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Complement factor H and factor H-related protein 3 levels during infectious meningococcal disease

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In preparation

Abstract

The causative agent of meningococcal disease (MD), *Neisseria meningitidis,* binds human complement factor H (FH) to evade complement-mediated clearance upon infection. The proposed antagonist of FH, FH-related protein 3 (FHR-3), also binds to *N. meningitidis* and decreases its serum resistance *in vitro*. However, due to the low levels of FHR-3 in human circulation compared to FH, it is unclear if FHR-3 plays a major role during MD. In order to elucidate the role of FHR-3 and FH during the acute phase of MD, we performed a retrospective study using serum samples of 90 MD patients. Levels of FH and FHR-3 were determined during the first days of infection and compared to severity markers and bacterial load. We show that both FH and FHR-3 levels are decreased during infection but that FH levels correlate more strongly with severity markers of MD. We could not elucidate a prominent role for FHR-3 as antagonist of FH in MD.

Introduction

Neisseria meningitidis is a Gram-negative human commensal carried in the nasopharynx by a large part of the population[1–3]. Upon infection, it can cause meningococcal meningitis and/or septicemia, collectively called meningococcal disease (MD). MD is a severe, debilitating and life-threatening disease with an occurrence of 2-20 per 100.000 in developed countries[4,5]. The complement system plays a major role in MD. Deficiencies in components of its terminal pathway have been associated with recurrent *N. meningitidis* infection[6,7]. Furthermore, *N. meningitidis* is known to exploit human complement regulator factor H (FH) by binding it to its surface via a specialized protein called factor H-binding protein (fHbp)[8]. This 'hijacking' of FH aids *N. meningitidis* in avoiding complement-mediated clearance, prolonging its survival in the human circulation[8–10]. Not surprisingly, a genome-wide association study identified an association between genetic variation in *CFH* and MD susceptibility[11]. This study also identified variations in *CFHR3*, a gene encoding for the FH homolog FH-related (FHR) protein 3, associating with MD susceptibility. This was the first indication that next to FH, the FHR proteins might also play an important role in MD.

FH is the major negative regulator of the alternative pathway of complement activation and is composed entirely of 20 complement control protein (CCP) domains. FH is crucial in protecting human cells from complement-mediated damage. FH contains two major regions involved in the binding to human cells, the first located in CCP6-8 and the second in CCP19-20[12]. Meningococcal fHbp binds FH at CCP6 and 7, mimicking the binding of FH as it would occur on a human cell while leaving the complement regulatory capabilities of FH intact[13].

There are six FHR proteins described, encoded by five genes which lay in tandem with *CFH* on 1q31.3. The FHRs are homologs of FH, with high sequence identity with the ligand binding regions of FH: CCP6-8 and CCP19-20. The FHRs lack CCP domains homologous to FH CCP1-4, known to be involved in complement regulation[14,15]. Thus, FHRs are hypothesized to act as antagonists of FH, allowing instead of inhibiting complement activation on the cell surface[16]. Of all FHRs, FHR-3 has the highest sequence identity with FH CCP6 and CCP7 (91% and 85%, respectively). Indeed, FHR-3 was found to also bind to fHbp *in vitro*, competing with FH for binding[17]. As FHR-3 lacks complement regulatory activity, binding of FHR-3 to *N. meningitidis* decreased its survival in human serum *in vitro*, which is suggested to explain the previously identified genetic association of *CFHR3* with MD. However, of note, the binding to and effect of FHR-3 on *N. meningitidis* was only demonstrated using concentrations that are 1,000-fold higher than the physiological FHR-3 concentration[18,19]. *In vivo* proof of competition between FH and FHR-3 at their physiological ratio or any indications for another role for FHR-3 during MD are still lacking.

Having recently established the first FHR-3-specific ELISA[18], we performed a retrospective study using samples of 90 MD patients drawn during the first days of infection to investigate the physiological role of FHR-3 during MD. We measured FH and FHR-3 levels during the acute phase of disease and compared those with levels after convalescence, *N. meningitidis* serogroup, diagnosis and severity parameters. When compared with FHR-3, the results of our current study suggest that FH plays a predominant role in MD severity although FH and FHR-3 seem to have equally low levels during the acute stage of infection.

Materials and Methods

Study cohort

The current study is a retrospective cohort study for which the samples have been collected at St. Mary's Hospital, London (UK) between 1992 and 2003, as part of previous studies. Full details of this cohort have been described previously[9,11,20–22]. All samples were obtained with informed consent of the parents or guardians of each patient according to the local ethics committee and the Declaration of Helsinki. Samples both at the time of acute infection and after recovery were stored at -80°C until used. Cases of confirmed meningococcal disease were selected based on the presence of at least one acute serum sample (admission or one day after admission) and a serum sample taken after convalescence.

Serology

C-reactive protein (CRP) levels, white cell count (WCC), platelet count, activated partial thromboplastin time (aPTT), international normalized ratio (INR), base excess and fibrinogen levels were all determined as part of routine diagnostics at St. Mary's Hospital, London, UK. Glasgow meningococcal septicemia prognostic score (GMSPS) and pediatric risk of mortality (PRISM) score were determined as previously described[23,24].

ELISAs

FH and FHR-3 levels in serum were determined by highly specific ELISAs as previously described[18]. In short, FH was measured by ELISA using an in-house developed, highly specific mouse monoclonal antibody (mAb) directed against CCP domains 16/17 of FH (clone anti-FH.16, Sanquin Research, Amsterdam, The Netherlands), as the capture mAb and using polyclonal goat anti-human FH (Quidel, San Diego, CA, USA), which was HRP-conjugated in house, as the conjugate. For the specific detection of FHR-3, an in-house developed mAb directed against FHR-3 and FHR-4A (clone anti-FHR-3.1, Sanquin Research) was used as the capture antibody. A biotinylated mAb directed against FHR-3 and FH (clone anti-FHR-3.4, Sanquin Research) was used as conjugate.

Detection and quantification of *Neisseria meningitidis*

Three sets of primers/probes widely used in molecular diagnostics laboratories targeting three different conserved genes (*metA*, *sodC* and *tauE*) in *N. meningitidis* were used for quantification by qPCR. Master mixes (LightCycler® 480 II Master mix, 2X conc. cat num. 04887301001, Roche, Basel Switzerland) contained 2.5 µL of primers (200 nM), 2.5 µL of probe (100 nM) and 20 µL of template DNA in a total volume of 50 µL reactions following manufacturer's recommendations. Initial denaturation of 95˚C for 7 min, followed by 50 cycles of 95˚C for 10 sec, 60˚C, for 40 sec and 72˚C for 1 sec) and a final cooling step at 40˚C for 10 sec was performed. Quantification was estimated using *N. meningitidis* DNA Standards copies/µL (dilution series of 1.15×10^{-6} to 1.15×10^{-3}) AMPLIRUN® DNA control (1.15×10^{-7} copies/µL). Amplification data were analysed by instrument software (Roche).

Statistical analysis

Data were analyzed and statistical analyses were performed using either GraphPad Prism, version 7.03 (GraphPad software, La Jolla, CA, USA) or R version 3.2.2[25]. Overall, $p < 0.05$ was regarded as statistically significantly different. Differences in protein levels between groups were assessed using the Mann-Whitney, Kruskal-Wallis, Wilcoxon rank sum or Friedman test when appropriate. Correlations between protein levels and continuous measures of severity were assessed using Spearman's rank-based measure of association, unless indicated otherwise.

Results

Study cohort

Of 90 pediatric meningococcal disease cases, an acute serum sample (admission or 1 day after admission) and a serum sample taken after convalescence were available (**Table 1**). Patient age ranged from 0-16 years at admission with a median age of 3 years and 42 out of 90 were male (46.7%). All 90 patients were Caucasian. The time at which samples were drawn after convalescence ranged from 10 to 2011 days after infection, with a median of 61 days

Table 1 Patients characteristics

	n	
Number of male cases (%)	90	42 (46.7)
Median age (y) at admission (IQR)	90	3.0 (1.3-7.9)
Median time interval (days) of convalescence sample drawn after admission (IQR)	90	61 (49-78)
Cases of meningococcal meningitis (%)	89	10 (11.2)
Cases of meningococcal septicemia (%)	89	63 (70.8)
Cases of meningococcal meningitis and septicemia (%)	89	16 (18.0)

n: number of informative cases; IQR: interquartile range

after infection. Of the 89 informative cases, 10 (11.2%) were diagnosed with meningococcal meningitis, 16 (18.0%) with meningococcal meningitis and septicemia and 63 (70.8%) with meningococcal septicemia. *N. meningitidis* serogroup was successfully typed in 60 cases, with serogroup B being the most prevalent (38 cases, 63.3%), followed by serogroup C (21 cases, 35%) and a single case of serogroup A infection (1.7%).

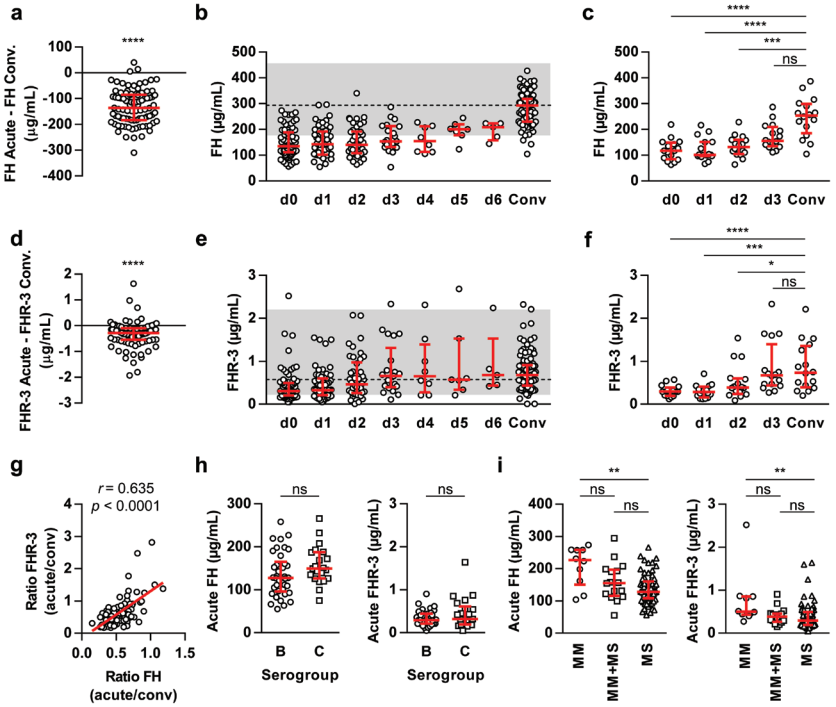

Figure 1 FH and FHR-3 levels during meningococcal disease. (**a**) Difference in FH levels between acute (day 0 or day 1) versus FH levels after convalescence (n = 90), calculated by subtracting paired levels after convalescence form acute FH levels. (**b**) FH levels during the first seven days of infection (day 0 to day 6). Shaded area indicates normal range in healthy donors, with dashed line indicating the median. (**c**) FH levels in paired samples (n = 16) during the first four days of infection (day 0 to day 3), compared with the FH concentration after convalescence. (**d-f**) Same samples and graphs as in (**a-c**) but with FHR-3 levels instead of FH. Shaded area in (**e**) indicates normal range in healthy donors with at least one copy of *CFHR3*, with dashed line indicating the median. (**g**) Scatter plot of acute/convalescent ratio in FH levels versus acute/convalescent ratio in FHR-3 levels. The two patients found to be deficient for FHR-3 were excluded from the analysis. Correlation was assessed using a Pearson correlation test (n = 88). (**h**) Acute FH (left graph) and FHR-3 (right graph) levels stratified by *N. meningitidis* serogroup B (n = 38) or C (n = 21) identified in the patients. (**i**) FH (left graph) and FHR-3 (right graph) associated with diagnosis. MM: meningococcal meningitis (n = 10); MS: meningococcal septicemia (n = 63); MM+MS: both (n =16). In all graphs except **g**, line with error bars indicate median with interquartile range. Statistical differences were assessed using a Wilcoxon Singed Rank test (**a** and **d**), Friedmann's test with Dunn's multiple comparison correction (**c** and **f**), Mann-Whitney (**h**) or Kruskal-Wallis test, with Dunn's multiple comparison correction (**i**). ns: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

FH and FHR-3 levels are decreased during acute infection

We measured FH and FHR-3 levels by ELISA in the selected 90 patients of which serum samples were present during the acute stage of disease and after convalescence. Convalescent serum levels of FH were within the normal range reported previously for healthy donors[26–29], with a mean FH concentration of 281.10 μg/mL (SD = 61.86 μg/mL). During the acute stage of disease (days 0 or 1 after admission), FH levels were significantly lower. On average, FH concentrations were 47% lower with a mean FH concentration of 148.90 μg/mL (SD = 54.79 μg/mL, $p < 0.0001$) (**Fig. 1a**). Samples were collected up to the first seven days of infection, allowing us to follow the FH concentration over time (**Fig. 1b**). The mean FH concentration remained low during the first seven days, but did show a slow increase over time. This became more apparent when looking at paired samples (n = 16) in the first four days of infection. Although FH concentrations could be considered low at day 3, they were no longer statistically different from the FH concentration at the convalescent stage (**Fig. 1c**).

Similar to FH, FHR-3 concentrations after convalescence were within the normal range[18,19] (mean 0.77 μg/mL, SD = 0.46 μg/mL). For two cases, no FHR-3 was detected in serum, presumably due to the relatively common deletion of *CFHR3*. Significantly lower FHR-3 levels were found during the acute phase of disease, with on average 43% lower FHR-3 with a mean concentration of 0.44 μg/mL (SD = 0.36 μg/mL, $p < 0.0001$) (**Fig. 1d**). However, FHR-3 levels over time show a different pattern compared to FH. Unlike FH, FHR-3 seemed to recover more quickly within the first seven days after infection (**Fig. 1e**). This was confirmed upon consideration of the paired samples only (n = 16), showing that FHR-3 levels had already fully recovered after three days (**Fig. 1f**).

Table 2 Correlation of acute FH and FHR-3 levels with clinical parameters

Parameters			MD patients	Acute [FH]		Acute [FHR-3]	
	Normal range	*n*	Median (IQR)	r_s	*p*	r_s	*p*
GMSPS	0	88	10 (6 - 12)	-0.366	<0.001	-0.149	0.166
PRISM	0	48	10 (6 - 15)	-0.302	<0.05	-0.283	0.051
CRP (mg/L)	5 - 10	81	96 (60.5 - 157.5)	0.341	<0.01	0.164	0.143
WCC (*10^9/L)	4.0 - 11.0	88	9.5 (3.8 - 21.9)	0.465	<0.00001	0.243	<0.05
Platelet count (*10^9/L)	150 - 400	88	198 (134.3 - 257.5)	0.262	<0.05	0.122	0.257
aPTT (sec)	30 - 40	83	45.5 (36.4 - 61.5)	-0.260	<0.05	0.010	0.927
INR	0.8 - 1.2	80	1.6 (1.3 -1.8)	-0.244	<0.05	-0.034	0.766
Fibrinogen (g/L)	1.5 - 4.0	73	3.1 (2.0 - 4.7)	0.461	<0.01	0.068	0.566
Potassium (mM/L)	3.5 - 5.0	87	3.5 (3.2 - 3.9)	0.100	0.357	0.002	0.982
BaseExcess (mEq/L)	-2 - 2	87	-6.8 (-10 - -4.9)	0.403	<0.001	0.309	<0.01

n: number of informative cases; GMSPS: Glasgow meningococcal septicemia prognostic score, ranges 0 - 15, with > 8 predicting fatal outcome[23]; PRISM: pediatric risk of mortality score, ranges 0 - 74 with higher values indicating higher risk[24]; CRP: C-reactive protein; WCC: white cell count; aPTT: activated partial thromboplastin time; INR: international normalized ratio; IQR: interquartile range. Correlations were assessed using a nonparametric Spearman's correlation test (rs).

FH and FHR-3 seemed to have equally decreased levels during the acute stage of infection. In fact, the relative decrease in FH and FHR-3 concentration at the acute stage compared to the convalescent sample correlated with each other (Pearson's r = 0.6351, n = 88, $p < 0.0001$) (**Fig. 1g**). We did not find any association of acute levels of FH or FHR-3 with serogroup B or C (**Fig. 1h**). For both acute FH and acute FHR-3 levels, lower levels did associate with meningococcal septicemia (n = 63) compared to patients diagnosed with meningococcal meningitis alone (n = 10) (**Fig. 1i**, $p < 0.01$).

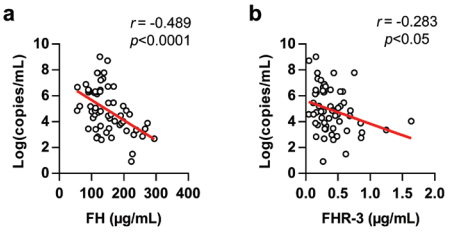

Figure 2 Acute FH and FHR-3 levels correlate with bacterial load. (**a**) Acute FH levels (Pearson's r = -0.489, *n* = 62, *p*<0.0001) and (**b**) acute FHR-3 levels (Pearson's r = -0.283, *n* = 62, *p*<0.05) plotted versus *N. meningitidis* copies/mL.

Low FH levels in the acute stage of disease correlate with severity and high bacterial load

Next, we investigated whether low acute levels of FH and FHR-3 are related to more severe meningococcal disease. To this end we assessed whether the acute levels correlated with several severity parameters previously determined at St. Mary's Hospital. These include the general prediction scores GMSPS and PRISM as well as more specific severity parameters such as white cell count, platelet count and CRP levels. FH correlated strongly with almost all severity parameters, most prominently with white cell count ($r_s = 0.465$, n = 88, $p < 0.00001$) and fibrinogen levels ($r_s = 0.461$, n = 73, $p < 0.01$) (**Table 2**). In general, low acute FH levels correlated with adverse values. Only for potassium levels no correlation with acute FH levels was found. In stark contrast with FH, FHR-3 acute levels only correlated with two severity markers, i.e. white cell count ($r_s = 0.243$, n = 88, $p < 0.05$) and base excess ($r_s = 0.309$, n = 87, $p < 0.01$).

Following the hypothesis that *N. meningitidis* binds FH and FHR-3 via fHbp, we investigated whether the decrease in FH and FHR-3 correlated with *N. meningitidis* bacterial load. Bacterial load was determined as average of the number of copies of *metA*, *sodC* and *tauE* per mL serum, measured by qt-PCR and ranged from $8.52*10^0$ to $1.04*10^9$ copies per mL serum (median = $5.68*10^4$). We found a negative correlation of both FH and FHR-3 with

bacterial load (Pearson's r = -0.487, n = 62, $p < 0.0001$ and Pearson's r = -0.305, n = 62, $p < 0.05$, respectively) (**Fig. 2a** and **b**).

Discussion

FH plays an important role in MD[30]. More recently, its smaller homologue, FHR-3, has also been implicated to play a role in (susceptibility for) meningococcal disease[11,17]. However, while FHR-3 has been suggested to compete with FH for the binding to *N. meningitidis* and may promote complement-mediated killing of the bacteria, it remains unclear whether this truly occurs *in vivo*, not in the last place due to the supra-physiological levels of FHR-3 required to affect meningococcal survival *in vitro*[17–19]. To date, no study has investigated FH and FHR-3 levels during the acute stage of meningococcal disease. By measuring FH and FHR-3 levels during MD in the first days after admission, we aimed to further elucidate the dynamics of the complement regulatory protein FH and its proposed antagonist FHR-3 during meningococcal disease in relation to disease severity.

For both FH and FHR-3, serum concentrations after convalescence in 90 pediatric MD patients were within the normal ranges previously established[19,26–29]. However, during the acute stage of disease, on day 0 (admission) or day 1 of infection, both FH and FHR-3 levels were markedly decreased. Compared to FH, FHR-3 levels seemed to recover more quickly, reaching normal levels within 4 days. This might indicate a difference in FH and FHR-3 consumption. However, the initial decrease in FH and FHR-3 was comparable (47% and 43% decrease, respectively) and correlated well. Alternatively, while FHR-3 does not seem to behave as an acute phase protein[18], FHR-3 expression might be increased more rapidly over several days during the infection. In addition, FH consumption might be more prolonged, possibly due to severe tissue damage during MD that activates complement, although one might expect this would also consume its antagonist FHR-3. Our observation that the decrease in FH and FHR-3 correlated well were unexpected in light of the current hypothesis that FHR-3 would compete with FH for binding to C3b or fHbp. However, it seems likely, that due to the ~140-fold excess of FH compared to FHR-3, such competition, if it occurs at all, is not measurable *in vivo*.

FH was found to correlate more dominantly with several severity markers than FHR-3. Lower FH levels during acute stage correlated with adverse values for the several severity markers. These include the more general disease prediction scores PRISM and GMSPS and highlight the pivotal role of FH in MD. In contrast, FHR-3 correlated only with some severity markers. These results show FH levels are more relevant during MD disease as compared to FHR-3, and question the proposed role of FHR-3 in MD.

Although this study allows for a better understanding of the role of FH and FHR-3 during MD, it has some limitations and raises several questions. For instance, we could not elucidate whether the lower FH and FHR-3 levels at the acute stage are causative or a consequence of a more severe disease. Furthermore, MD is a very severe disease in which tissues and several organs are affected, including the kidneys. It therefore cannot be excluded that (part of) the decrease seen in FH and FHR-3 levels during MD is not caused by binding to the meningococci but by secondary consumption due to tissue damage and subsequent complement activation. This seems to be reflected by the association of lower acute FH and FHR-3 levels with meningococcal septicemia, which is a more systemic disease compared to meningococcal meningitis. Alternatively, the decrease in FH and FHR-3 might be caused by leakage as a result of increased vascular permeability[31]. Indeed, total IgG levels showed similar decreases in 15 patients as seen for FH and FHR-3, indicative of increased vascular permeability (**Supplemental Fig. 1**). Furthermore, this study was performed retrospectively, including only pediatric MD patients who survived. We therefore cannot conclude anything on FH and FHR-3 levels in non-survivors. It may be expected that, as decreased FH levels correlated with severity, non-surviving patients have even lower FH levels during the acute stage of disease compared to the surviving patients reported here. A prospective study on infectious diseases in children including MD is ongoing to further address this.

For both FH and FHR-3 acute levels, we found a negative correlation with *N. meningitidis* bacterial load. Of note, it is unknown if we measure only freely circulating FH and FHR-3 (not bound by fHbp) or whether fHbp-bound FH and FHR-3 could still be detected by the antibodies used. Assuming we only measure freely circulating FH and FHR-3, the correlation with bacterial load could reflect consumption through binding by fHbp. More *N. meningitidis* would result in more binding of FH and FHR-3 and thus lower protein levels. This assumes that (a majority of) the decrease in FH and FHR-3 levels is indeed directly caused by binding to *N. meningitidis*. Furthermore, while this explains the correlation with FH, as binding of FH would also promote survival of *N. meningitidis* in circulation[9,10], binding of FHR-3 is expected to result in a decrease of survival and thus lower bacterial load[17]. Thus, this might indicate that FHR-3 binding to *N. meningitidis* does not have detrimental effects on its survival *in vivo*. Alternatively, if we do measure all FH and FHR-3 (unbound and bound by fHbp), the observed correlation with FHR-3 is explained by the theoretical function of FHR-3 but does not correspond with the known functional consequences of FH binding. More likely, these correlations are not directly linked to FH and FHR-3 binding to fHbp and further understanding of what happens with FH and FHR-3 after being bound by *N. meningitidis* in serum, and possible effects on their quantification, is necessary to fully interpret these results. As bacterial load does reflect severity[32], the found correlations are in line with the above described associations regarding acute FH and FHR-3 levels with severity markers.

In conclusion, we describe for the first time both FH and FHR-3 levels in MD patients during the acute stage of disease. Both proteins are significantly decreased in the same proportion during the acute stage of MD compared to their stable levels after convalescence. We could not find evidence of competition between FH and FHR-3 for binding to fHbp, nor did we find strong evidence for a major role for FHR-3 during MD. FH correlated with severity, underlining the pivotal role of FH in MD.

Author contributions

Study concept and design: RBP, DW, ML and TWK. Data acquisition and analysis: RBP, CH, VJW, MCB, JT and LC-B. Interpretation of data: all authors. Drafting of manuscript: RBP, DW and TWK. All authors critically revised the manuscript and contributed to the final version.

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Conflict of interest disclosure

All authors declare no relevant conflict of interest.

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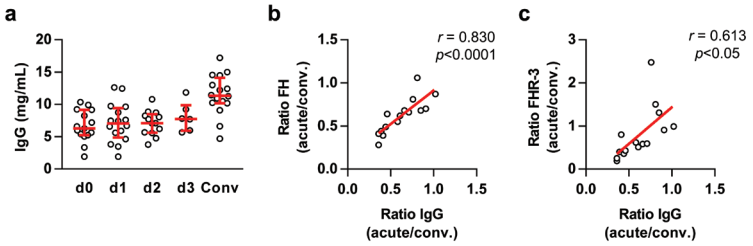
Supplemental materials



Supplemental Figure 1 IgG levels are decreased during meningococcal disease and correlate with FH and FHR-3 levels. (**a**) IgG levels during the first days of MD (n = 15). (**b**) Acute/convalescent ratio in IgG levels plotted against acute/convalescent ratio in FH levels. Correlation was assessed using a Pearson correlation test (n = 15). (**c**) Acute/convalescent ratio in IgG levels plotted against acute/convalescent ratio in FHR-3 levels. Correlation was assessed using a Pearson correlation test (n = 15).