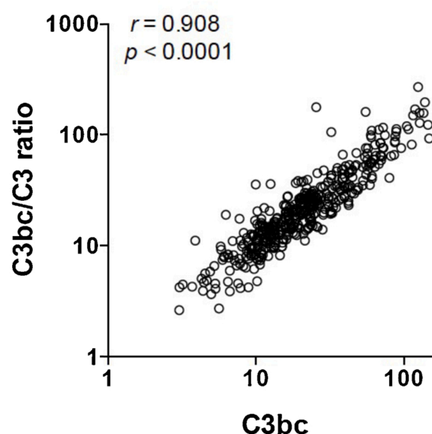


Fig. 2. The correlations between C3bc and C3 and between C3bc and the C3bc/C3 ratio in the STEMI (upper panel) and OHCA (lower panel) population were performed in logarithmic scale using the non-parametric spearman correlation. A p value < 0.05 was considered statistically significant.

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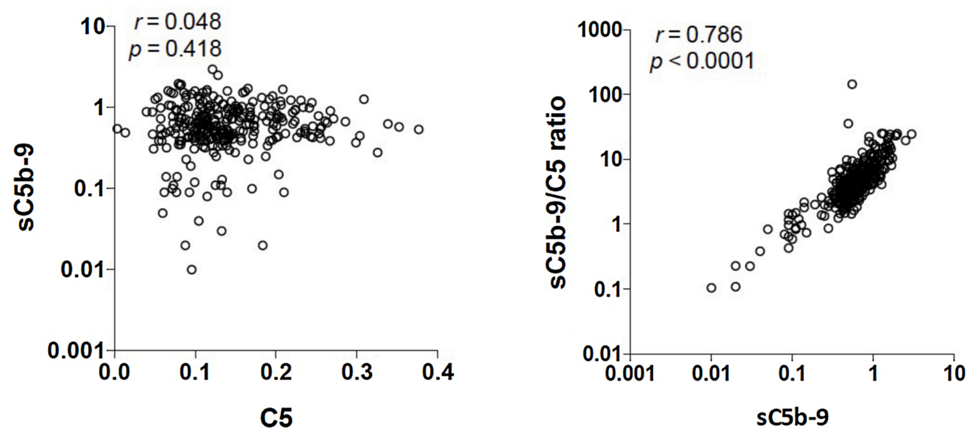

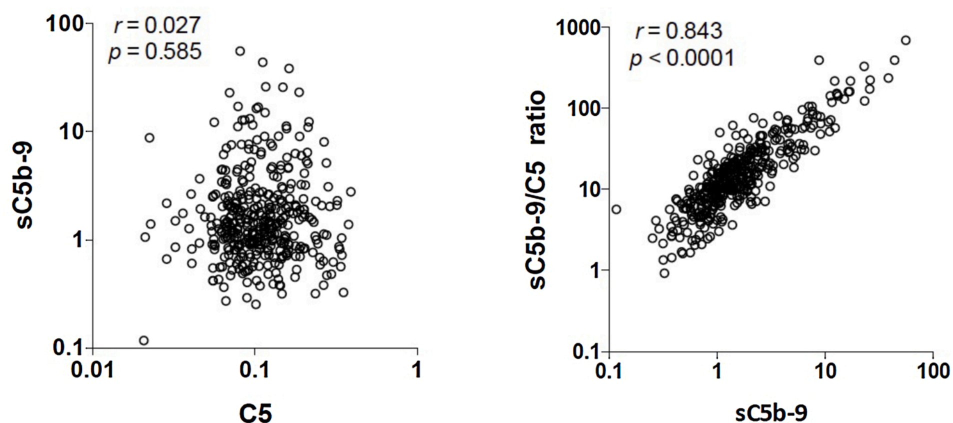

Fig. 3. The correlations between sC5b-9 and C5 and between sC5b-9 and the sC5b-9/C5 ratio in the STEMI (upper panel) and OHCA (lower panel) population were performed in logarithmic scale using the non-parametric Spearman correlation test. A p value < 0.05 was considered statistically significant.

C5 ($r = 0.05$, $p = 0.84$), however, the correlation between sC5b-9 and the ratio of sC5b-9/C5 was strong ($r = 0.743$, $p = 0.0002$) (data not shown).

4. Discussion

The present study demonstrates strong correlations between the complement activation products C3bc and sC5b-9, and the ratio to their native components, C3 and C5, in two clinical cohorts comprising patients with STEMI complicated with acute heart failure and comatose OHCA patients. Importantly, the study documents that the ratio of the activation products, C3bc/C3 and sC5b-9/C5, were not more sensitive in detecting complement activation systemically than the activation products itself. In fact, the increased ratios of C3bc/C3 and sC5b-9/C5 were almost exclusively dependent on increased levels of C3bc and sC5b-9. Of particular interest was that normal or low C3 or C5 concentrations caused increased ratios in only 3/600 (0.5 %) and 6/319 (1.8 %) of the samples, respectively. No correlations were found between C3bc and C3 or sC5b-9 and C5. In STEMI-patients developing cardiogenic shock, and in OHCA-patients with poor outcome, sC5b-9 was indeed a more sensitive marker of complement activation compared to the sC5b-9/C5 ratio.

The complement activation products are normally present in traces, but may increase log-folds when activation occurs, not least due to the alternative pathway amplification loop, responsible for 80 % of the magnitude of the activation irrespective of the activation pathway (Harboe et al., 2004, 2006). In particular, sC5b-9 is a stable activation product, highly resistant to thawing and freezing and demonstrate low intra- and inter-assay variability (Bergseth et al., 2013). Both C3bc and sC5b-9 are excellent markers to detect complement activation systemically. Thus, a slightly increased concentration of the activation product imbalances the ratio, reflected by the convincingly strong correlation between the activation product and the ratio. This is in fact not surprising given that approximately 1 % of the components are activated under physiological condition, a 10-fold increase in the activation product will only give a reduction by 10 % of the parent molecule, and hardly influence the ratio. If the corresponding C3bc concentration is increased from 5 to 50 AU/mL, it will considerably increase the ratio by 10 times, consistent with the finding in this study.

Thus, positive ratios were almost solely dependent on increased activation products and rarely on low concentration of the native components. The samples included in the present study were from patients with acute, critical illness, OHCA or acute heart failure after STEMI with or without cardiogenic shock, both incidents likely to cause regional or global ischemia/reperfusion injury and complement activation (Banz and Rieben, 2012). However, the study cohorts comprise a

wide range of complement activation patterns, from no activation to excessive activation, thus elucidating the value of complement-activation ratios over a broad range of complement activation in a comprehensive context.

Previous studies have suggested that ratios may be useful and more sensitive than activation products in measuring ongoing complement activation (Nurnberger and Bhakdi, 1984; Kim et al., 2019). These reports refer to systemic lupus erythematosus (SLE), a chronic condition where the disease activity is related to complement consumption and decreased concentration of C3 and C4. Recently, it was demonstrated that the relative changes in iC3b/C3-ratio discriminated between active and inactive form of SLE (Kim et al., 2019). Thus, the usefulness of a ratio might be greater in chronic diseases characterized by complement consumption, but in acute disease like in the present study, the ratio does not appear to be better or a more useful marker, even with very low levels of native components.

The control levels of C3 and C5 used in this study correspond to the levels referred to in the literature (Bergseth et al., 2013). However, the C5 level was significantly higher in both patient populations compared to the control group, whereas the level of C3 was significantly higher in the STEMI population only. Previous reports document that an increased level of C3 is associated with increased arterial stiffness, cardiovascular disease and obesity (Engstrom et al., 2005; Muhammad et al., 2017; Kirschfink and Mollnes, 2003; Nilsson et al., 2014) which may explain the elevated level of C3 observed in the STEMI population. The observation of increased levels of C5 in both patient cohorts, whereas C3 was increased only in the STEMI population is of interest and may have several explanations. The different levels in C3 and C5 may reflect different mechanisms of low-grade chronic inflammation among these patients, or alternatively a rather specific C3 increase as an acute response to the insult. Mechanistically, this could be explained by a differential efficacy and stability of the C3- and the C5 convertases. Such a phenomenon is known from other conditions like certain nephritic factors (autoantibodies to the convertases) where some stabilize only the C3 convertase and C3 is activated without a consecutive activation of C5 (Mollnes et al., 1986). We recently showed the same phenomenon by activation complement by air bubbles, leading to a huge activation of C3 and the alternative pathway, with a limited activation of the terminal pathway (Storm et al., 2021). This interesting differential activation of the convertases could under some conditions be explain by the density of C3b molecules generated by the C3 convertase (Mannes et al., 2021). Finally, it was recently shown that properdin binding to the convertase to a great extent explained the shift towards C5 activation after alternative pathway activation (Michels et al., 2021). In any case, the increased levels of the native components, C3 and C5, reduce the likelihood for a positive ratio as the denominator increases, thus making the ratio a less sensitive marker of complement activation.

sC5b-9 is a robust marker of terminal complement pathway activation due to its detection of complement activation to the final stage and the relatively long half-life compared to other complement activation products (Deppisch et al., 1990; Mollnes, 1985). The sC5b-9/C5-ratio has previously not been investigated, and in the present study, increased sC5b-9/C5-ratio was vastly dependent on increased formation of sC5b-9. Interestingly, when looking at subgroups, increased formation of the activation product sC5b-9 occurred more frequently than the ratio, both among STEMI patients with cardiogenic shock and OHCA patients with poor outcome. It is therefore tempting to suggest that the activation product sC5b-9 might be a more robust marker than the ratio itself, at least in critically ill patients.

In conclusion, the present study demonstrates a strong correlation between the complement activation products and their ratios to native components in critically ill patients with complement activation detected systemically. Compared to the activation products, the ratios were less sensitive in detecting complement activation systemically than the activation products alone. Both C3bc and sC5b-9 are sensitive markers of complement activation and sufficient for screening of clinically significant complement activation.

Data availability

The present study is based on clinical data and plasma samples provided from two clinical trials, the NORCAST (Nakstad et al., 2020) and Leaf trial (Nielsen et al., 1996). The samples were taken snap-frozen, stored properly, and analyzed in our lab.

Data will be made available on request.

Declaration of Competing Interest

The authors report no declarations of interest.

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