UNIVERSITY OF MEDICINE AND PHARMACY „CAROL DAVILA”, BUCHAREST
DOCTORAL SCHOOL
MEDICINE

EFFECTS OF INTRAVENOUS IRON ADMINISTRATION ON ENDOTHELIUM AND SOME OXIDATIVE STRESS PARAMETERS IN PATIENTS WITH CHRONIC KIDNEY DISEASE

Ph.D. THESIS SUMMARY

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2023

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RESEARCH HYPOTHESES AND GENERAL OBJECTIVES

Anemia has an increased prevalence globally (22.8% in 2019), despite a decreasing trend over the last 20 years [1]. Iron deficiency is the first cause of anemia, even in developed countries, and is commonly observed both in the general population and in patients with chronic diseases (CKD, heart failure, inflammatory bowel disease, neoplasia) [2].

Chronic kidney disease is a global public health problem with a prevalence of 10-15%. Iron deficiency - absolute, functional - or due to immobilization by inflammation in macrophages is present in 20-40% of patients with CKD, and anemia is one of the most common complications in these patients. Anemia is more prevalent as kidney function declines, and is even more pronounced in those with high proteinuria and those with diabetes. Iron deficiency is the main mechanism involved, even in non-dialysis patients [3].

The clinical impact of anaemia in CKD has been extensively studied. In addition to affecting quality of life, other effects associated with anemia are progression of CKD, increased incidence of cardiovascular events and mortality [4]. However, there are few data regarding the clinical impact of iron deficiency in CKD. An association between iron deficiency and reduced quality of life by decreasing components of physical activity, independent of hemoglobin levels, has been reported recently [5]. Moreover, iron deficiency predicted all-cause mortality in non-dialysis CKD patients [6].

The KDIGO guidelines for anemia management in CKD recommend iron supplementation for iron deficiency anemia, targeting increasing hemoglobin and reducing the need for erythropoiesis-stimulating agents [7]. Recent data supports that pro-active intravenous iron therapy was clinically beneficial for haemodialysis CKD patients, an advantage that appears to be due to correction of iron deficiency independent of correction of anaemia, in other words due to the non-haematological effects of iron [8].

In conclusion, iron deficiency and anemia are common in patients with CKD, their presence being associated with worsening prognosis. At the same time, the correction of iron deficiency and anemia in CKD have a favorable clinical impact as not only

significantly improves hematological parameters, but iron therapy also has non-hematological benefits.

Treatment options are oral and intravenous iron therapy.

Oral iron therapy could be considered in non-dialysis patients with CKD, the limitations being mainly due to the reduced tolerance and degree of restriction of erythropoiesis. In addition, the reduced absorption of oral iron preparations makes parenteral iron therapy to be chosen, even in non-dialysis patients. In these patients, venous iron was superior to oral iron therapy in achieving and maintaining a target haemoglobin level and in reducing erythropoietin requirements [9]. Similar benefits have been reported in subgroups of patients with CKD in studies in patients with acute or chronic heart failure [10-12].

Regarding hemodialysis patients, parenteral iron therapy is the preferred route, as it is easily performed through the hemodialysis circuit, avoiding additional venous punctures [13]. A large number of cotrolled studies support the superiority of intravenous versus oral iron therapy in increasing hemoglobin levels and reducing erythropoietin doses in both hemodialysis and peritoneal dialysis patients. Of the formulations, iron sucrose is the best studied [13, 14].

Intravenous iron preparations are nanoparticles consisting of a polynuclear iron(III) oxyhydroxide nucleus stabilized by various carbohydrates. Depending on carbohydrate type, nanoparticle size and iron reactivity, these compounds have different antigenicity, kinetic stability and thermodynamic stability [15]. Based on the type of the nanoparticle, it is possible that after parenteral administration, a small amount of biologically active iron (labile iron) is released into circulation. Small nanoparticles such as iron sucrose hold iron less tightly and can release larger amounts of free iron compared to larger molecules such as iron carboxymaltose or isomaltose [16].

In vitro evidence showed that non-transferin bound iron catalyzes the formation of oxygen free radicals, generating oxidative stress [17]. Through this mechanism, labile iron can reduce endothelial synthesis of nitric oxide and induce endothelial dysfunction [18]. Endothelial dysfunction plays a central role in atherogenesis, and cardiovascular diseases are highly prevalent in patients with CKD, prevalence explained incomplete by the presence of classical cardiovascular risk factors [19].

Data from observational and randomised studies in CKD patients, dialysed or not, as well as in the general population show lipid peroxidation and attenuation of flow-dependent vasodilation within minutes of parenteral iron administration [20-22]. In contrast, other reports have not associated endothelial dysfunction with intravenous iron administration in hemodialysis or peritoneal dialysis patients [23, 24]. Most of the data evaluating the potential of intravenous iron to generate oxidative stress and endothelial dysfunction come from patients treated with iron sucrose, with limited comparative data between the effects of different formulations [25].

In conclusion:

- Iron deficiency is frequent in patients with CKD, it first has non-hematological consequences, unfortunately little studied in patients with CKD, and only subsequently hematological consequences: iron deficiency anemia, through iron deficiency, absolute or functional, the last one by blocking iron in macrophages and inflammation;
- Parenteral iron therapy has been extensively studied and effectively corrects iron deficiency anemia in CKD;
- Since parenteral iron preparations can release catalytically active iron into circulation, increased oxidative stress may produce endothelial dysfunction, which may have clinical consequences. The few studies – especially under experimental conditions – that have investigated this, have had conflicting results.

Starting from these premises, we structured the research in four studies:

1. A retrospective observational pilot study with the target of describing the morbidity (frequency, duration and causes of hospitalisations) associated with long-term iron sucrose treatment in haemodialysis patients with CKD, as this is the best documented parenteral iron formulation, and haemodialysis patients are frequently exposed to it;
2. A prospective crossover study, aiming to investigate the changes in oxidative stress during haemodialysis sessions, in patients in whom intravenous iron was or was not administered;

3. A prospective crossover study with the goal of investigating acute changes in endothelial function comparing iron sucrose with iron carboxymaltose administered in non-dialysis CKD patients;
4. A prospective crossover study with the purpose of assessing the effect of iron carboxymaltose on endothelium and some parameters of oxidative stress.

Study 1 - Long-term intravenous iron therapy and morbidity in hemodialysis patients

1. Objectives

To describe morbidity (number of hospitalisations, total number of days of hospitalisation, duration of each hospitalisation, main diagnoses of admission) in haemodialysis CKD patients with or without iron sucrose therapy.

2. Materials and methods

This observational retrospective cohort study was conducted at "Dr.Carol Davila" Teaching Hospital of Nephrology, with the approval of the local Ethics Committee.

All patients aged over 18 years who were on maintenance hemodialysis therapy and were admitted in our hospital on the course of one year, were screened. Those with hemodialysis vintage of at least 12 months were included.

Diabetic nephropathy as primary kidney disease (because there were only 3 cases available in the study period), pregnancy and subjects with incomplete data regarding study aims, were excluded.

Study parameters

Data was recorded from the Electronic Database of our hospital, discharge abstracts (admissions in other hospitals) and hemodialysis patient's files.

Main study parameters

Outcomes measures: number of hospitalizations, total number of days of hospitalization, length of stay per admission, main admission diagnoses.

Intravenous iron therapy characteristics were as follows:

- Drug: iron sucrose (Venofer® 20 mg iron/mL, 1 vial = 100 mg), infusion diluted with 100 ml 0.9% sodium chloride (1mg/mL concentration) during the last hour of hemodialysis session, via the venous line of the extracorporeal circuit.
- Dosage: 100 mg iron sucrose at 1-4 weeks, in order to achieve and maintain the target for renal anemia treatment according to the Romanian Society of Nephrology Guidelines [26];

- Duration: at least 12 months.

Statistical analysis

All statistical analysis were performed using IBM® SPSS® Version 23 and Microsoft Office Professional Excel®2003 +Analyse-it® (the same programs were used for statistical analysis of the data of the 4 studies). Normalitatea variabilelor continue a fost testată prin testul Shapiro-Wilk.

Normally distributed data were expressed as mean ±standard deviation (SD) and non-normally distributed data, as median and interquartile range. Categorical variables were described as percentages. Incidence of hospitalizations was calculated as new cases/(population x timeframe).

Differences between groups were assessed using Chi-square and Kruskal-Wallis H tests.

A p value <0.05 was considered statistical significant.

3. Results

A total of 220 patients were enrolled. Mean age was 53±13 years and 56% were males. Median hemodialysis vintage was 5 (1-26) years.

Two thirds of them were iron-treated. In almost a half, low doses of 100 mg monthly were used. Only 20% of patients received higher doses (400 mg monthly) (**Table 1.I**).

Number of hospital admissions

A total of 119 hospitalizations, with an incidence of 54/100 patient-year, was noted. Eighty four admissions were recorded in the iron-treated group (56/100 patients-year), in contrast with 35 in the iron-untreated group (50/100 patients-year), p=0.1.

No differences were observed regarding the number of hospital admission (no admission, 1, 2 or 3-5 admissions) between the two groups.

Days of hospitalization

The length of stay per admission and the total days of hospitalization was similar, irrespective of iron therapy.

Tabel 1.I. Characteristics of patients according to the presence of iron therapy

Characteristics	Iron-treated group n=150	Iron-untreated group n=70	p
Age (years)	53 (27-86)	53 (22-81)	0.8
Male, n (%)	86 (57)	38 (54)	0.7
Primary cause of ESKD (%)			
• Glomerular nephropathy	53	59	0.6
• TIN	22	26	0.3
• Polycystic kidney disease	12	10	0.8
• Vascular nephropathy	11	4	0.1
• Unknown	3	1	0.5
HD vintage (years)	4 (1-24)	6 (1-26)	0.2
Iron therapy, monthly (%)			
100 mg	46%		
200 mg	36%		
400 mg	18%		
Data are expressed as median (interquartile range) or percentages. ESKD- end stage kidney disease; HD-hemodialysis; TIN- tubulointerstitial nephropathies			

Causes of hospital admissions

Hospitalization rates due to infectious and cardiovascular diseases were similar for both groups (12/100 patients-years vs 5.7/100 patients-years, p=0.3 and 11.3/100 patients-years vs 4.3/100 patients-years, p=0.2, respectively).

Relationship between iron dosing and hospitalization requirements

The hospitalization rate significantly increased with the administered iron dose (**Figure 1.1)**.

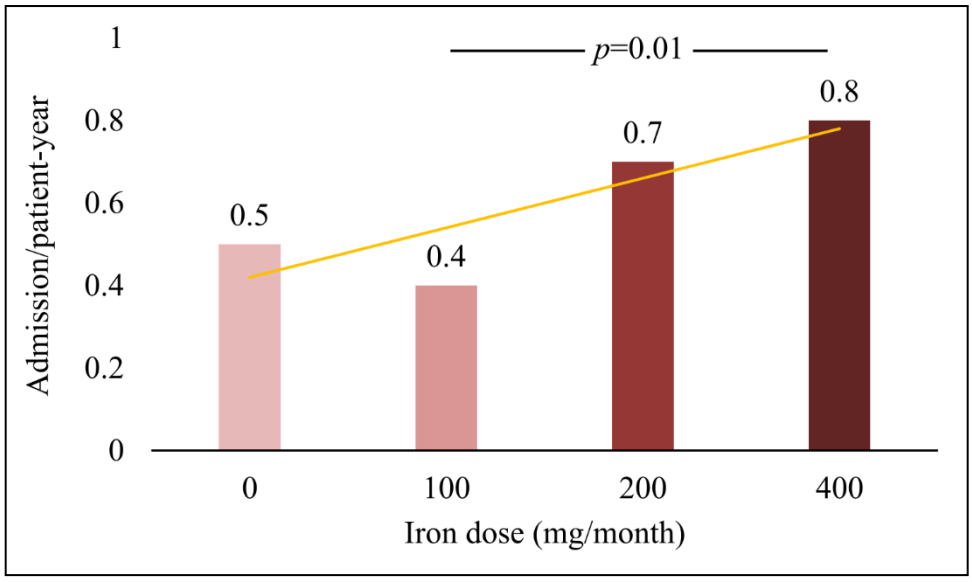

Figure 1.1. Hospitalization rate based on iron dose

Study 2 - Acute effect of iron sucrose on oxidative stress in hemodialysis CKD patients

1. Objectives

1. To evaluate the acute influence of IV iron sucrose infusion on the antioxidant status by comparing intradialytic changes in erythrocyte antioxidant parameters, in chronic hemodialysis CKD patients.

2. To evaluate the acute influence of IV iron sucrose infusion on the antioxidant status by comparing intradialytic changes in plasma antioxidant parameters, in chronic hemodialysis CKD patients.

3. To evaluate the acute influence of IV iron sucrose infusion on oxidative stress by comparing intradialytic changes in parameters of increased free radical production, in chronic hemodialysis CKD patients.

2. Material and methods

A prospective, crossover study was conducted Nephrology Department of "Dr. Carol Davila" Teaching Hospital of Nephrology "Dr. Carol Davila".

Inclusion criteria: age >18 years, HD vintage >6 months, a stable dose of ESA for at least one month before the beginning of the study and serum hemoglobin >9 g/dl.

Exclusion criteria were: a history of iron allergic reactions, iron supplementation during the last month before enrolment, signs of iron overload (ferritin >500 ng/mL and/or transferrin saturation >50%), other causes of anemia (hemoglobinopathies, vitamin B12 and/or folic acid deficiency), active inflammation and/or infection, diabetes mellitus, active liver diseases, severe malnutrition, malignancies, pregnancy or breastfeeding, active smoking and ongoing pro- or antioxidant therapy.

Study protocol

Hemodialysis session

Blood samples were drawn at two mid-week HD sessions, 7 days apart:

- first one without iron administration (HD-Fe(-)) and
- the second one with an IV infusion of 100 mg iron sucrose (Venofer® Vifor Pharma),

diluted with 100 ml 0.9% saline, over the first 20 minutes of the session in the venous line of the extracorporeal circuit (HD-Fe(+)).

All blood samples were collected after overnight fasting, at three moments during the HD sessions:

- at the beginning of the session (0 min.);
- at 45 minutes (i.e., 25 min. after the end of iron infusion, corresponding to the maximal reactive species generation induced by HD procedure [27] and to the iron sucrose dose alpha phase of elimination [28]);
- at the end of the session (270 min.).

Stduy parameters

Data was recorded from the Electronic Database of our hospital, discharge abstracts (admissions in other hospitals) and hemodialysis patient's files.

Main study parameters:

- *erythrocyte antioxidant parameters*: (superoxide dismutase activity - SOD, catalase activity - CAT, non-protein thiols level - ESHnp, total thiols level - ESHt, and total antioxidant capacity of erythrocytes – TEAC (assessed by a modified ABTS method for serum) were measured by spectrophotometry, using methods previously described [29-33].
- *plasma antioxidant parameters*: total plasma free thiols (Pt-SH), after Ellman's method [33], total serum antioxidant capacity (TSAC) by ABTS method [32], residual serum antioxidant activity (RAC) [34].
- *increased free radical production parameters*: plasma thiobarbituric acid reactive substances [35] and oxidized LDL (ox-LDL), as parameters for lipid peroxidation, plasma reactive dicarbonyl compounds (CDC, using Girard-T reagent [36]) and plasma Amadori products (Pamad, fructosamine, measured by Furdh's method [37]) determined by spectrophotometry as carbonil stress indicators.

Statistical analysis

In order to correct for intradialytic hemoconcentration, ratios of iron status parameters and oxidative stress parameters to plasma total protein, hemoglobin and cholesterol were analyzed.

All data were normally distributed. Results were expressed as mean±SD and were compared using Student's t test, paired or not. Linear regression and Pearson's r correlation coefficient were used to evaluate the relationship between pairs of variables.

A p value less than 0.05 was considered significant.

3. Results

Twenty clinicaly stable chronic hemodialysis CKD patients (13 men, mean age 56±13 years), were included. Mean HD vintage was 71±7 months.

Erythrocyte antioxidant parameters

There were no significant differences regarding neither baseline values of erythrocyte antioxidant parameters, nor those of iron metabolism parameters between the two hemodialysis sessions.

Total antioxidant capacity of erythrocytes decreased during both hemodialysis sessions, but notably, the reduction seen at 45 minutes was more important in those with iron sucrose therapy (**Table 2.I**).

The level of ESHt increased at 45 minute and at the end of the HD-Fe(+) session, comparative with baseline (115 and 108 versus 80 μmol/g Hb, p= 0.019), but dind't chanced significantly in control session (**Table 2.I**).

Nivelul de ESHnp a scăzut numai la sfârșitul ședinței HD-Fe(+) (233 versus 262 nmol/g Hb, p= 0.038) (**Table 2.I**).

Enzimatic antioxidant erythrocyte parameters (SOD, CAT) did not change during the haemodialysis session, regardless of iron administration (**Table 2.I**).

Table 2.I. Erythrocyte antioxidant status and iron metabolism during hemodialysis sessions

	HD-Fe(-)*			HD-Fe(+)*		
	0min.	45min.	270min.	0min.	45min.	270min.
Hemoglobin (g/dl)	10.3±0.98	10.61±0.97	†‡12.18±1.6	10.29±0.95	10.42±0.9	†‡11.85±1.4
Hematocrit (%)	31.3±2.7	32.25±2.7	†‡36.7±4.7	31.7±2.8	31.9±2.5	†‡35.4±4.4
TSAT (%)	38.5±8.2	38.4±8.8	40.68±9	34.86±9.5	†§74.4±10.7	†§75.7±10.7
sFerr <200ng/ml (%)	40	-	-	37	-	-
sFe/protein (µg/g)	13.3±3.6	12.8±3.5	13.8±4	12.1±3.2	§†51.4±8.6	§†‡36.6±8
sFerr/protein (ng/g)	34±17	-	40±20	37±19	-	42±21
TSAT/protein (%/g)	5.6±1.2	5.3±1.2	†4.9±0.9	5.3±1.5	§†10.8±1.4	§†‡9.8±1.7
ESHt (µmol/g Hb)	97.5±29	96±46.7	100.5±36	80±46	†115±54	†108±46
ESHnp (nmol/g Hb)	241±45	237±50	239±64	262±52	260±57	†233±50
SOD (U/g Hb)	934±189	914±310	820±297	864±286	886±311	903±365
CAT (k/g Hb)	354±69	363±57	344±76	346±69	368±66	356±61
TEAC (µmol/g Hb)	1.64±0.1	1.58±0.17	†‡1.22±0.2	1.63±0.5	§1.45±0.3	†‡1.15±0.2

*Mean ± DS; p<0.05 † vs. 0min.; ‡ vs. 45min.; § vs. HD-Fe(-) at the same moment.
CAT- catalase activity; ESHnp – unbound thiol; ESHt – total free thiol; Hb - hemoglobin; HD-Fe(-) – hemodialysis session without iron therapy; HD-Fe(+) – hemodialysis session with iron therapy; SOD – superoxide dismutase activity; sFe - sideremia; sFerr – serum ferritin; TEAC- total antioxidant capacity of erythrocytes; TSAT- transferrin saturation

Plasma antioxidant parameters

Total free plasma thiol augmented after 40 minutes, in both investigated HD sessions. There were no notable differences in total plasma thiol concentration in the presence of intravenous iron therapy compared to its absence.

Total serum antioxidant capacity (TSAC) did not change during HD session, regardless of iron administration (**Table 2.II**).

Table 2.II. Plasma antioxidant status and iron metabolism during hemodialysis sessions

	HD-Fe(-)*			HD-Fe(+)*		
	0min	40min	270min	0min	40min	270min
Pt-SH/protein (mcmol/g)	11.9±1.4	11.8±1.4	†‡14.2±1.3	11.3±1.8	11.6±1.3	†‡14.1±1.3
TSAC/protein (mmol/g)	2.1±0.3	2.0±0.2	2.1±0.2	2.2±0.2	2.2±0.3	2.1±0.2
RAC/protein (mmol/g)	1.0±0.3	1.1±0.3	†‡1.2±0.2	1.1±0.3	1.2±0.3	1.3±0.3
Uric acid (mg/dl)	6,39	4,72†	2,07‡	6,44	4,69†	1,75‡
sFe/protein (µg/g)	13±3.6	12.8±3.5	13.8±4	12.1±3.2	§†51.4±8.6	§†‡36.6±8
sFerr/protein (ng/g)	34±17	-	40±20	37±19	-	42±21
TSAT/protein (%/g)	5.6±1.2	5.3±1.2	†4.9±0.9	5.3±1.5	§†10.8±1.4	§†‡9.8±1.7

*Mean ± DS; p<0.05 † vs. 0min.; ‡ vs. 45min.; § vs. HD-Fe(-) at the same moment.
HD-Fe(-) – hemodialysis session without iron therapy; HD-Fe(+) – hemodialysis session with iron therapy; Pt-SH- plasma thiol concentration; RAC- residual serum antioxidant activity; sFe - sideremia; sFerr – serum ferritin; TSAC- total serum antioxidant capacity; TSAT- transferrin saturation

Increased free radical production parameters

Plasma concentration of TBARS increased at 40 minutes during both HD sessions. However, a sustained rise was noted only during the HD-Fe(+) session, at the end of which higher values were reached compared to the HD-Fe(-) session (**Table 2.III , Figure 2.1**).

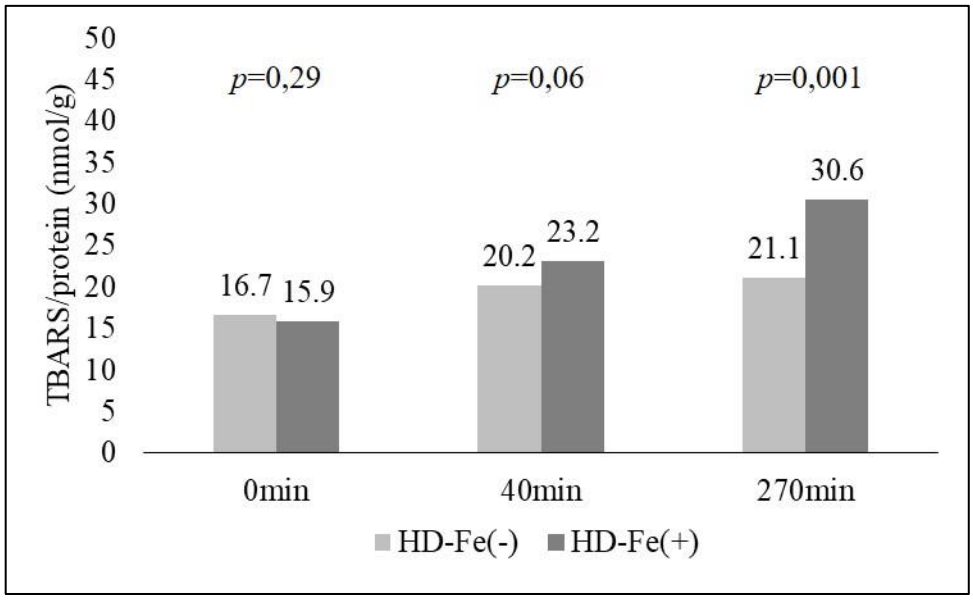

Figure 2.1. Plasma thiobarbituric acid reactive substances (TBARS) during studied hemodialysis sessions. HD-Fe(-) - hemodialysis session without iron therapy; HD-Fe(+) - hemodialysis session with iron therapy

Oxidized-LDL was markedly increased only during HD-Fe(+) session, at 40 minutes (**Table 2.III**).

Table 2.III . Free radical dynamic during hemodialysis sessions

	HD-Fe(-)*			HD-Fe(+)*		
	0min	40min	270min	0min	40min	270min
TBARS/protein (nmol/g)	16.7±4.4	†20.2±5.9	†21.1±5.2	15.9±4.8	†23.2±5.4	†‡§30.5±6.7
ox-LDL/chol(U/mg)	0.93 ± 0.12	0.91 ± 0.11	†‡0.78 ± 0.11	0.90 ± 0.12	§†1.08 ± 0.21	§‡0.91 ± 0.18
TSAT/chol(%/mg)	0.23 ± 0.10	0.23 ± 0.10	0.2 ± 0.07	0.20 ± 0.08	§†0.42 ± 0.09	§†‡0.36 ± 0.08
LDL-c/chol (mg/mg)	0.61 ± 0.05	0.63 ± 0.07	0.6 ± 0.06	0.61 ± 0.07	0.64 ± 0.07	0.61 ± 0.07

*Mean ± DS; p<0.05 † vs. 0min.; ‡ vs. 45min.; § vs. HD-Fe(-) at the same moment.
chol-cholesterol; HD-Fe(-) – hemodialysis session without iron therapy; HD-Fe(+) – hemodialysis session with iron therapy; ox-LDL-oxidized-LDL; Pamad- Amadori produts; TBARS- plasma thiobarbituric acid reactive substances; TSAT- transfferin saturation

Oxidized-LDL was positively associated with transferrin saturation, at 40 minutes after the start of the HD-Fe(+) (i.e., 20 minute after iron infusion), r =0,66, *p*=0,002.

Plasma reactive dicarbonyl compounds significantly decreased during HD, irrespective with the presence of iron therapy (7.6vs 13.4vs 14,4mcmol/mg at 270vs 40vs 0min during

HD-Fe(-), and 9.7vs 15.2vs 16.1mcmol/mg at 270 vs. 40 vs. 0min during HD-Fe(+) (**Table 2.IV.**).

Amadori products behaved similarly (146 vs. 203 vs. 221mmol/mg at 270 vs. 40 vs. 0 min during HD-Fe(-), and 156 vs. 214 vs. 238 la 270 vs. 40 vs. 0 min during HD-Fe(+) (**Table 2.IV**).

Table 2.IV. Carbonil stress status parameters during studied hemodialysis sessions

	HD-Fe(-)*			HD-Fe(+)*		
	0min	40min	270min	0min	40min	270min
RDC/chol (mcmol/mg)	14.4 ± 4.4	13.4 ± 4.5	†‡7.6 ± 3.0	16.1 ± 5.0	15.2 ± 5.4	†‡9.7 ± 5.0
PAmad/chol (mmol/mg)	221 ± 46	203 ± 38	†‡146 ± 34	238 ± 47.3	214 ± 57.5	†‡156 ± 45.4
TSAT/chol(%/mg)	0.23 ± 0.10	0.23 ± 0.10	0.2 ± 0.07	0.20 ± 0.08	§†0.42 ± 0.09	§†‡0.36 ± 0.08
ox-LDL/chol (mg/mg)	0.61 ± 0.05	0.63 ± 0.07	0.6 ± 0.06	0.61 ± 0.07	0.64 ± 0.07	0.61 ± 0.07

*Mean ± DS; p<0.05 † vs. 0min.; ‡ vs. 45min.; § vs. HD-Fe(-) at the same moment.
chol-cholesterol; HD-Fe(-) – hemodialysis session without iron therapy; HD-Fe(+) – hemodialysis session with iron therapy; ox-LDL-oxidized-LDL; Pamad- Amadori products; RDC- plasma reactive dicarbonyl compounds; TSAT- transfferin saturation

Study 3 - Acute effects of two intravenous iron preparations on endothelial function in non-dialysis CKD patients

1. Objectives

To investigate the acute changes in endothelial function after single IV iron infusions of iron sucrose (IS) and ferric carboxymaltose (FCM), in nondialysis CKD patients.

2. Materials and methods

This was a prospective crossover study conducted at "Dr. Carol Davila" Teaching Hospital of Nephrology, Bucharest. Patients with CKD stages 3–5 not on renal replacement therapy, who required IV iron as part of their routine medical care as judged by the in-charge physician were enrolled in a period of 6 months.

After applying the exclusion criteria, 31 stage 3–5 nondialysis CKD iron-deficient patients not on erythropoietin or iron, were included.

Medical history of iron allergic reactions, signs of iron overload (ferritin >500 ng/mL and/or transferrin saturation >50%), other causes of anemia (hemoglobinopathies, vitamin B12 ±folic acid deficiency), active infection, imunosupresion, malignancy, and pregnancy or breastfeeding were **exclusion criteria.**

Study parameters

Patient data were recorded from the hospital electronic database.

Main study parameter

Was the variation in flow-mediated vasodilation of the brachial artery as an endothelial dysfunction indicator (see below **Therapeutic intervention**).

Therapeutic intervention

The study intervention consisted of 3 infusions over 30 minutes each, 72 hours apart, as follows:

- 250ml 10% glucose solution as comparator;
- 500-mg FCM (Ferinject; Vifor Pharma, Opfikon, Switzerland), in 250-mL 0.9% saline solution;
- 200-mg IS (Venofer; Vifor Pharma), in 250-mL 0.9% saline solution (**Figure 3.1**).

The 10% glucose solution was chosen as comparator due to its close to the sucrose iron solution osmolarity (505 vs. 564 mOsm/L), allowing to avoid the compounding effect of the high osmolarity on the endothelium.

Assessment of endothelial function

The endothelial function was assessed 15 minutes before and after each infusion by measuring floe-mediated vasodilatation (FMD). The measurements were performed 15 minutes before and after each infusios (**Figure 3.1**).

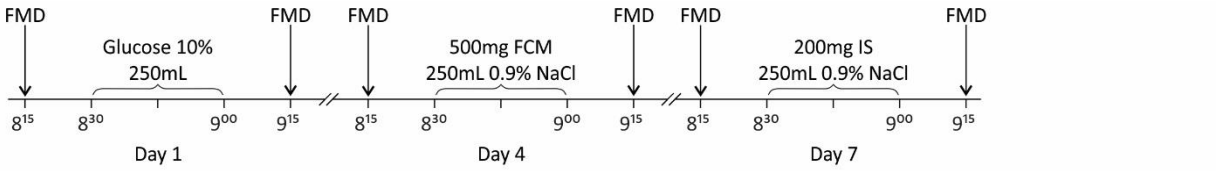

Figure 3.1. Study schedule. The infusions were administered over 30 minutes, 72 hours apart, the comparator (10% glucose) first, followed by FCM and IS. Flow-mediated vasodilatation (FMD) was measured 15 minutes before and after infusions

FMD was calculated as the percentual variation in brachial artery diameter 60 seconds after release of the occluding cuff, using the formula: FMD(%) = (maximum diameter – baseline diameter)/ baseline diameter.

Statistical analysis

Normal distribution of data was assessed by the Shapiro–Wilk test. Data were expressed as mean/median and 95% confidence interval. Categorical variables were described as percent, and comparison between groups was performed with the Pearson x2 test. The post/preinfusion differences (D) for each parameter were compared by the Wilcoxon paired test. A p value <0.05 indicated statistical significance.

3. Results

Median age of the cohort was 60 (54–66) years, 20% were men, 23% had diabetes mellitus, 31% were obese, 94% had arterial hypertension, and 45% had cardiovascular disease.

Eighty percent were in CKD stages 3–4, and median estimated glomerular filtration rate was 24 (19–34) mL/min. Glomerular diseases followed by vascular nephropathies were the most common CKD causes.

The anemia was predominantly mild (10% with hemoglobin lower than 10 g/dL), as was nflammation (19% with CRP over 5 mg/L). Almost all had iron deficiency, 41% absolute or functional 35%.

Endothelial function after a single dose of IV iron

FMD decreased only after IS administration from 16.0 (12.0–20.9) to 12.3 (10.3–16.6)% (**Figure 3.2**).

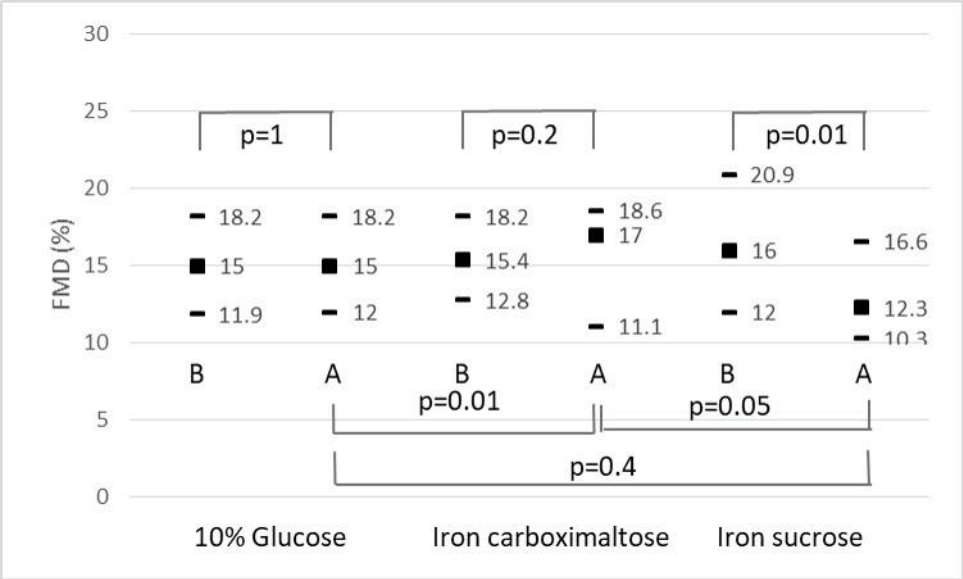

Figure 3.2. Flow-mediated vasodilation (FMD; %) before (B) and after (A) 10% glucose, iron carboxymaltose, and IS infusions

Moreover, FMD was altered significantly (median downward change) only after IS as compared to FCM and 10% glucose (-2.3 (0.4 to -5.7) versus 1.0 (-2.0 to -2.5)%.

Study 4 - Endothelial dysfunction and oxidative stress after a single intravenous dose of iron carboxymaltose in patients with non-dialysis CKD

1. Objectives

To investigate the effect on endothelial function and oxidative stress, of 1000mg iron carboxymaltose compared with a control-solution (0.9% NaCl), in nondialysis CKD patients with iron deficiency and anemia.

2. Materials and methods

This was a unicentric, prospective, crossover study, conducted in Nephrology Department of "Dr. Carol Davila" Teaching Hospital of Nephrology "Dr. Carol Davila". After applying the exclusion criteria, 41 subjects were included.

Exclusion criteria were: contraindications of intravenous iron administration: history of iron allergic reactions, hemochromatosis, signs of iron overload (ferritin >500 ng/mL and/or transferrin saturation >50%); iron supplementation or treatment with erythropoiesis stimulating agents during the last 6 month before enrolment; active smoking; pro- or antioxidant therapy during the last 3 months before enrolment; active bleeding; other causes of anemia (hemoglobinopathies, vitamin B12 and/or folic acid deficiency; multiple myeloma and other paraproteinemias); severe anemia (Hb<7g/dl); baseline FMD<7% (noticing the existence of atherosclerosis which limits arterial reactivity); malignancies (last 6 months); active inflammation and/or infection; active liver diseases, pregnancy or breastfeeding.

Main study parameters:

Endothelial function indicators:

- Clinically - variation of flow-mediated vasodilatation (FMD) of brachial artery.
- Paraclinically - variation of circulating level of nitrate/nitrite (NO_x) and L-arginase activity.

Oxidative stress indicators - variation of circulating level of total plasma antioxidant capacity (TEAC) and of advanced oxidation protein products (AOPP).

Study protocol

The study procedures were performed after a fasting period of approximately 12 hours.

The intervention:

- Control: 250 mL 0,9% NaCl;
- The intervention: 1000 mg of iron carboxymaltose in 250 mL 0,9%NaCl;

in this order, infusions over 30 minutes each, 24 hours apart.

At the study moments (before and after the intervention), the procedures described in **Figure 4.1.** were performed.

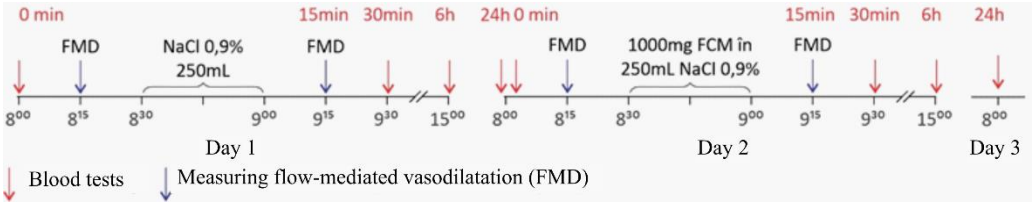

Figura 4.1. Study schedule. FCM – iron carboxymaltose; FMD – flow-mediated vasodilatation; NaCl0.9% - isotonic saline

Statistical analysis

Study parameters – FMD; NO_x, activitatea arginazei, TEAC, AOPP – before-after the intervention and control, as well as variation before-after the intervention and control, both in absolute number (Δ = the post-infusion value- pre-infusion value) and percentage (value post-perfusion-value pre-perfusion%), were compared.

Asymmetric data were expressed as median and 95% confidence interval. For comparing medians between repeated measurements of a variable in the same subject, the nonparametric t-paired test and Wilcoxon signed rank sum test were used. For comparison of medians between two groups, the nonparametric Kruskal-Wallis test was used.

Categorical variables were described as percentages, and the comparison beteen two groups was performed using Chi^2 test or Fisher's Exact Test (for a small number of subjects). Differences were expressed as OR and confidence intervals.

The relationship between two variables was analized by bivariate correlation, using Spearman test.

For the analysis of the relationship between NO_x and the investigated parameters, subjects were divided into subgroups defined according to the median of the entire group.

For the analysis of the relationship between AOPP and the investigated parameters, patients were divided into subgroups defined according to the median of the entire group. Parameters where significant differences were identified were introduced into binomial logistic regression models.

The differences identified from comparisons were considered statistically significant at an accepted threshold of 95%, i.e. p=0.05.

3. Results

Median age was 68 (58-70) ani, 61% were women, 90% had hypertension (more than a half with target-organ involvement), 41% had diabetes (most of them complicated), and 42% had cardiovascular diseases.

Median eGFR was 24 (16-34) ml/min, 39% had G5 category of non-dialysis CKD. Median albuminuria was moderate: 309 (177-553) mg/g creatinine. Inflamation had a low prevalence: PCR 3 (2-6) mg/l.

Anemia was mild: only 15% had hemoglobin less than 10g/dl. Most of the patients (68%), had absolute iron deficiency.

Endothelial function

Flow mediated vasodilatation

There were no differences between FMD (%) neither before, nor at 15 minutes after each of the infusions (NaCl 0,9%, and iron carboxymaltose) (**Figure 4.2**).

Plasma nitrates/nitrites (NO_x)

Plasma level of nitrates/nitrites NO_x) was similar before both infusions, but decreased at 30 minutes after both infusions, with a greater drop after iron carboxymaltose (**Figure 4.3**).

NO_x levels were lower after iron carboxymaltose administration versus 0.9% NaCl at all study moments. Moreover, in the case of carboxymaltose iron, the decline from basal level persisted after 6 and 24 hours (**Figure 4.3**).

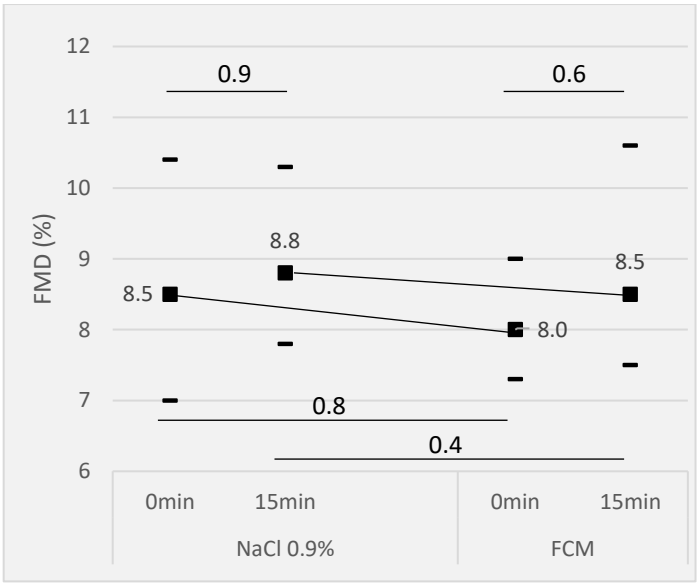

Figure 4.2 Flow-mediated vasodilatation expressed in percentages (FMD%), before and at 15 minutes after 0.9% NaCl and iron carboxymaltose (FCM)

NO_x variation 30 minutes after FCM administration was not related to variations in FMD, arginase or total anti-oxidant capacity (r=-0,1) p=0,5; r=-0,3 p=0,8; r=-0,04 p=0,8).

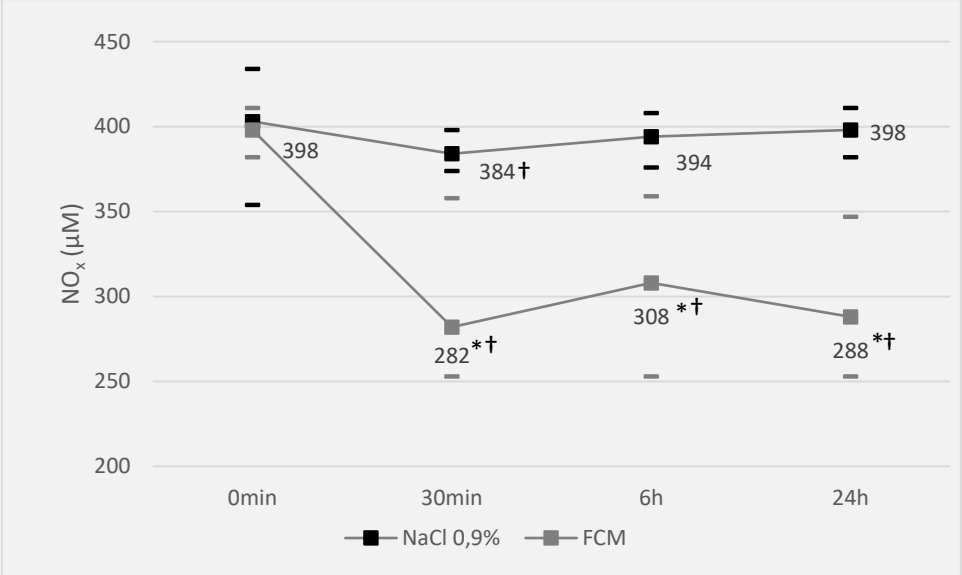

Figure 4.3. Plasma level of nitrates/nitrites (NO_x) at study moments, according to intervention: 0.9% NaCl and iron carboxymaltose (FCM). *p<0.05 versus NaCl 0,9%; † p<0.05 vesus moment 0min

Analyzing subgroups defined according to the median of NO_x at 30 minutes after FCM infusion of the entire group, those with lower than the median of NO_x variation, had lower eGFR, higher albuminuria, and lower total cholesterol and serum ionic calcium.

Other studied parameters, including FMD variation, arginase plasma activity, and total serum anti-oxidant capacity, were similar in both subgroups.

Arginase plasma activity

Arginase activity was no different before the two infusions (**Figure 4.4**).

The kindey function was not associated with arginase activity at baseline (r=-0,04, p=0,8), which was expected given the similarly marked reduction in eGFR in all investigated subjects.

There were no differences in plasma arginase activity or variation after iron carboxymaltose infusion compared to 0.9% NaCl at any moment of the study (**Figure 4.4**).

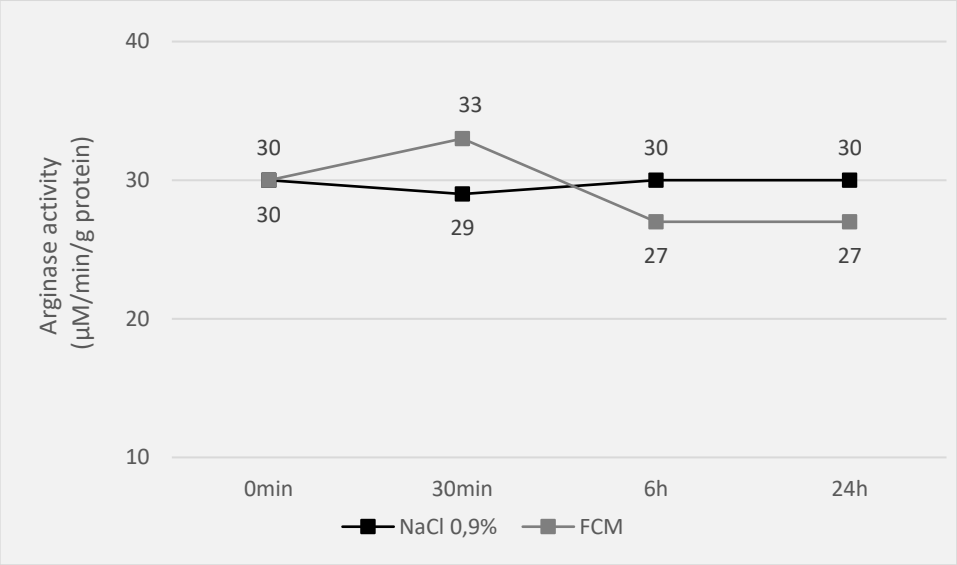

Figure 4.4. Plasma arginase activity at study moments according to the intervention: : 0.9% NaCl and iron carboxymaltose (FCM)

Oxidative stress

Total serum antioxidant capacity

Total antioxidant activity was similar before both infusions and did not change at any of the study moments, for none of the interventios (**Figure 4.5**).

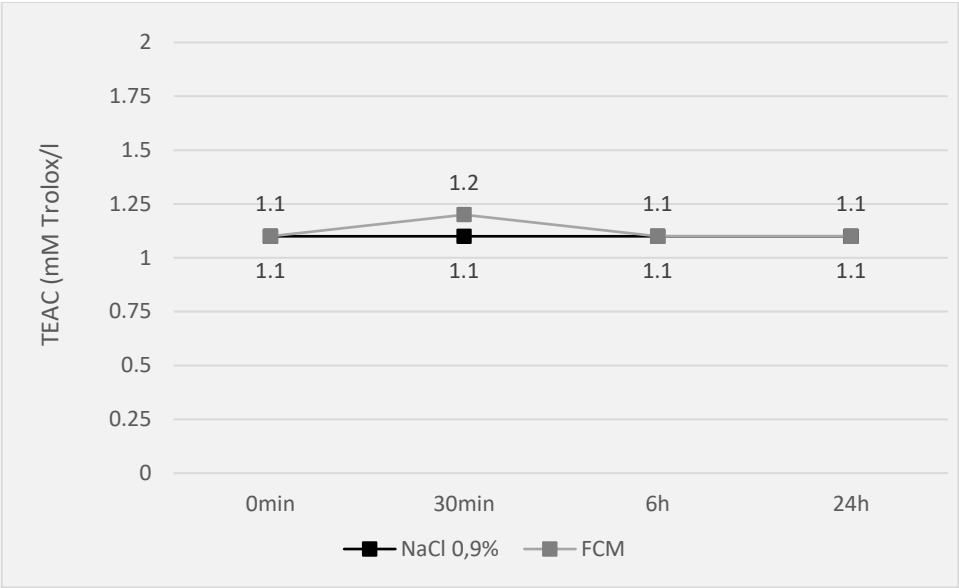

Figure 4. 5. Total serum antioxidant capacity (TEAC) at study moments according to the intervention: 0.9% NaCl and iron carboxymaltose (FCM)

Advanced oxidation protein products (AOPP)

Were measured in 22 subjects. Kidney function was not associated with baseline AOPP level.

AOPP level increased after iron carboxymaltose in comparison with 0.9% NaCl infusion, at all study moments. The rise was significant at 30minutes after iron carboxymaltose infusion, and continued at 6 hours versus 30minutes. Then, AOPP level decreased after 24 hours compared to 6 hours, but without returning to the baseline (**Figure 4.6**).

In subgroups defined according to median AOPP 30 minutes after FCM administration of the entire group, those with higher than median AOPP levels had more pronounced anaemia, higher total serum cholesterol, lower serum total phosphatase and body mass index.

Larger variations in AOPP were independently associated with more pronounced anaemia, higher serum cholesterol levels, and lower serum total alkaline phosphatase levels.

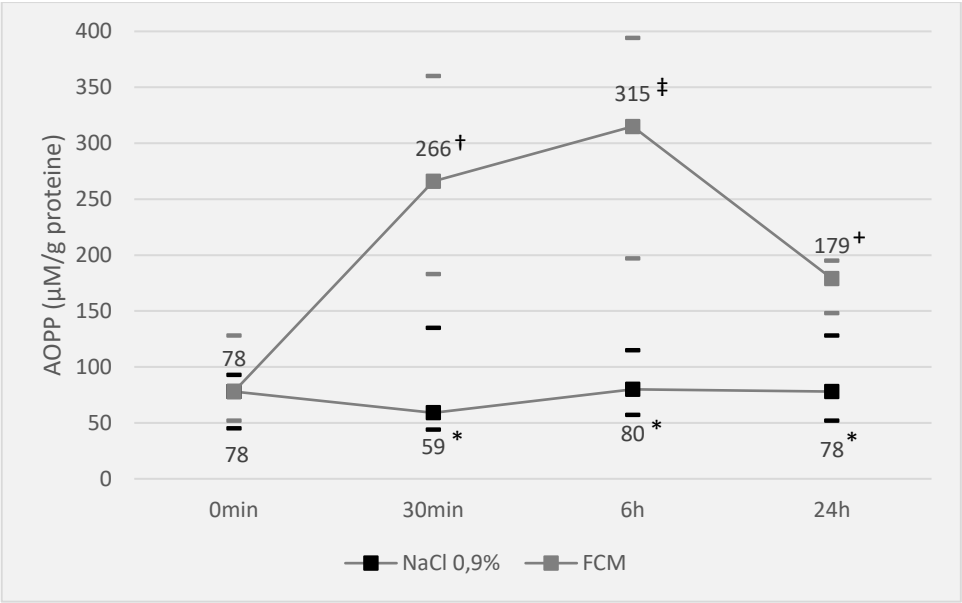

Figure 4.6. Advanced oxidation protein products (AOPP) at study moments, according to intervention: 0.9% NaCl and iron carboxymaltose (FCM). † p<0,05 vs. 0min; ‡ p<0,05 vs. 30min; + p<0,05 vs. 6h

10. Conclusions and personal contributions

Anemia has an increased prevalence globally (22.8% in 2019), despite a decreasing trend over the last 20 years. Iron deficiency is the first cause of anemia, not only in the general population, but also in patients with chronic diseases (CKD, heart failure, inflammatory bowel disease, neoplasia), even in developed countries.

Up to 20-40% of patients with CKD has iron deficiency, and anemia is one of the most frequent complications, its prevalence increasing as glomerular filtration rate (GFR) declines. Iron deficiency plays a central role in the pathogenesis of anemia in patients with CKD. The clinical impact of anaemia concerns quality of life, progression of CKD, incidence of cardiovascular events and mortality.

Iron therapy is commonly indicated in CKD, and parenteral iron formulations are indicated even in non-dialysis CKD patients, since oral iron therapy has limitations given both by the slower response of hematopoiesis and, especially, by reduced adherence to treatement due to poor divestive tolerance.

Intravenous iron therapy is effective in achieving and maintaining hemoglobin levels, meanwhile reducing erythropoietin requirements, and is superior to oral iron. Particularly in hemodialysis patients, parenteral iron is preffered, as additional venous punctures are avoided.

Intravenous iron formulations are nanoparticles consisting of a oxyhydroxide nucleus covered in a carbohydrate shell. Depending on nanoparticle's characteristics, different products, act unique. Thus, after parenteral administration, it is possible that an variable amount of iron to be released directly into the blood, being unbound to transferrin. This "free" plasma iron is redox active and cand produce free radicals and oxidative stress.

In vitro and *in vivo* studies assessed the effect of parenteral iron preparations in various categories of iron deficiency patients with different methodologies, formulations and dosages. The results are discordant: some support adverse effects mediated by oxidative stress and endothelial dysfunction, even with long-term impact, others argue for reducing cardiovascular morbidity.

Since reported data are contradictory, we designed this paper in order to deepen the effect of two common parenteral iron formulations - iron sucrose and iron carboxymaltose -, one of which, iron carboxymaltose, is considered extremely safe. We structured the research into four studies.

After analysing data from the first pilot study, in which we aimed to describe morbidity (frequency, duration and causes of hospitalisations) associated with long-term iron sucrose therapy in haemodialysis CKD patients, we concluded that long-term administration of iron sucrose seems to minimally influence general and specific causes of morbidity in non-diabetic haemodialysis CKD patients. However, high doses of iron sucrose appear to be associated with increased risk of hospitalization. As a result, given that data from previous studies are conflicting, even if lower doses (100-200mg iron sucrose/month) are safe for the treatment of anemia in dialysis patients with CKD, caution is needed at higher doses.

Continuing the research with the second, prospective crossover study, we aimed to investigate changes in oxidative stress during hemodialysis sessions in patients receiving or not, intravenous iron therapy.

We noticed that a usual dose of iron sucrose (100 mg) may acutely worsen erythrocyte antioxidant activity in chronically hemodialysis patients, as suggested by reduced glutathione consumption and greater decrease in total erythrocytes antioxidant capacity after iron infusion. However, iron sucrose appears to have limited influence on extracellular antioxidant status. At the same time, iron sucrose augments pre-existing oxidative stress in hemodialysis patients without carbonyl stress being influenced, which is supported by increased lipid peroxidation (TBARS, ox-LDL), while dicarbonyl compounds and fructosamine remained unchanged.

Nevertheless, the clinical significance of these findings and their potential impact on long-term health require further studies.

The validity of the results is supported by the fact that this study is a prospective and cross-over one.

Given that comparative data regarding the effect of different parenteral iron preparations on endothelial function are limited, this paper makes contributions to an area of ongoing research. Thus, in the third prospective crossover study, we aimed to investigate acute changes in the endothelial function induced by iron sucrose versus iron carboxymaltose in patients with non-dialysis CKD.

Arterial reactivity was acutely altered by intravenous administration of iron sucrose, but not iron carboxymaltose, to non-dialysis CKD patients with a high prevalence of iron deficiency. Although there are limitations to this study, as biological indicators of endothelial dysfunction have not been determined, the results support previous data in which iron sucrose, rather than iron carboxymaltose, is involved in acute endothelial dysfunction.

Continuing the research we examined the intravenous parenteral formulation, iron carboxymaltose. This preparation is the least investigated in terms of acute impact on oxidative stress and endothelial dysfunction, being considered to have a very good safety profile.

In this study, we observed the effect of administering 1g iron carboxymaltose to non-dialysis CKD patients. The study benefit from an appropriate methodology. Firstly, patients were carefully selected to allow assessment of flow-mediated vasodilation. Thus, subjects with altered flow-mediated vasodilation (less than 7%) were excluded. Secondly, biochemical parameters of endothelial function and indicators of oxidative stress were measured simultaneously with easily determinable clinical parameters (flow-mediated vasodilation). So far, only one other study has been published with similar goals, but with a smaller number of patients.

Iron carboxymaltose did not influence flow-mediated vasodilation at any moment of the study (30 min, 6h, 24 hours after administration), given that, paradoxically, it persistently reduces (24 hours) plasma levels of nitric oxide metabolites. The persistent reduction in plasma levels of nitric oxide metabolites can have two causes. Either as a consequence of FCM-induced endothelial dysfunction, i.e. the reduction of NO production by inhibition of endothelial synthase by oxidative stress, or as a consequence of NO_x consumption for NO formation by reduction, via NO-synthase independent pathways. Since FCM

administration did not alter flow-mediated vasodilation under the study conditions, it is likely that the reduction in circulating oxidised metabolites of NO was caused by their reduction to NO via the independent route of NO synthases, and the NO thus produced prevented altered vascular reactivity. This hypothesis is a priority of the research within the doctoral thesis.

Iron coraboxymaltose does not alter the anti-oxidant capacity of serum, which suggests, consistent with other studies, the insignificant effect of FCM on oxidative stress by releasing free iron into circulation. However, FCM persistently increased plasma levels of advanced protein oxidation products. Highlighting for the first time the generation of advanced protein oxidation products after parenteral iron carboxymaltose therapy is another priority of this doctoral thesis.

Therefore, although the oxidative stress generated by intravenous administration of FCM seems low, it is sufficient to produce protein oxidation. Previous studies have shown associations between the amount of iron sucrose administered parenterally, the level of advanced protein oxidation products and preclinical indicators of atherosclerosis (increase in mean intimate thickness at the common carotid).

As controlled studies evaluating the usefulness and safety of parenteral administration of iron preparations, including FCM, did not have survival as a primary parameter, the present study highlights the possibility of increasing endothelial damage over time succeeding intravenous treatment with FCM.

In conclusion, the personal contributions of the research in this doctoral thesis consist in demonstrating:

1. the predominant pro-oxidant effect on red blood cells of intravenous administration of iron sucrose, the parenteral formulation of considerable age and the most widely used. At the same time, iron sucrose increases oxidative stress, resulting in lipid peroxidation (including LDL);
2. for the first time, that iron carboxymaltose does not alter flow-mediated vasodilation, while stable oxidised metabolites of NO decrease. To explain this

result, the original hypothesis of reducing the level of stable oxidized metabolites of NO to nitric oxide by NO synthase pathways was hypothesized;

3. for the first time, that the level of advanced protein oxidation products increases persistently after parenteral administration of iron carboxymaltose, suggesting the possibility of initiation or aggravation of endothelial lesions.

The research within this doctoral thesis opens the way to exploring, under clinical conditions, other effects of parenteral iron formulations both on endothelium (expression of adhesion molecules) and metabolism of fibroblastic growth factor 23, the main factor controlling phosphaturia. Also, validating the hypothesis of maintaining an adequate level of nitric oxide by NO-synthase independent pathways would open new perspectives in understanding nitric oxide metabolism, with therapeutic implications.

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PUBLISHED SCIENTIFIC PAPERS

Articles:

1. **Mehedinti** AM, Lipan M; Stancu S, Mircescu G, Capusa C. **Acute Effects of Iron Sucrose and Iron Carboxymaltose on Endothelial Function in Nondialysis Chronic Kidney Disease Patients**. American Journal of Therapeutics. 2020 April, 28;29(2):e175-e181. doi: 10.1097/MJT.0000000000001091. PMID: 35389571. ISI; IF2.68; https://journals.lww.com/americantherapeutics/Abstract/2022/04000/Acute_Effects_of_Iron_Sucrose_and_Iron.2.aspx. (**Capitolul 8, pag. 93-106**)
2. **Mehedinti** AM, Capusa C, Andreiana I, Popa O, Mircescu G. **Long-Term Intravenous Iron Therapy and Morbidity in Hemodialysis Patients**. Maedica (Bucur). 2021 June;16(2):194-199. doi: 10.26574/maedica.2021.16.2.194. PMID: 34621339; PMCID: PMC8450661. BDI; https://www.maedica.ro/articles/2021/2/2021_16(19)_No2_pg194-199.pdf. (**Capitolul 6, pag. 51-59**)
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Abstracts:

1. **AM Mehedinti**, L Iosif, I Stoian, S Stancu, G Mircescu, C Capusa. **Acute Effects of a Single Intravenous Iron Dose on Endothelial Function and Oxidative Stress in Non-Dialysis Chronic Kidney Disease Patients. Protocol for a Cross-Over, Single-Center Study.** Abstracts of the 30th Meeting of the European Renal Cell Study Group (ERCSG). Nephron 2018;139:83-111. doi: 10.1159/000488293; https://karger.com/nef/article/139/1/83/211623/Abstracts-of-the-30th-Meeting-of-the-European.

2. **AM Mehedinti**, L Iosif, I Stoian, I Andreiana, C Capusa, G Mircescu, **Acute impact of a single high dose of ferric carboxymaltose on endothelial function in CKD non-dialysis patients.** Mini Orals of the 58th ERA-EDTA Congress 2021. Nephrol. Dial. Transplant 2021; 36(1), gfab085.0025, https://doi.org/10.1093/ndt/gfab085.0025.

Projects:

Privately funded project obtained as a result of **The competition for funding a scientific research grant organized by SC Sanador SRL, Contract 10488/19.04.2016, from 1 mai 2016-31 oct. 2017 "Prospective crossover study on effects of single intravenous iron administration on endothelial dysfunction and oxidative stress parameters in patients with non-dialysis chronic kidney disease".**

Position within the project: member.