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## Complement activation in chronic kidney disease and dialysis

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# CHAPTER 7

## **Distinct *In-vitro* Complement Activation by Various Intravenous Iron Preparations**

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## Abstract

### Background

Intravenous (IV) iron preparations are widely used in the treatment of anemia in patients undergoing hemodialysis (HD). All IV iron preparations carry a risk of causing hypersensitivity reactions. However, the pathophysiological mechanism is poorly understood. We hypothesize that a relevant number of these reactions are mediated by complement activation, resulting in a pseudo-anaphylactic clinical picture known as complement activation-related pseudo allergy (CARPA).

### Methods

First, the *in-vitro* complement-activating capacity was determined for 5 commonly used IV iron preparations using functional complement assays for the 3 pathways. Additionally, the preparations were tested in an *ex-vivo* model using the whole blood of healthy volunteers and HD patients. Lastly, *in-vivo* complement activation was tested for one preparation in HD patients.

### Results

In the *in-vitro* assays, iron dextran, and ferric carboxymaltose caused complement activation, which was only possible under alternative pathway conditions. Iron sucrose may interact with complement proteins, but did not activate complement *in-vitro*. In the *ex-vivo* assay, iron dextran significantly induced complement activation in the blood of healthy volunteers and HD patients. Furthermore, in the *ex-vivo* assay, ferric carboxymaltose and iron sucrose only caused significant complement activation in the blood of HD patients. No *in-vitro* or *ex-vivo* complement activation was found for ferumoxytol and iron isomaltoside. IV iron therapy with ferric carboxymaltose in HD patients did not lead to significant *in-vivo* complement activation.

### Conclusions

This study provides evidence that iron dextran and ferric carboxymaltose have complement-activating capacities *in-vitro*, and hypersensitivity reactions to these drugs could be CARPA-mediated.



## Introduction

A majority of patients with chronic kidney disease (CKD) receive intravenous (IV) iron for the treatment of anemia.<sup>1</sup> However, controversy exists regarding the safety of IV iron preparations since hypersensitivity reactions have been reported for all iron drugs.<sup>2</sup> Although these reactions appear sporadic, they can be acute and life-threatening. The exact frequency of the hypersensitivity reactions is unknown. This is attributed to a lack of data, due to underreporting and differential reporting.<sup>3</sup>

The underlying mechanism of hypersensitivity reactions by IV iron remains unclear. However, elucidating the pathophysiology is critical to improve prediction, prevention, and management of these adverse events. In contrast to the IgE-mediated anaphylaxis seen with the older IV iron compounds, hypersensitivity reaction by new IV iron preparations are thought to result from complement activation-related pseudo-allergy (CARPA).<sup>4,5</sup> Nonetheless, this has not been tested systematically. CARPA is an adverse event seen after administration of monoclonal antibodies, intravenously administered drugs, and nanoparticle-containing drugs.<sup>5,6</sup> CARPA was postulated since all available preparations consist of iron-carbohydrate nanoparticles.<sup>7</sup>

Activation of the complement system occurs via three pathways: the Classical Pathway (CP), the Lectin Pathway (LP) and the Alternative Pathway (AP). The CP is activated by antibody-antigen complexes, the LP by carbohydrates and the AP by microbial surfaces. This results in the formation of the C3- and C5-convertases and the generation of anaphylatoxins. Subsequently, the terminal pathway activation leads to the formation of the membrane attack complex (C5b-9).<sup>8</sup> In CARPA, such a cascade is initiated firstly by the generation of complement activation products; leading to the stimulation of mast cells and basophil granulocytes resulting in secretion products; which cause various responses in effector cells such as platelets, endothelial cells, and smooth muscle cells. Clinically, these processes may give rise to bronchospasm, laryngeal edema, tachycardia, hypo- or hypertension, and hypoxia.<sup>6</sup>

The aim of this study was to determine the effect of five currently available IV iron preparations on the complement system. By evaluating different IV iron drugs in an *in-vitro* and *ex-vivo* model for complement activation we intended to test for the probability of CARPA by IV iron drugs. Lastly, *in-vivo* complement activation was tested for one IV iron preparation in hemodialysis patients.

## Materials and methods

### Subjects

We recruited two groups:

1. Control subjects (5 to 10 per experiment, as indicated below).
2. Patients on maintenance hemodialysis (n=8). During one dialysis session, blood samples were taken at times 0, 120, and 240 minutes during dialysis. Patients received 100 mg/2 mL ferric carboxymaltose (Ferinject<sup>®</sup>) intravenous over 1h at 120 minutes into the dialysis session.

## Reagents

Iron sucrose (Venofer<sup>®</sup>) and ferric carboxymaltose (Ferinject<sup>®</sup>) were purchased from Vifor Nederland, Breda, The Netherlands. Ferumoxytol (Rienso<sup>®</sup>) from Takeda Nederland, Hoofddorp, The Netherlands. Low molecular weight iron dextran (CosmoFer<sup>®</sup>) and iron isomaltoside 1000 (Monofer<sup>®</sup>) from Cablon Medical, Leusden, The Netherlands.

For the whole blood experiments, lepirudin (Refludan<sup>®</sup>, Hoechst, Frankfurt am Main, Germany) was used as anti-coagulant.

## Normal human serum

Blood was taken from 10 healthy volunteers and directly stored on ice. Samples were centrifuged, then pooled and stored at -80°C until further analysis.

## Complement pathway activity in human serum

Functional assays were used to allow quantification of complement activation via the CP, the LP and AP in human serum. These assays were previously described.<sup>9</sup> In brief, 96-well plates were coated overnight with human IgM for the CP, mannan for the LP or LPS for the AP. Plates were washed three times after each step with PBS containing 0.05% Tween-20. Plates were blocked with 1% BSA in PBS for 1h at 37°C. Serum was diluted in gelatin veronal buffer (GVB) buffer adapted specifically for each pathway. For the CP and LP, serum was diluted in GVB with Ca<sup>2+</sup>-Mg<sup>2+</sup>. For the AP, serum was diluted in GVB with magnesium only. After 1h at 37°C, deposition of properdin, C4, C3, or C5b-9 was detected using rabbit anti-human properdin (obtained from the lab. of Nephrology, Leiden, The Netherlands), mouse anti-human C4 (obtained from the lab. of Nephrology, Leiden, The Netherlands), RfK22 (anti-human C3, obtained from the lab. of Nephrology, Leiden, The Netherlands) and AE11 (anti-human C5b-9, DAKO, Glostrup, Denmark), respectively. Binding of antibodies was detected using the appropriate primary and secondary antibody. For visualization TMB and H<sub>2</sub>SO<sub>4</sub> were added before the absorption was measured at 450 nm. Prior to incubation on the ELISA plate, all serum samples were pre-incubated at 37°C for 30 min with iron in a dose ranging from 0.0625 to 0.5 mg/ml. Next, samples were further diluted to the final concentration with the appropriate buffer.

## Complement activation assays by IV iron

For the complement activation assay, iron preparations or BSA were coated overnight on a 96-well plate followed by blocking with 1% BSA/PBS at 37°C for 1h. The wells were exposed to pooled human serum diluted in adapted GVB (see Normal human serum) or with EDTA (20 mM) for 1h at 37°C. The plate was then incubated with antibodies against properdin, C3 or C5b-9 (see Normal human serum). Detection was completed using appropriate primary and secondary antibody. The plate was washed with PBS Tween-20 (0.05%) between each step. Visualization was similar as described in Normal human serum.

## Complement pathway activity in human whole blood

The experimental set-up has previously been described.<sup>10</sup> In short, blood was drawn in LPS-free tube with 50 µg/ml lepirudin. Whole blood was then incubated for 0 min or 90 min at 37°C with IV iron

(0.5 mg/ml ferrous iron) while continuously rotated. PBS was added to the negative controls. The reaction was stopped with EDTA (final concentration of 20mM). Samples were then centrifuged and plasma was stored at -80°C until further analysis.

### **Quantification of the antigenic levels of C1q, C3d, C3 MBL, properdin and C5b-9**

The ELISA for C1q, C3d, C3 MBL, properdin, and C5b-9 were performed as described previously.<sup>11-13</sup>

### **Statistics**

Statistical analyzes were performed using BM SPSS Statistics Version 22 and P-values<0.05 were considered statistically significant. The Kruskal-Wallis test and Mann-Whitney U test were used to assess differences between groups of non-parametric data and one-way ANOVA and t-test for normally distributed data. If needed, data were ln-transformed for normality.

### **Ethics**

All participants gave informed consent. The Medical Ethical Committee of the University Medical Center Groningen has reviewed the study design and it was confirmed that an official approval of this study by the committee is not required since the Medical Research Involving Human Subjects Act (WMO) does not apply.

## **Results**

### ***In-vitro* effect of IV iron preparations on complement activity**

The interaction of the different IV iron drugs with complement was determined using functional complement assays for each pathway. Normal human serum (NHS) was pre-incubated with different IV iron drugs prior to the assay; subsequently, residual complement activity was measured. In this assay decreased residual activity reflects either activation or inhibition of complement by the IV iron compound during the pre-incubation period.

### ***Decreased residual activity of the Classical Pathway by iron sucrose***

First, residual complement activity was tested for the CP after incubation with the IV iron drugs (Table 1). Iron sucrose was the only preparation that significantly reduced residual complement activity. Furthermore, the effect of iron sucrose on CP activity (P=0,016) was dose-dependent (Figure 1). At a concentration of 0.5 mg/mL, iron sucrose reduced C4, C3 and C5b-9 deposition by 92%, 88% and 96%, respectively (P<0.001). For iron dextran, ferric carboxymaltose, iron isomaltoside and ferumoxytol, there was no change in residual complement activity, indicating low to no effect on the CP.

### ***Decreased residual activity of the Lectin Pathway by iron sucrose***

Next, residual complement activity for the LP was assessed (Table 1). Once again, iron sucrose significantly reduced residual complement activity in a dose dependent manner (P<0.001) indicating

prominent activation of the LP during the pre-incubation (Figure 1). Deposition of C4, C3 and C5b-9 were lowered by 88%, 95%, and 95% at 0.5 mg/mL for iron sucrose ( $P < 0.001$ ). For iron dextran, ferric carboxymaltose, iron isomaltoside and ferumoxytol, there was no change in residual complement activity for the LP.

Table 1 | Activation of complement components of the Classical, Lectin and Alternative Pathway by IV iron drugs

Residual complement activity <sup>1</sup>	IV iron preparations, %					
	control	iron dextran	ferric carboxy-maltose	iron isomaltoside 1000	ferumoxytol	iron sucrose
CP	100 ± 5.5	126.8 ± 23.4	94.3 ± 13.8	108.2 ± 3.9	110.8 ± 4.2	10.4 ± 4.5***
LP	100 ± 5.2	88 ± 4.4	88.7 ± 3.5	84.3 ± 2.4	91.4 ± 3.3	4.7 ± 0.4***
AP	100 ± 4.6	6.3 ± 0.9***	62.3 ± 11.8*	75 ± 14.5	89.2 ± 16.5	80 ± 16

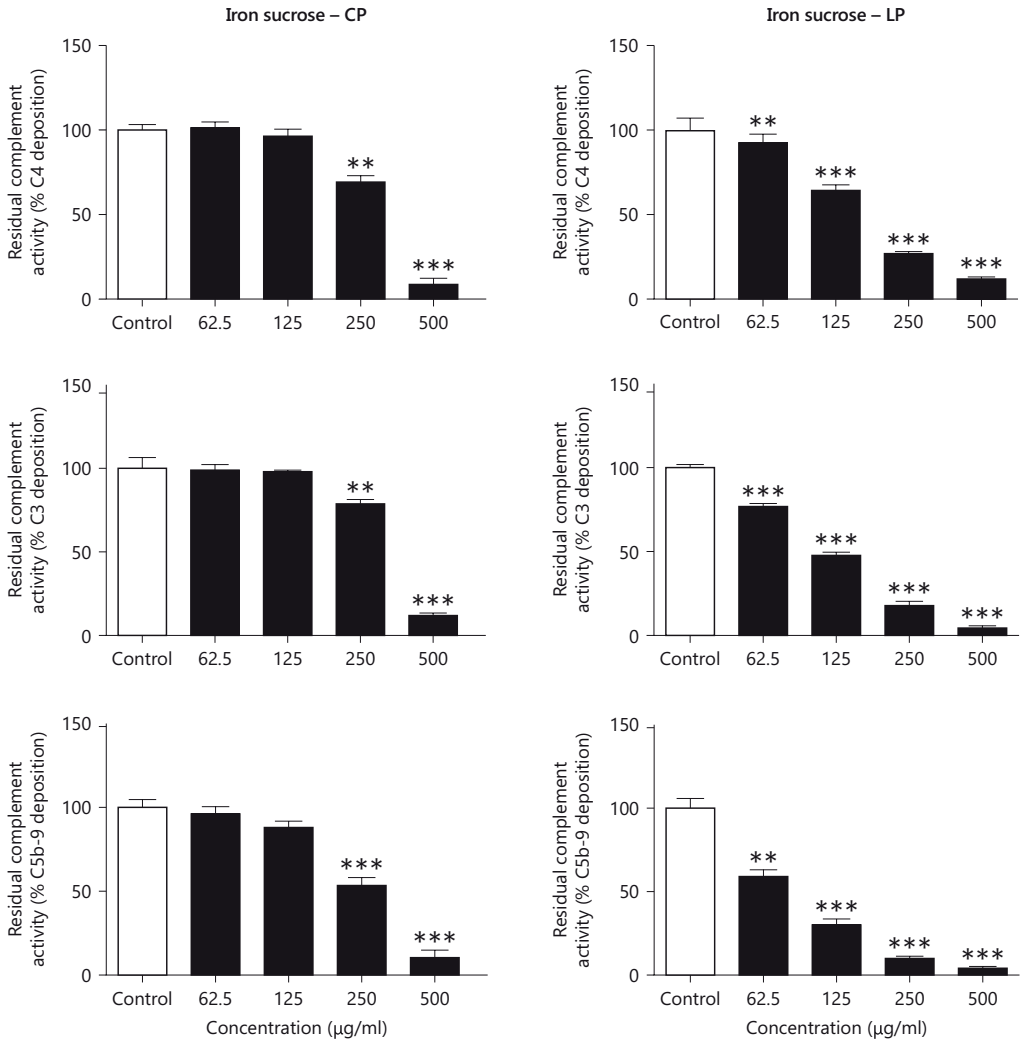
Complement activation <sup>2</sup>	IV iron preparations, %						
	positive control	iron dextran	ferric carboxy-maltose	iron isomaltoside 1000	ferumoxytol	iron sucrose	BSA
CP	100 ± 0.5	2.9 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	3.0 ± 0.1	3.0 ± 0.0	2.9 ± 0.1
LP	100 ± 3.4	3.1 ± 0.1	3.0 ± 0.1	4.1 ± 0.1	3.8 ± 0.3	4.0 ± 0.1	4.0 ± 0.1
AP	100 ± 8.1	138.5 ± 5.5***	122.2 ± 10.9**	9.1 ± 0.4	8.6 ± 0.3	8.1 ± 0.4	9.7 ± 2.1

<sup>1</sup> Pooled serum was pre-incubated with 0.5 mg/mL ferrous iron for 30 minutes at 37°C. PBS was used for the controls. The serum was then used in the functional assay for the classical, lectin or alternative pathway to measure residual activity. Deposition of C5b-9 was used as readout and the amount obtained in the control was set at 100% (y-axis).

<sup>2</sup> Iron preparations were coated overnight on a 96-well plate. The wells were exposed to pooled human serum diluted in a buffer adapted specifically for each pathway. Deposition of C5b-9 was used as readout and the amount obtained in the positive control was set at 100% (y-axis). Data are shown as mean ± SEM of three experiments (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

### Decreased residual activity of the Alternative Pathway by iron dextran and ferric carboxymaltose

Lastly, residual activity of the AP was analyzed (Table 1). The addition of iron dextran and ferric carboxymaltose caused a significant reduction in residual complement activity at the level of C5b-9 generation (Figure 1). In accordance, pre-incubation with iron dextran and ferric carboxymaltose resulted in a significant dose-dependent reduction of residual complement activity at the level of properdin and C3 deposition ( $P < 0.01$ ). For iron dextran, deposition of properdin, C3 and C5b-9 were lowered by 71%, 85% and 94% at 0.5 mg/mL ( $P < 0.01$ ) and lowered by 34%, 24% and 30% at 0.5 mg/mL ( $P < 0.01$ ) for ferric carboxymaltose. Ferumoxytol, iron sucrose and iron isomaltoside did not affect complement activity of the AP.



**Figure 1 | The dose-dependent decrease of residual activity of the Classical, Lectin and Alternative Pathway by iron sucrose, iron dextran, and ferric carboxymaltose. Pooled serum was pre-incubated with increasing concentrations of iron sucrose (x-axis, log<sub>2</sub> scale) for 30 minutes at 37°C. PBS was used for the controls. The serum was then used in the functional assay for the Classical Pathway, Lectin Pathway, and Alternative Pathway to measure residual activity. Deposition of C4, Properdin, C3, and C5b-9 were used as readout and the amount obtained in the control was set at 100% (y-axis). Data are shown as mean ± SEM of three experiments (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001).**



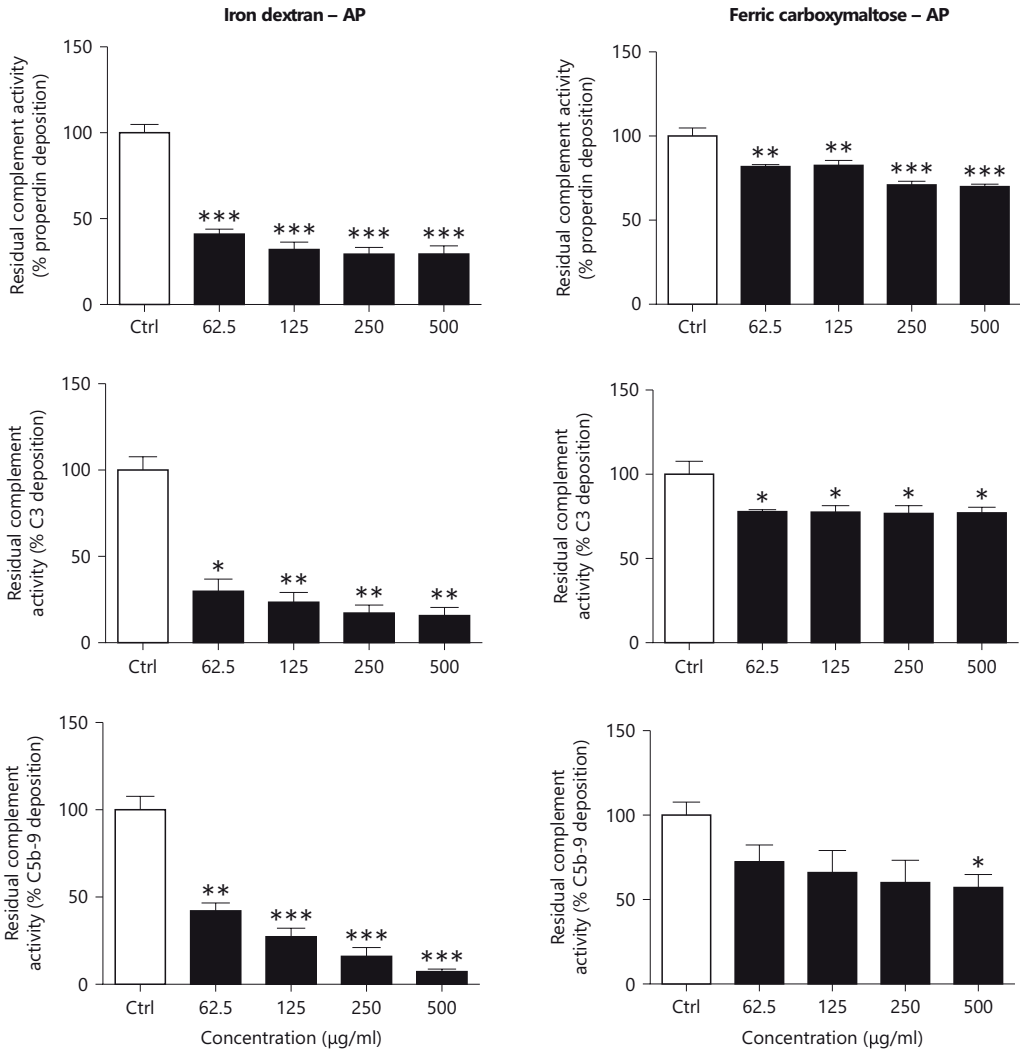


Figure 1 | Continued

### ***In-vitro* testing of complement activation by IV iron drugs**

Next, we investigated whether IV iron preparations can directly activate the complement system. In an ELISA-based set-up, we immobilized the IV iron drugs on the plate and added NHS diluted in buffers that allow the specific activation of CP, LP or AP. Under these conditions, iron dextran and ferric carboxymaltose had the capacity to activate the AP. Ferumoxytol and iron isomaltoside showed no complement activation for all pathways (Table 1). In this set-up, iron sucrose failed to show complement activation for the LP or the CP.

### ***Alternative Pathway activation by iron dextran and ferric carboxymaltose***

We further determined conditions required for iron dextran and ferric carboxymaltose mediated complement activation. An ELISA plate was coated with iron dextran, ferric carboxymaltose or BSA and then exposed to 15% pooled human serum diluted in either MgEGTA or EDTA. Subsequently, C5b-9 deposition was assessed. Iron dextran and ferric carboxymaltose coating caused strong C5b-9 depositions compared to BSA controls (Figure 2A). The addition of EDTA completely inhibited complement deposition. Hence, complement deposition was the result of calcium and magnesium-dependent complement activation. The degree of complement activation was dependent on the concentration of iron dextran and ferric carboxymaltose immobilized on the plate (Figure 2B). Furthermore, we titrated NHS in MgEGTA and showed that C5b-9 depositions were dose dependent when compared to the negative control, BSA (Figure 2C). Lastly, we tested whether AP activation also involves deposition of other complement components of the AP. We found that similar to C5b-9 deposition, C3 (Figure 2D) and properdin deposition (Figure 2E) occurred in a dose-dependent manner, while no C4 deposition was seen (data not shown). Altogether, these results show that dextran and ferric carboxymaltose-mediated complement activation is only possible under AP conditions.

### ***Ex-vivo analysis of the effect of IV iron drugs on complement activation in healthy volunteers***

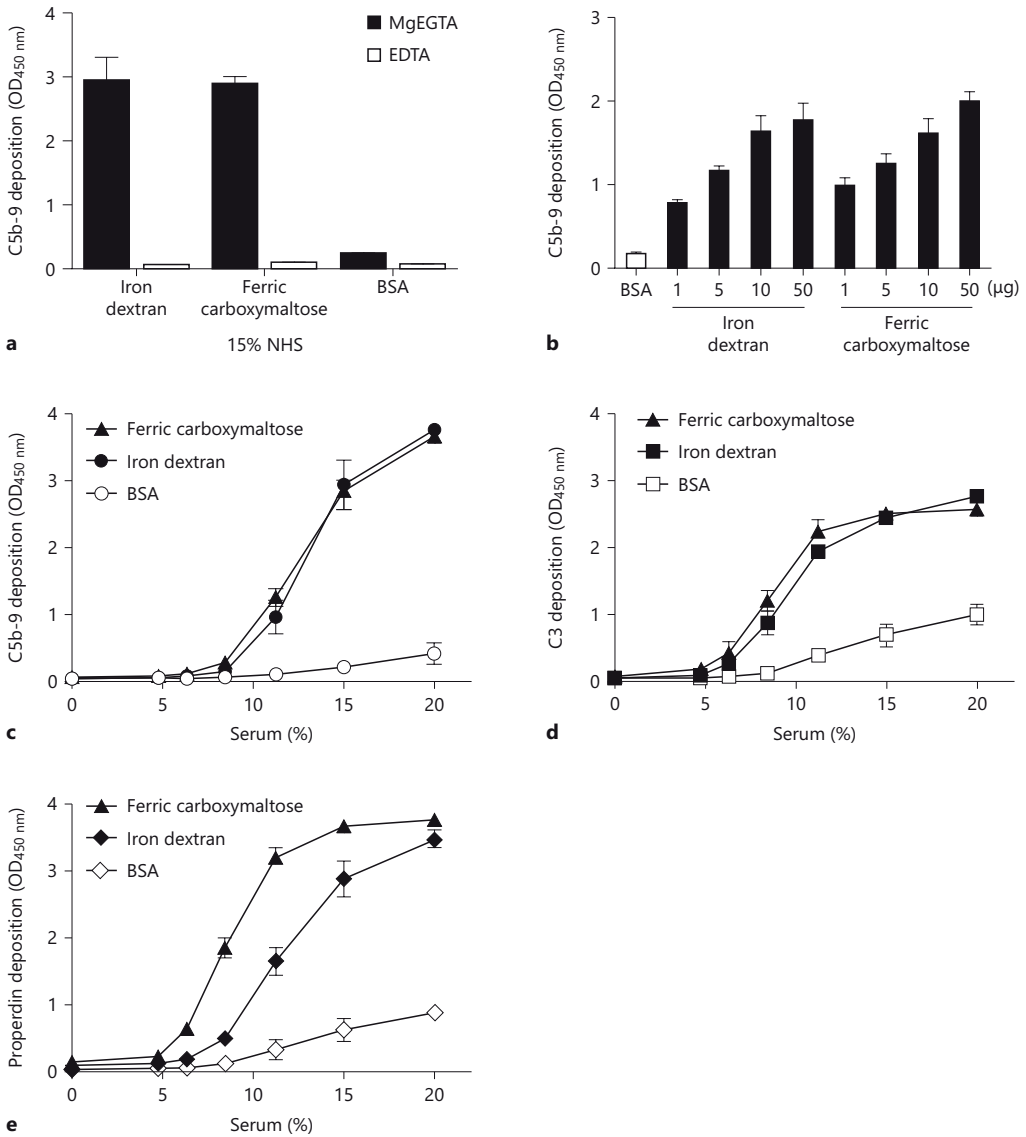
The effect of IV iron drugs on fluid-phase complement activation was determined by incubating IV iron preparation (0.5 mg/mL ferrous iron) for 90 min in human whole blood. Subsequently, complement activation in the samples was determined by measuring sC5b-9 levels. Increased sC5b-9 levels demonstrate complement activation. Additionally, properdin, MBL, and C1q levels were measured to determine which pathway was involved.

### ***Ex-vivo terminal pathway complement activation by iron dextran***

The addition of iron dextran to whole blood samples of healthy volunteers led to vast terminal pathway activation (Figure 3a). Levels of sC5-b9 were 13-fold higher than in the controls ( $P<0.001$ ). Incubation with iron sucrose, ferric carboxymaltose, iron isomaltoside or ferumoxytol did not lead to significant complement activation.

### ***Ex-vivo complement activation by iron dextran is mediated via the Alternative Pathway***

In order to determine which complement pathway was activated, C1q, MBL, and properdin were measured at 0 min and 90 min (supplementary data). For iron dextran, a significant decrease in properdin concentration of 42% was found compared to control ( $P=0.032$ ). The concentration of C1q and MBL remained largely unchanged (Figure 3b). No significant alterations of in C1q, MBL, and properdin concentration were found for iron sucrose, ferric carboxymaltose, iron isomaltoside and ferumoxytol.



**Figure 2 | Alternative Pathway mediated complement activation on iron dextran and ferric carboxymaltose.** (A) ELISA wells were coated with iron dextran, ferric carboxymaltose at 50 µg and 1% BSA as a negative control. Wells were blocked by incubating with 1% BSA/PBS for 60 min at 37°C. A fixed concentration of 15% pooled human serum diluted in GVB++ MgEGTA or EDTA was added to the wells with detection by mouse anti-human C5b-9 antibody. Data are shown as mean ± SEM of three experiments. (B) Iron dextran and ferric carboxymaltose at various concentrations or 1% BSA were coated to the wells. All coated wells had 1% BSA/PBS added for 60 min at 37°C as a blocking agent. 15% pooled human serum diluted in GVB++ MgEGTA was added followed by detection using mouse anti-human C5b-9 antibody. (C–E) Iron dextran and ferric carboxymaltose were coated at 50 µg and 1% BSA as a negative control to ELISA wells. The plate was blocked using 1% BSA/PBS at 37°C for 60 min. Increasing concentrations of pooled human serum diluted in GVB++ MgEGTA were added to the wells followed by measuring deposition for C5b-9, C3 or Properdin.

### Effect of IV iron drugs on complement in whole blood from hemodialysis patients

We next analyzed whether the observed effects of iron dextran can be extrapolated from control subjects without CKD to HD patients with severe CKD and whether other iron preparations induce complement activation similar to iron dextran.

#### *Ex-vivo terminal pathway complement activation by iron dextran*

Similar to healthy controls, iron dextran led to significant complement activation in whole blood from HD patients ( $P < 0.001$ ), indicated by the marked sC5b-9 generation (Figure 3c). Surprisingly, Ferric carboxymaltose and iron sucrose also led to significant complement activation in HD whole blood but not in healthy controls. However, the complement activation by ferric carboxymaltose and iron sucrose was 2- to 3-fold lower than iron dextran. Iron isomaltoside or ferumoxytol did not lead to significant complement activation.

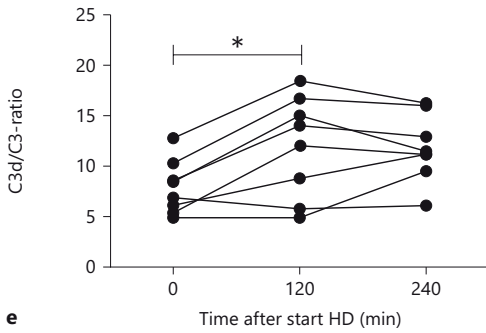
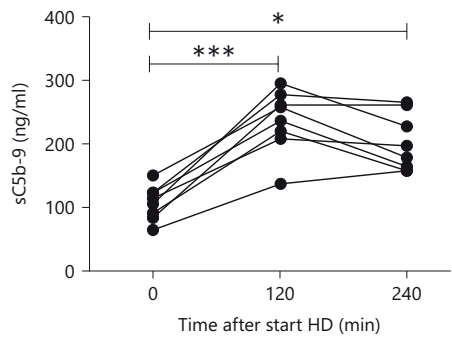
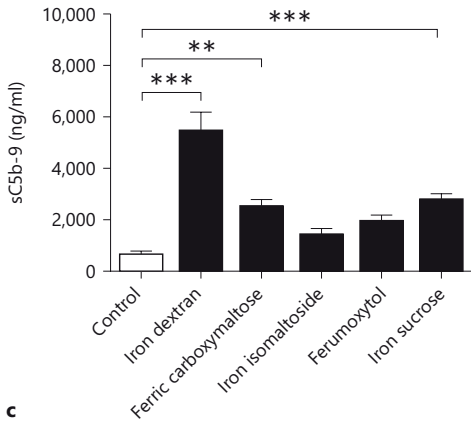
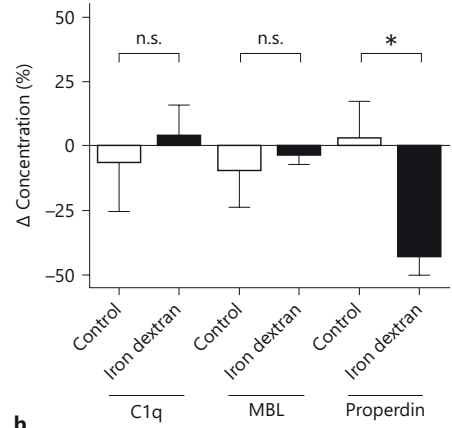
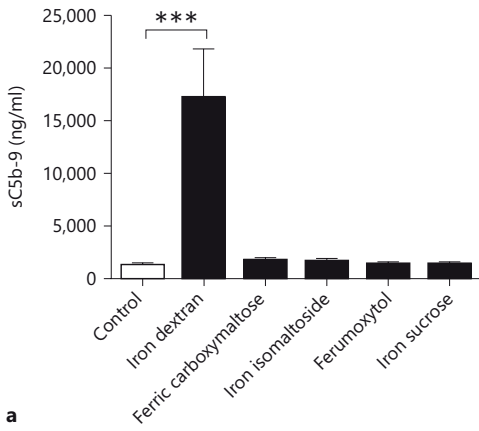
#### No *in-vivo* complement activation by current IV iron treatment in hemodialysis patients

Lastly, we checked if the current intravenous iron therapy, used in our dialysis unit, leads to *in-vivo* complement activation in HD patients (Figure 3d–3e). Prior to iron therapy, all patients already displayed strong complement activation within the first 120 minutes. The sC5b-9 levels (Figure 3d) increased from 109 ng/mL (IQR: 85–122) to 247 ng/mL (IQR: 211–274), while the C3d/C3-ratio (Figure 3e) almost doubled from 7.68 (IQR: 5.52–9.92) to 13.04 (IQR: 6.55–16.32). Patients then received 100 mg of Ferric carboxymaltose intravenously throughout 1 hour at 120 minutes into the dialysis session. At the end of the dialysis, complement levels remained higher than baseline but did not increase significantly compared to levels at 120 minutes. Median sC5b-9 levels at 240 minutes were 252 ng/mL (IQR: 188–264), while C3d/C3-ratio were 15.22 (IQR: 11.40–16.29).

## Discussion

Current EMA-approved intravenous (IV) iron drugs have markedly better safety profiles than the traditional IV iron compounds. However, hypersensitivity reactions still occur and have led to controversy regarding the safety and the risk-benefit ratio of these preparations.<sup>2</sup> Unlike the IgE-mediated reactions by older IV iron compounds, the majority of hypersensitivity reactions by the new IV iron preparations are thought to be caused by CARPA.<sup>5-7</sup> The results of our study are the first, to our knowledge, to support this hypothesis by demonstrating the capacity of several IV iron preparations to activate complement in *in-vitro* and *ex-vivo* models using blood samples of healthy volunteers and HD patients.

Initially, an *in-vitro* assay was used to investigate a possible interaction between IV iron and complement in serum. In this set-up, interaction (binding) and complement activation cannot be distinguished. During pre-incubation, the IV iron drug reacts with the complement system. If the IV iron preparation activates complement, this consequently leads to decreased residual complement activity and therefore deposition on the ELISA plate will be reduced. However, if IV iron binds complement proteins than this effect will also reduce complement deposition as the drug is diluted but not removed after the pre-incubation step.





**Figure 3 | The ex-vivo effect of iron preparations and in-vivo effect of ferric carboxymaltose on complement activation.** Whole blood was incubated with 0.5 mg/mL of iron dextran, Iron sucrose, ferric carboxymaltose, iron isomaltoside and ferumoxytol (x-axis) for 90 minutes at 37°C. PBS was used for the controls. (A) The concentration of soluble C5b-9 (sC5b-9) was determined in plasma from healthy controls and used as a read-out for complement activation (y-axis). Data are mean and SEM of five experiments using different donors each time. (B) The concentration of C1q, MBL and Properdin was determined in samples from healthy controls with 0.5 mg/mL of iron dextran at 0 and 90 minutes. The difference in concentration was calculated by dividing the concentration at 90 minutes, by the concentration at 0 minutes and then minus 100% (y-axis). (C) Concentration of soluble C5b-9 (sC5b-9) was determined in plasma from hemodialysis (HD) patients (y-axis). Data are mean and SEM of eight experiments using different donors each time. (D) sC5-9 levels and (E) C3d/C3-ratio were determined in HD patients during one dialysis session, in which they received 100 mg of ferric carboxymaltose at 120 minutes into the dialysis session. Data are mean and SEM of eight subjects (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

In order to distinguish between true IV iron-mediated activation and other forms of interaction, ELISA plates were coated with different concentrations of IV iron preparations and fixed concentrations of NHS were added. Complement activation was increased in a dose-dependent manner by iron dextran and ferric carboxymaltose under AP-specific conditions. Combining these results we can conclude that the reduced complement deposition after incubation with iron dextran and ferric carboxymaltose in NHS in the functional assays was indeed due to complement activation. However, for iron sucrose, we have to consider an alternative explanation such as a direct effect of iron sucrose on C2, C4 or the serine proteases.

We next tested the capacity of each drug to activate complement in an *ex-vivo* model. By incubating whole blood with iron, the preparations were not only exposed to serum components but also blood cells and membrane-bound complement regulatory factors. In line with the previous *in-vitro* experiments, iron dextran induced significant complement activation, while, surprisingly, ferric carboxymaltose did not. This might be because the functional assays measure complement deposition on a plate and thereby test solid phase activation while the whole blood model tests fluid phase activation by measuring soluble complement activation products. A similar discrepancy has been found for LPS and IgA.<sup>14</sup> Furthermore, the whole blood model and the functional assays differ in sensitivity. While coating with the iron preparation and exposing it to NHS serum is a very sensitive test, the whole blood model does not involve dilution of the blood sample and is, therefore, a more physiological approach.

Subsequently, we analyzed the effect of IV iron in a group of hemodialysis (HD) patients who are regularly receiving IV iron. In the *ex-vivo* experiments, whole blood from HD patients showed similar activation trends as whole blood from healthy volunteers. In both groups, iron dextran caused a significant increase in sC5b-9 generation. However, the overall complement activation was lower compared to healthy volunteers. This can be considered a sign of pre-existing chronic complement activation, which is well described in HD patients.<sup>15</sup> Concordantly, in our *in-vivo* experiments elevated C3d/C3-ratio and C5b-9 serum levels were measured in blood samples taken from these patients prior to dialysis.

The IV infusion of ferric carboxymaltose did not lead to significant additional complement activation in HD patients. Both, sC5b-9 levels as well as the C3d/C3 ratio rose during the first half of the dialysis session and then remained consistently elevated from the start of the IV iron administration till the end of the HD session. While these measurements were performed in a small patient group, the results are in line with the *ex-vivo* findings, which did not indicate strong complement activation capacity for ferric carboxymaltose. Moreover, the slow administration as a continuous infusion over 1 h reduces the risk of massive complement activation.<sup>6,16</sup> Lastly, vast complement activation and subsequently relative depletion of complement factors has taken place during the first half of the HD session. We would, therefore, expect to see more complement activation in non-dialysis CKD patients after IV iron. In addition, we would hypothesize that bolus injection would lead to more complement activation than slow administration. This is supported by previous studies, showing that the rate of infusion is crucial for both the risk of hypersensitivity reactions and complement activation.<sup>17</sup> As none of our patients are currently treated with iron dextran we were unfortunately not able to test the complement activating properties of this iron preparation *in-vivo*. To further unravel the effects of different iron preparations *in-vivo*, a trial comparing the *ex-vivo* and *in-vivo* effects of different IV iron drugs in various patient populations would be needed. Nonetheless, since these trials will not be able to observe and compare the very rare clinical severe adverse events, data of observational cohorts including adequate sampling need to be gathered. In addition, further *in vitro* studies may help to better understand the mechanism behind hypersensitivity reactions by IV iron preparations.

Clear guidelines exist regarding the maximum dose and minimal duration of administration per IV iron drug.<sup>1</sup> For iron dextran and iron sucrose, the recommended dose is 100–200 mg administered intravenously over 2–5 minutes for 5–10 consecutive HD sessions. Considering an average post-dialysis blood volume of  $3755 \pm 941$  ml, final blood concentrations would vary between 42–71  $\mu\text{g/ml}$ .<sup>18</sup> Other IV iron drugs are given in higher doses or administered more rapidly, resulting in much higher local concentrations at the site of injection than concentrations measured in the peripheral blood.<sup>19</sup> In addition to that, Geisser and Burckhardt found higher IV iron blood concentrations after repetitive dosing.<sup>20</sup> Thus, concentrations chosen for the experiments are considered physiologically reasonable.

A limitation of our study is the extrapolating of our findings into the clinical setting. Hypersensitivity reactions to intravenous iron are rare and not in line with the complement activation seen in the *in-vitro* and *ex-vivo* results. Thus, an extremely important question that remains to be answered is what explains the difference in frequency of clinically observed adverse events and the frequency and magnitude of complement activation in our *in vitro* experiments. Factors such as route and rate of administration and patient characteristic (conditions of pre-existent complement activation) determine the magnitude of complement activation. However, mere activation of the complement system is not sufficient to cause CARPA, but it is a crucial first step in this reaction. In addition, beyond the acute effects, it has been hypothesized that repetitive complement activation, inflammation, and oxidative stress may cause endothelial dysfunction and vascular remodeling. Indeed in an observational study, Bailie et al. report an 18% increase in mortality in HD patients

receiving high doses of IV iron. However, due to the observational study design, no conclusion could be drawn regarding the causal relation between IV iron and mortality.<sup>21</sup>

Previous studies defined a 5 to 10-fold increase of complement activation as a realistic predictor for clinical reactions.<sup>17</sup> Given this information, it can be assumed that iron dextran carries a risk of causing CARPA mediated hypersensitivity reactions. In accordance with our findings, it has been shown that dextran-coated magnetic iron nanoparticles activate the complement system via the AP. These agents are used as an MRI contrast agent and are able to cause severe hypersensitivity reactions in patients. The chemical structure of the iron dextran preparation is similar to this contrast agent.<sup>27</sup> We hypothesize that the iron-carbohydrate nanoparticles are complement-activating and not the iron itself, since ferric chloride didn't cause significant complement activation (data not shown). In addition, there are several clinical studies stating the higher risk of serious adverse events after administration of iron dextran formulations.<sup>25,26</sup> Recently, Wang et al. investigated the risk of adverse events among the different IV iron drugs. A three times higher rate of adverse events was found for iron dextran compared to other IV iron. Also, more anaphylactic reactions were seen after the first administration of IV iron compared to repeated administration.<sup>23</sup> This phenomenon is in line with our results and the description of CARPA.<sup>24</sup> Ferric carboxymaltose also showed complement activating capacity and could shift the regulatory balance in predisposed individuals towards unregulated complement activation.

In conclusion, the present study shows that different IV iron formulations have the *in-vitro* capacity to activate complement in healthy individuals as well as in HD patients undergoing long-term IV iron treatment. The major finding of this study is that iron dextran significantly activates complement via the AP *in-vitro* and *ex-vivo*. In addition, ferric carboxymaltose also activated complement *in-vitro* via the AP. Furthermore, iron sucrose may interact with complement proteins of the LP and CP but did not activate complement. Notably, slow infusion of ferric carboxymaltose during HD did not lead to additional complement activation. Our results indicate that current guidelines are efficient at avoiding CARPA by IV iron and explain why these routinely administered drugs show a limited number of adverse events. Our results are the first to our knowledge, to provide proof of concept of complement activation by IV iron and therefore provide new insights into the pathophysiological mechanism for a well-described adverse reaction to IV iron. Mere activation of the complement system is not sufficient to cause CARPA, but it is a crucial first step in this reaction. Furthermore, long-term complement activation is known to cause free radical generation and accelerate arteriosclerosis. These findings warrant further translational studies in HD and iron naïve patients in order to gain new insights into the pathophysiological mechanism of these clinical adverse events and to develop a safer treatment.

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