Sample processing time but not storage time affects complement activation markers C4a, C4d, C3a, iC3b, Bb, C5a, and sC5b-9 levels in EDTA-plasma of individuals at clinical high-risk for psychosis

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ABSTRACT

The complement system is an important part of the innate immune system and plays a key role in inflammatory processes. Concentrations of complement activation fragments in plasma are markers of systemic activation and have been found to be altered in a wide range of diseases. Some plasma activation marker levels can be influenced by sample processing and storage time. We quantified seven complement activation markers (C4a, C4d, C3a, iC3b, Bb, C5a, and sC5b-9 (TCC)) in EDTA-plasma as part of a multi-centre clinical study analysing complement activation in individuals with clinical high-risk (CHR) for psychosis compared with healthy controls. Samples had been collected, processed, and subsequently stored at -80°C over a period of 9.5–13.6 years, according to a standard operating protocol (SOP). Complement activation markers were quantified using commercially available and standardised enzyme-linked immunosorbent assays (ELISA). In a post hoc analysis of variables affecting the analyses we investigated the impact of EDTA-to-freezer processing time (<1–7.35 hours) and freezer storage time (9.5–13.6 years). EDTA-to-freezer processing time moderately correlated positively with C4a, C3a, iC3b and sC5b-9 levels. Storage time at -80°C was not significantly correlated with any complement activation marker. This study provides valuable insight into the impact of sample processing and long-term sample storage in complement activation marker studies. The results suggest that storage time in -80°C is not a confounding factor affecting non-specific complement activation in EDTA-plasma. Sample-processing time does moderately affect the levels of some complement activation markers. This should be considered as a co-variate when analysing complement activation marker levels. Further, the impact may vary for healthy or clinical samples where immune activation is part of the pathology. These findings are important when planning large-scale clinical studies that include quantification of complement components and its activation fragments as biomarkers. It supports the collection of EDTA-plasma and fast sample processing to be included into a study standard operating procedure.

1. Introduction

The complement pathways are central to host defense against pathogen infection and injury. Complement is an important part of innate immunity in the blood and plays a key role in inflammatory processes. This blood-based innate defense system is composed of proteins that are proteolytically activated, leading to the generation of active molecules essential to immunity. The complement system consists of about 50 complement components and regulators in the plasma fluid phase, as well as cell membrane-bound, which work together to fight infection and promote inflammation (Merle et al., 2015b). Concentrations of complement components and their activation fragments in plasma have been found to be altered in a wide range of different diseases, including cancer, atherosclerosis, diabetes, inflammatory bowel disease, neurodegenerative disorders, autoimmune diseases and infections (Ekdahl et al., 2018). Consequently, complement components are increasingly being tested as potential plasma, serum, or cerebrospinal fluid (CSF) biomarkers, and as treatment targets in a variety of conditions (Pouw

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and Ricklin, 2021). Several assays (e.g., functional, qualitative, or quantitative) are now commonly used to analyse the levels of complement markers (Mollnes et al., 2007).

Often, clinical blood samples, like EDTA-plasma, are stored for several months or years in the freezer prior to analysis. Large cohort studies often use samples that are collected in different centres and from different clinical labs, and while a standard operating procedure is in place, differences in processing times occur.

Complement is activated via three distinct pathways, the classical (CP), lectin (LP) and alternative pathway (AP) (Carroll and Sim, 2011), primarily in response to vascular injury (Kerr and Richards, 2012) and infection. The classical pathway is activated via antibody-antigen complexes, the lectin pathway by sugar moieties on the surfaces of bacteria, and the alternative pathway is constitutively activated by a "tick-over" mechanism (Merle et al., 2015a). All pathways converge on the generation of the C3 convertase enzymes, which cleave the central component of the complement system, C3 (Fung et al., 2016), and subsequently progressing into the terminal pathway, and lytic pore formation (Bubeck, 2014; Serna et al., 2016), as summarized in Fig. 1. Classical pathway activation requires both Ca^{2+} and Mg^{2+} ions, whereas the alternative pathway requires only Mg^{2+}. EDTA, a chelator of both Ca^{2+} and Mg^{2+}, inhibits complement activation in plasma by all three pathways, and is more efficient than citrate and heparin in inhibiting in vitro

activation. However, non-specific complement activation due to processing or storage conditions has been observed (Stöve et al., 1995), which may affect the measured levels of complement activation markers affecting the measurement accuracy. This may be particularly important for the interpretation of results where complement activation marker levels are associated with disease.

Levels of complement components and activation markers can be inconsistent between different laboratories due to - in part - lack of standardisation or use of different quantification methods, with variation also occurring in sample collection, handling, and storage (Baatrup et al., 1992; Lippi et al., 2006; Mollnes et al., 1988; Nilsson and Ekdahl, 2012; Woodhams et al., 2001). In our study, we analysed complement activation markers in EDTA-plasma samples using standardised commercially available ELISA kits for complement activation markers C4a, C4d, C3a, iC3b, Bb, C5a, and sC5b-9 (TCC). There are few studies reporting on the impact of sample processing or storage time on complement activation fragment levels; and those are often underpowered or in vitro analyses. For instance, the use of EDTA that chelates any Calcium or Magnesium, essential for activation of complement, was reported to support the stability of complement markers in whole blood (Yang et al., 2015). The effect of storage time as a potential confounder has been reported with largely non-significant effects on biomarker stability. This has been assessed in samples stored up to 6 years (Mollnes
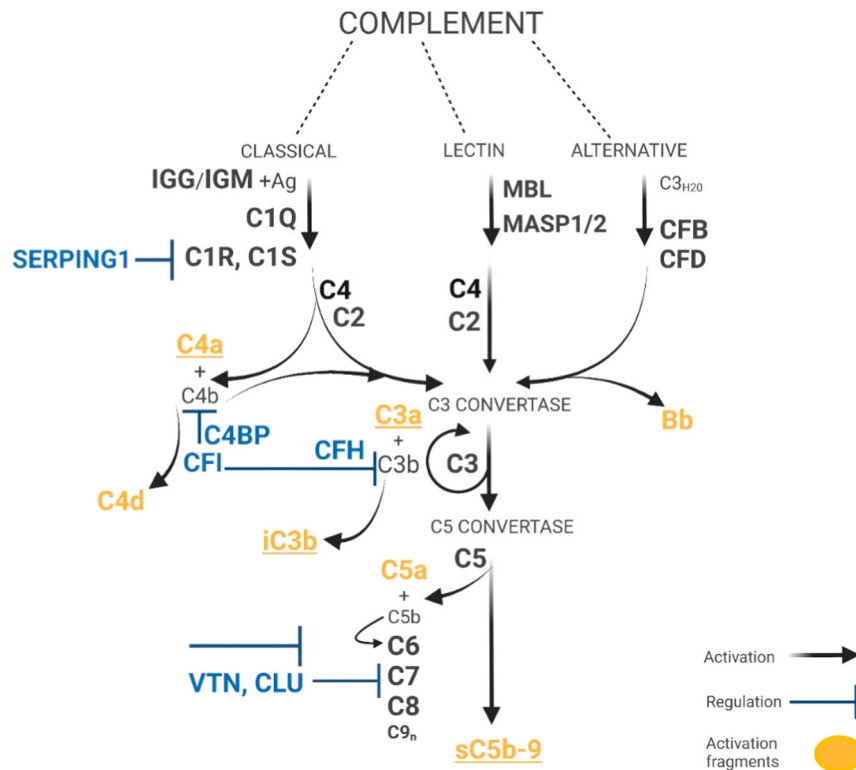
Fig. 1. Overview of the complement pathways. The complement system is activated (→) by three distinct pathways, classical (CP), lectin (LP) or alternative pathway (AP). Lectin and classical pathway activation leads to the cleavage of C4 and C2, resulting in the formation of the C3 convertase (C4b2a). The alternative pathway is continuously activated by the "tick-over" mechanism of C3 hydrolysis into $C3_{H2O}$, which is able to bind factor B (FB) and factor D (D) to form an initial C3 convertase ($C3_{H2O}$Bb). The C3 convertases cleave C3 into anaphylatoxin C3a and opsonin C3b, which can bind FB and FD to form the alternative pathway C3 convertase C3bBb. All pathways converge at the level of C3 convertase formation and cleavage of many C3 molecules, which is amplified by each C3b molecule able to form more C3bBb, known as the alternative pathway amplification loop (indicated by a circular arrow). The terminal pathway (TP) of complement commences with the formation of the C5 convertases and cleavage of C5 into anaphylatoxin C5a and C5b, which subsequently binds C6, C7, C8, and multiple C9 molecules forming the membrane attack complex (MAC, C5b-9). Several complement regulators (blue) tightly control complement activation and amplification. As part of complement activation and regulatory mechanisms, several activation and enzymatic cleavage fragments are generated that can be quantified in blood plasma or serum as markers of complement activation. Complement C4 is cleaved into C4b and C4a. C4d is generated by the FI/C4BP-mediated cleavage of C4b. C3 and C5 are cleaved into C3b, C3a and C5b, C5a, respectively. The soluble terminal complement complex (TCC) is sC5b-9. iC3b is generated, e.g. by the FI/FH-mediated cleavage of C3b. Bb is a component of the alternative pathway C3 convertase (C3bBb), which dissociates naturally over time. The complement activation and cleavage fragments quantified in this study were C4a, C4d, C3a, iC3b, Bb, C5a, and sC5b-9 (TCC) (shown in yellow). Activation markers that correlate with EDTA-to freezer time in the CHR sample cohort (but not healthy controls) are shown as underlined. Blunt arrows (⊥) indicate inhibition while sharp arrows (→) indicate activation.

et al., 1988; Yang et al., 2015), but one study found that long term storage over 6 years (6.6 – 10.6 years at -80°C) resulted in increased levels of complement activation markers (Morgan et al., 2017). This suggests some degree of activation during storage. Our samples are from matched case and control cohorts, adjusting for non-disease related factors such as age and sex relevant to plasma biomarker levels or stability (Gaya da Costa et al., 2018b). Our study population (n=180) included individuals aged 12–28 years as part of a multi-centre study of clinical high risk (CHR) individuals at risk of developing psychosis compared to healthy controls (HC) (n=80). From these, data points for the EDTA-to-freezer time analyses were available for n=169 CHR (including primary outcome data on transition to psychosis) and n=76 HC, whereas for the storage time analyses, the data were available for n=178 CHR individuals and all HC (n=80). It was previously shown by proteomics analyses that CHR individuals present with altered complement component levels and complement is thought to be a contributing factor for progression to disease (Heurich et al., 2022). To accurately measure complement activation in plasma samples, our data assessed complement activation marker levels by statistical association with relevant co-variables, which could influence these levels, such as sample processing and freezer storage time.

2. Methods

2.1. Study population

This study included 180 participants from the multi-site North American Prodrome Longitudinal Study (NAPLS2) study. Clinical High-Risk (CHR) or Ultra High Risk (UHR) criteria aimed at identifying individuals at risk for psychosis and treating them before their first episode. Plasma-EDTA samples at baseline (n=180 CHR; n=80 healthy controls (HC)) were obtained as part of a secondary analysis of the NAPLS2 study, which recruited individuals who met criteria for CHR and followed them over time. Our study included samples from CHR participants who later transitioned to psychosis compared with those who did not transition (outcome). The aims and methods of the NAPLS2 study, as well as inclusion/exclusion criteria, demographics and clinical presentation, were described previously (Addington et al., 2012). In brief, those CHR were between 12 and 35 years old and meet diagnostic criteria for a prodromal syndrome as per the COPS criteria (McGlashan et al., 2010). The CHR participants are help-seeking individuals and as such present not only with subthreshold symptoms, but also a wide range of comorbidity (Addington et al., 2012). Informed consent was obtained for all subjects according to the Declaration of Helsinki (1991) and protocols and procedures were approved by the relevant institutional review board at each collection site. Further information regarding the cohort has been previously described (Perkins et al., 2015). The work described herein included all CHR samples, independent of outcome.

2.2. Sample collection

In brief, the standard operating procedure used in the study collection centres defined that plasma samples are collected at the baseline visit into Becton Dickenson P100 blood collection tubes with ethylene diamine tetra-acetic acid (EDTA) as anticoagulant, proprietary protein stabilizers, and a mechanical separator. Plasma was harvested, aliquoted and then frozen at –80°C (Perkins et al., 2015).

2.3. ELISA quantification of complement activation markers

We received the samples frozen on dry ice; these had been aliquoted and undergone two freeze-thaw cycles, consistent for all samples. The levels of complement activation markers in EDTA-plasma samples were measured in duplicates according to manufacturer instructions (Quidel) for ELISAs:C4a (A036), C4d (A008), C3a (A032), iC3b (A006), Bb (A027), C5a (A025), and sC5b-9 (TCC) (A029). Fig. 1 shows an overview of the complement pathways, highlighting the complement activation markers quantified in this study. Plasma standard (P9523, Sigma, UK) was used to determine intra-assay and inter-assay coefficients of variation (CV) for each ELISA. CV limits were deemed acceptable at <10%.

2.4. Statistical analysis

The clinical cohort characteristics were analysed with either chi square or Mann-Whitney U test. Complement activation marker concentrations were determined from standard curves plotted using nonlinear regression. The units of concentration for all complement markers shown are nanogram (ng) or microgram (µg) per millilitre. The complement marker values of the samples were not normally distributed for all markers tested except Bb, therefore Spearman correlation was used to identify correlations between complement activation marker levels and EDTA-to-freezer time or freezer storage time. Analyses were conducted for data available for the two variables with n=169 samples for the EDTA-to-freezer time analysis, and n=178 samples for the storage time analysis for the CHR cohort, and n=80 for the storage time analysis and n=76 for the EDTA-to-freezer time analysis for the healthy controls (HC). Testing the difference between two correlations for HC and CHR groups was done by equality of independent Spearman rho correlation (Supplementary methods). All analysis were done in GraphPad Prism version 9 or IBM SPSS Statistics.

3. Results

Demographics and sample characteristics of participants are summarized in Table 1. No significant difference in age, sex, or cannabis use was observed. HC had significantly lower smoking status, antidepressant and antipsychotic medication use relative to CHR. There was no difference in EDTA-to freezer time (hours) or sample storage time (months) when comparing HC with CHR.

Processing time (EDTA-to-freezer) ranged for CHR samples <1–7.4 hours, and for HC samples <1–6.6 hours. The storage time for plasma samples in the -80°C freezer ranged from 9.5 to 13.6 years for CHR and 9.75–13.6 years for HC.

The intra-assay CV was <5% for all ELISA assays (C4a (3.78%), C4d (3.8%), C3a (3.02%), iC3b (2.113%), Bb (2.1%), C5a (2.5%), and sC5b-9 (TCC) (2.8%)), and the inter-assay CV (n=12 plates) was under 10% for all assays (C4a (5.48%), C4d (6.62%), C3a (3.45%), iC3b (5.449%), Bb (2.25%), C5a (3.13%), and sC5b-9 (TCC) (4.67%)).

Complement activation fragment plasma levels are shown as concentration mean, median, and standard deviation in Table 2.

Table 1
Study cohort characteristics.

Characteristic	CHR	HC	
	N=180[a,b]	N=80[a,b]	p
Age, mean (sd), years	17.9 (3.4) (9.8)	18.5 (3.6)	0.313
Sex, % (n) male	55 (99)	44 (35)	0.0896[a]
Smoking status, % (n)	25(46)	5(4)	**<0.0001[a]**
Cannabis use, % (n)	24(45)	16.25(13)	0.117776
Antidepressants use, % (n)	28(51)	1.25(1)	**<0.0001[a]**
Antipsychotics use, % (n)	19.4(35)	0(0)	**<0.0001[a]**
EDTA-to freezer time (hours), mean (sd)	1.01 (0.9)	0.97 (1.2)	0.7728[b]
Sample storage time (months), mean (sd)	11.9 (1.01)	11.9 (0.88)	1.000[b]

CHR = Clinical high-risk, HC = Healthy Controls. *Significant at p<0.05, **Significant at p<0.01, ***Significant at p<0.001; aChi-square test was performed for differences in sex and smoking status, Cannabis, Antidepressant and Antipsychotic use. Data available for smoking status, CHRn=178, HCn=79 due to missing data; bfor EDTA to freezer time, CHR n=169, HC=76 and storage time CHR n=178, HC n=80 due to missing data points.

Table 2

Plasma concentration means and range for complement activation markers. Protein levels (ng/ml and µg/ml) of complement activation markers (C4a, C4d, Bb, C3a, iC3b, C5a, and sC5b9 (TCC)) were quantified in EDTA-plasma samples in clinical high-risk (CHR) individuals (n=180) as aof secondary analysis of the NAPLS2 study, and compared to healthy controls (HC), n=80. Mean±Std. deviation and median with interquartile range are shown for each complement activation marker.

	C4a (ng/ml)	C4d (µg/ml)	Bb (µg/ml)	C3a (ng/ml)	iC3b (µg/ml)	C5a (ng/ml)	sC5b-9 (ng/ml)
Clinical high-risk individuals (CHR) - NAPLS2 (n=180)							
Minimum	286.0	1.55	0.3399	33.72	4.029	1.711	116.2
25% Percentile	412.2	3.72	0.9683	87.10	13.78	5.591	193.8
Median	516.1	5.06	1.163	148.8	32.00	8.243	234.0
75% Percentile	715.3	7.66	1.368	208.1	57.71	10.90	281.2
Maximum	4228	20.94	7.833	2614	494.4	19.92	2775
Mean	737.9	6.23	1.241	246.9	47.03	8.419	262.4
Std. Deviation	632.8	3.81	0.6006	387.7	60.05	3.757	209.2
Std. Error of Mean	47.17	0.28	0.04477	28.90	4.476	0.2801	15.59
Healthy controls (n=80)							
Minimum	333.1	1.899	0.6461	127	19.82	3.119	130.7
25% Percentile	413.5	3.758	0.942	194.3	35.87	6.212	190
Median	504.8	6.013	1.056	233.1	45.05	8.953	244.4
75% Percentile	694.7	8.177	1.244	294.9	61.71	11.04	304.8
Maximum	1785	26.39	2.652	1265	189.2	18.28	3514
Mean	655.5	6.552	1.098	298.3	53.96	8.799	311.6
Std. Deviation	401.6	3.862	0.2759	222.1	31.1	3.412	387.2
Std. Error of Mean	44.9	0.4317	0.03085	24.84	3.478	0.3814	43.29

Spearman correlation was used to correlate EDTA-to-freezer time, as a measure of sample processing time, with the concentration of each activation marker. All samples combined (CHR and HC) showed a weak, but statistically significant, correlation for C4a (r=0.1579, p=0.0133), C3a (r=0.1371, p=0.0319) and sC5b-9(TCC) (r=0.1345, p=0.0354). Within the groups, CHR samples showed a significant positive correlation between EDTA-to-freezer time and plasma concentration of complement C4a (r=0.2514, p=0.001), C3a (r=0.2570, p=0.0007), iC3b (r=0.1795, p=0.0196), and sC5b-9 (TCC) (r=0.1894, p=0.0137). We further noted differences in EDTA-to-freezer correlation with complement concentration when differentiating between CHR with different outcome of CHR transition to psychosis (Supplementary Table 1). In the HC samples, no significance correlation was found with EDTA-freezer time, but near significance - or a similar trend - was observed for C3a, as shown in Table 3. When comparing the correlations between groups (CHR, or HC), only C4a showed a difference between group correlations (α=0.047487572).

Next, we analysed the correlation of storage time (months) at -80°C with each complement activation marker concentration. Neither the combined sample (CHR and HC), nor individual CHR and HC groups, showed a significant correlation of storage time with any of the complement activation markers, as summarised in Table 4.

Overall, our results show that storage time at -80°C over a period of 9.5–13.6 years was not affecting complement activation marker levels in our EDTA-plasma samples. On the other hand, the initial sample processing (EDTA-to-freezer) time did correlate with complement activation markers, C4a, C3a, iC3b and sC5b-9(TCC) in the CHR sample, also highlighted in Fig. 1. No EDTA-to-freezer time correlation was observed in the HC plasma, nor in the subgroup analysis when differentiating by outcome.

4. Discussion

We measured seven complement activation markers spanning all complement pathways in EDTA-plasma samples of CHR individuals and healthy controls as part of a post hoc analysis of extrinsic factors (co-variables) to test if these would affect the measurements of complement activation markers in plasma. In the CHR samples we only found a

Table 3

Correlation analysis of complement activation markers with EDTA-to-freezer time. Spearman correlation analysed the relationship of complement activation markers (C4a, C4d, Bb, C3a, iC3b, C5a, and sC5b9 (TCC)) levels with sample processing time in all NAPLS2 study cohort samples, clinical high-risk (CHR) samples, and health controls (HC). The EDTA-to-freezer time ranged for the full cohort min-max: 0–7.350 hours, mean: 0.9970±1.062 hours, for CHR min-max: 0–7.350 hours, mean: 1.010±0.9846 hours, for HC min-max: 0–6.600 hours, mean: 0.9691±1.224 hours).

NAPLS2 cohort	EDTA to freezer vs. C4a (ng/ml)	EDTA to freezer vs. C4d (µg/ml)	EDTA to freezer vs. Bb (µg/ml)	EDTA to freezer vs. C3a (ng/ml)	EDTA to freezer vs. iC3b (µg/ml)	EDTA to freezer vs. C5a (ng/ml)	EDTA to freezer vs. sC5b-9(TCC) (ng/ml)
Pathway	CP/LP	CP/LP	AP	Common pathway	Common pathway	TP	TP
Clinical high-risk individuals (n=169)							
r	0.2514	0.1202	0.08411	0.2570	0.1795	-0.05170	0.1894
95% confidence interval	0.1000–0.3915	-0.03581–0.2705	-0.07219–0.2364	0.1059–0.3965	0.02480–0.3257	-0.2054–0.1045	0.03507–0.3349
P (two-tailed)	**0.0010*****	0.1195	0.2769	**0.0007*****	**0.0196***	0.5044	**0.0137***
Healthy controls (n=76)							
r	-0.02965	0.009671	0.1111	0.219	0.2107	-0.06771	0.04816
95% confidence interval	-0.2598–0.2036	-0.2227–0.2410	-0.1240–0.3343	-0.01355–0.4291	-0.02225–0.4220	-0.2950–0.1668	-0.1858–0.2770
P (two-tailed)	0.7993	0.9339	0.3394	0.0573	0.0677	0.5611	0.6795
All samples combined (n=245)							
r	0.1579	0.07654	0.1168	0.1371	0.1148	-0.07541	0.1345
95% confidence interval	0.02952–0.2812	-0.05298–0.2035	-0.01237–0.2422	0.00825–0.2615	-0.01439–0.2403	-0.2024–0.05411	0.00561–0.2590
P (two-tailed)	**0.0133***	0.2326	0.068	**0.0319***	0.0728	0.2396	**0.0354***

EDTA-to-freezer time was available for n=169 (CHR) and n= 76 (HC) samples. P<0.05 were considered statistically significant and are indicated as * P ≤ 0.05 ** P ≤ 0.01 *** P ≤ 0.001 and highlighted in bold, ns: not significant. CP: Classical pathway, LP: Lectin pathway, AP: Alternative Pathway, TP: Terminal Pathway.

Table 4

Correlation analysis of complement activation markers with storage time. Spearman correlation analysed the relationship of complement activation fragment (C4a, C4d, Bb, C3a, iC3b, C5a, and sC5b9(TCC)) levels with storage time at -80°C (months since collection) in all NAPLS2 study cohort samples, clinical high-risk (CHR) samples, and health controls (HC).

NAPLS2 cohort	Months since collection vs. C4a (ng/ml)	Months since collection vs. C4d (µg/ml)	Months since collection vs. Bb (µg/ml)	Months since collection vs. C3a (ng/ml)	Months since collection vs. iC3b (µg/ml)	Months since collection vs. C5a (ng/ml)	Months since collection vs. sC5b9(TCC) (ng/ml)
Pathway	CP/LP	CP/LP	AP	Common pathway	Common pathway	TP	TP
Clinical high-risk individuals (n=178)							
r	0.00005854	-0.04324	-0.0156	-0.01785	-0.03708	-0.05801	-0.09257
95% confidence interval	-0.1513–0.1514	-0.1933–0.1088	-0.1666–0.1361	-0.1688–0.1339	-0.1874–0.1149	-0.2076–0.09418	-0.2406–0.05963
P (two-tailed)	0.9994	0.5666	0.8362	0.8131	0.6232	0.4418	0.2191
Healthy controls (n=80)							
r	0.1609	0.09027	0.1871	0.1113	0.08896	0.043	0.1202
95% confidence interval	-0.06757–0.3733	-0.1385–0.3099	-0.04061–0.3963	-0.1176–0.3291	-0.1398–0.3087	-0.1848–0.2664	-0.1087–0.3371
P (two-tailed)	0.154	0.4258	0.0965	0.3255	0.4326	0.7049	0.2881
All samples combined (n=258)							
r	0.0476	-0.02215	0.06742	-0.07297	-0.05081	-0.03956	-0.04865
95% confidence interval	-0.07857–0.1723	-0.1474–0.1038	-0.05878–0.1915	-0.1969–0.05321	-0.1754–0.07537	-0.1644–0.08657	-0.1733–0.07752
P (two-tailed)	0.4465	0.7233	0.2806	0.2428	0.4164	0.527	0.4365

Storage time data was available for n=178 CHR and n=80HC. P<0.05 were considered statistically significant and are indicated as * P ≤ 0.05 ** P ≤ 0.01 *** P ≤ 0.001 and highlighted in bold, ns: not significant. CP: Classical pathway, LP: Lectin pathway, AP: Alternative Pathway, TP: Terminal Pathway.

moderate (r<0.26), but significant, positive correlation with processing time (EDTA-to-freezer) for C4a, C3a, iC3b and sC5b-9 (TCC), but no correlations with long term freezer storage. No impact of sample processing time nor storage time was seen in the healthy control samples.

A similar study reporting a correlation of storage time with complement components and activation fragment concentrations in EDTA-plasma was reported by Morgan and colleagues (Morgan et al., 2017). They quantified complement C3, FI, FB, FD, C5, sCR1 and activation markers C3a, iC3b, Bb and TCC and found a strong positive correlation with -80°C storage time, while FH, C1q, and C1inh correlated negatively. Long-term storage time (6–10 years) was similar to our study, with no reported freeze-thaw cycles. In contrast, our study quantifying the same complement activation markers (C3a, iC3b, Bb and TCC/sC5b-9), showed no correlation with -80°C storage time. While different ELISA assays were used for the quantification, a key difference may be the age of the study population. While our study generally comprised samples from young individuals aged 12–35, Morgan et al. analysed complement markers in elderly individuals (>70 years), many with significant pathology (n=262 with Alzheimer's disease, n=199 with mild cognitive impairment, and n=259 elderly controls with no dementia). Aging, in healthy individuals has been shown to increase the levels of complement proteins (Gaya da Costa et al., 2018a). It has been suggested that increased complement levels alone may make plasma samples more vulnerable to ex-vivo complement activation due to the higher presence of complement activators or other sample dysregulating factors (Prohászka et al., 2018). Our analysis of the healthy cohort (HC) samples resulted in no correlation for either EDTA-to-freezer, nor storage time. However, the effect size and trend in p-value may suggest that this could be due to power and sample size. Nevertheless, another study using samples from healthy individuals did see similar results to ours, observing no significant correlation of storage time with complement activation markers C3a, C4d, C5a, TCC(C5b-9) and Bb (Yang et al., 2015) in long-term freezer storage of 88 plasma samples from 51 larger sample (n=180) and assessing a wider range of complement markers. While increased complement levels may contribute to ex-vivo complement activation (Prohászka et al., 2018), factors that affect increased complement component or activation marker levels, such as age of the patients and their particular pathology may need to be considered when assessing storage time as a possible co-variable for data analysis in

cohort studies.

4.1. Evidence from in vitro or mechanistic studies

When investigating complement levels in relation to pre-storage and post-storage factors (Mollnes et al., 1988), such as different anti-coagulants or specimen preparations, this did not impact total C3 levels. In our hands, total C3 levels was measured as part of further analysis (data not shown) and were not affected by EDTA-to-freezer time, nor storage time. While our samples were stored at -80°C post processing, others have reported that storage conditions can affect complement activation over 1–2 days when the sample was stored at different temperatures 4 °C or 37 °C. The addition of EDTA, heparin, and citrate plasma showed no differences, while serum had increased baseline values (Mollnes et al., 1988). Serum and plasma, depending on their storage and handling, can have different uses when it comes to complement activation studies (Moghimi and Simberg, 2022), while plasma is more preferred for studies where triggering clotting may affect complement (). Overall, EDTA is more effective in preserving the integrity of C3 at 37 °C (Mollnes et al., 1988). Focusing on the terminal pathway, it was found that when the samples are stored at 4 °C, there is no increase in TCC for up to 10 days, however serum and plasma concentrations still differed, and serum sC5b-9 values were double as high as plasma. When looking at long-term storage time, samples of 40 healthy volunteers were stored for 3 years at -80°C, and then tested versus a similar healthy cohort for C3 activation products, namely C3b, iC3b, and C3c. Neither storage time at -80°C, nor (up to 4) freeze-thaw cycles affected the levels of TCC and C3 activation products (Mollnes et al., 1988). This agrees with our findings regarding storage time and their effects on sC5b-9. However, that study was only measuring one activation marker and the conditions of pre-storage sample handling differed by monitoring the effects of both temperature and time (days) in 3 differently prepared plasma samples.

4.2. Evidence from sample collection and storage condition studies

Sample processing has also been shown to affect complement levels. We have found moderate, but positive and significant correlation of sample processing time (EDTA-to-freezer time within a range of

0–7.35 hours affecting complement markers C4a, C3a, iC3b and sC5b-9 (TCC). Another study (Dufresne et al., 2017) focusing on C4B, showed that tryptic peptides are cleaved from C4B at room temperature conditions over an hour. On the contrary, samples stored immediately at -80°C did not show any cleaving and peptide releasing. Further, another study (Yang et al., 2015) showed that plasma and serum differ in their stability of complement markers C3a and C4d. The same study found that a pre-storage time of around 4 hours did not affect C4a, C3a, Bb, C5a, or TCC if these were stored in EDTA tubes with a final EDTA sample concentration of >10 mM. It was also shown that freeze-thaw cycles, when thawing at 37 °C, affected markers depending on the anticoagulant used, with citrate plasma showing higher complement activation than EDTA-plasma. If the freeze/thaw cycles were done on ice or room temperature, there were no substantial differences except for C4d. which increased in all sample types (serum, citrate plasma, EDTA-plasma) after two freeze/thaw cycles, whereas if the thawing was done on ice C4d levels increased after 3 freeze/thaw cycles. In our study, sC5b-9 (TCC), C3a, and C4a in EDTA-plasma samples were moderately affected by processing time in CHR, and underwent less than 3 freeze/thaw cycles prior to analysis. The same complement activation markers were not influenced in the HC samples, which could be due to sample size, or differences in other complement component levels, which in turn may influence the levels of the activation markers. In addition, factors such as the tube type used for collection of the initial whole blood sample and the thawing temperature of samples may influence the stability of the circulating proteins (Lee et al., 2015).

The effects of EDTA-to-freezer time can theoretically be modelled and accounted for by introducing a correction factor for storage time. This however does not guarantee the removal of the confounding factors (depending on subsequent data transformations (Qiu et al., 2005)), and may in fact introduce other sources of bias (Pain et al., 2018). Conversely, removing a sample could affect downstream analysis such as reducing the power of the sample and may result in the accidental removal of natural variation assumed to be an outlier.

4.3. Strengths and limitations

This study has its strengths and limitations. The number of samples used is robustly provide evidence of an effect of a potentially confounding factor affecting complement activation marker levels. This is the first study that quantified complement activation markers relevant to all pathways in a large multi-centre clinical high-risk for psychosis cohort as well as healthy controls samples that were taken at the same time. The results we present here did not undergo removal of potential outliers or normalisation versus other confounders to represent real-world samples. No other complement components were included in the measurements, as our study focused on complement activation markers alone (although C3 was quantified in a secondary analysis, which did not show any correlations). Finally, rank order analyses (Spearman) were used to ensure that our investigations were not subject to skewing from such data points.

The study we report here is not without its limitations. We had one sample per individual at a single time point available and we tested complement activation marker levels using a distinct ELISA kit for each marker. Further, our data focused on one cohort of clinical-high risk individuals (and healthy controls collected at the same time) only and our data do not exclude the possibility that complement activation fragment levels are different in other cohorts. Additionally, within our study cohort we may expect pathophysiological differences in baseline complement components levels, which may affect their activating capacity through specific or non-specific mechanisms and therefore overall complement activation fragment levels (Heurich et al., 2022).

We found different complement activation fragment levels between CHR and HC in our study, which implies that these samples are inherently different, but independent of EDTA-freezer time (p=0.0703, CHR: HC).

The demographics of CHR and HC, including the NAPLS2 cohort are further discussed in Healy et al. (Healy et al., 2024). Complement levels and activity could be influenced by age and sex (Gaya da Costa et al., 2018a). In this study, we observe no differences in age or sex comparing HC with CHR. No significant differences at baseline in cannabis use was seen, although there are differences in medication exposure and smoking status, and these may impact complement protein levels and activation (Kokelj et al., 2021; Susai et al., 2023; Wyatt et al., 1981). Differences in sample numbers between the CHR and HC were noted and may have resulted in the HC results to be underpowered. This could affect the correlation of markers C3a and iC3b with sample processing, as these groups show a similar r value, but wider Cis in the HC, which may be contributing to p being above the cut-off point. The effects seen for C4a and sC5b-9 could be due to a different immune profile between CHR and HC, which has been shown for other inflammatory markers (Mondelli et al., 2023), but also for complement markers (Zhang et al., 2023). Furthermore, neither the EDTA-to-freezer time data nor the storage time data were equally distributed among the samples, which may mask or over-emphasize their effect on complement markers.

CHR individuals are a specific subgroup of the general population with unique characteristics (Yung et al., 2004), and it is important to point out that CHR individuals who do not transition to psychosis differ clinically from healthy controls (Addington et al., 2019). Indeed, a recent similar study of complement markers in CHR as well as those individuals with first episode psychosis also found that storage time was not associated with complement levels in serum but was negatively associated in cerebrospinal fluid (CSF) (Cropley et al., 2023). They showed that protein levels in CSF did not statistically differ between groups, but were affected by sample storage time. Across the whole sample, serum and CSF protein levels were not correlated. While this study did not focus on complement activation markers and was conducted in serum samples, their findings overall support our conclusions that complement activation markers in plasma are largely unaffected by prolonged freezer storage time.

Further, the limited age range of the cohort is due to the young age range during which most CHR individuals are diagnosed (Fusar-Poli et al., 2013). In a larger cohort analysis, we (Healy et al., 2024) observed a small, but significant difference between the HC and CHR in terms of age, but with small effect sizes. No significant age difference was observed in our NAPLS2 samples. Therefore, our findings in relation to CHR may only be relevant to similarly aged cohort studies.

To be able to evaluate the impact of sample processing and long-term sample storage in complement studies, we therefore strongly recommend that standard operating procedures include sample collection and processing time, as well as freeze-thaw cycles and long-term storage time, when possible. Sample collection for complement analysis should aim for sample processing time below one hour and using the same conditions (e.g. temperature of sample preparation and processing, same EDTA concentration in tubes etc.). EDTA-to-freezer time should be considered for inclusion in the data analysis when assessing co-variables analysing complement component and activation markers levels in plasma samples, in particular a correlation analysis with storage characteristics. Serum and different plasma preparations (e.g. EDTA, citrate, etc.) can either be used for complement markers analysis, but when quantifying complement activation markers, EDTA-plasma should be used whenever possible to reduce non-specific activation (Moghimi and Simberg, 2022). Overall, it is acceptable for a variable to be associated with the exposure, provided it is not associated with the outcome, nor does it influence the exposure-outcome relationship. Finally, when assessing co-variables for data analysis in large cohort studies, factors that affect intrinsic complement component or activation marker levels, such as age of the study cohort and the particular pathology may need to be considered when assessing storage time as a co-variable. Further, investigating comorbidities and symptom profiles in relation to complement markers would be a useful addition in the future in a larger sample cohort. The relationship between medication exposure with

complement activation was not accessible in our samples. However, this an interesting question; we have shown that treatment response to amisulpride in first-episode psychosis was associated with some complement proteins being elevated (Susai et al., 2023), and this should be addressed in future cohort studies.

5. Conclusions

When measuring complement activation markers in clinical samples, factors such as sample processing and storage time should be included in the analysis. We report that sample processing times (EDTA-to-freezer time) prior to freezer storage at -80°C moderately influence the levels of some complement activation markers in a sample cohort of clinical-high risk individuals of psychosis. This is not necessarily reflected in a healthy cohort, where we did not observe any correlation with any complement marker levels. Further, our data suggest that there is no significant complement activation in our EDTA-plasma samples after long-term storage at −80°C.

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CRediT authorship contribution statement

Meike Heurich: Conceptualization, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. **David Cotter:** Writing – review & editing. **Diana O Perkins:** Writing – review & editing. **Mary Cannon:** Writing – review & editing. **Melanie Foecking:** Writing – review & editing. **Jonah F Byrne:** Writing – review & editing. **Colm Healy:** Writing – review & editing. **Eleftheria Kodosaki:** Formal analysis, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bionps.2024.100097.

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