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Doctoral dissertation (article-based)

SCREENING OF NOVEL CHELATORS OF MICROBIOGENIC METALS

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Hradec Králové 2020   Maria Carmen Catapano, MSc

I hereby declare that this thesis is my original work which I solely composed by myself under the supervision of Assoc. Prof. Přemysl Mladěnka. All used literature and other sources are summarized in the list of references and properly cited. This work has not been submitted for any different or equal degree.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně pod vedením svého školitele doc. Přemysla Mladěnky. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

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Maria Carmen Catapano, Msc

Lovingly dedicated to my family

"The scientist is not a person who gives the right answers,
he is one who asks the right questions"

"Lo scienziato non é colui che sa dare le giuste risposte,
ma colui che sa porre le giuste domande"

(Claude Levi-Strauss)

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I finish today with much more than a Ph.D. degree, with much more than a huge set of technical skills and publications. I leave as a new human being.

ABSTRACT

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Title of Doctoral Thesis: Screening of novel chelators of microbiogenic metals

Iron, copper and zinc are microbiogenic elements which play crucial roles in a series of physiological processes in human organism. Homeostasis of these transition metals is strictly regulated since, among others:

  a) free or loosely bound iron or copper can catalyse the production of hydroxyl radical;

  b) lack of zinc but also of the previously mentioned metals is associated with significant impairments.

Hereditary hemochromatosis, transfusion-induced secondary iron overload and Wilson´s disease are known as pathological conditions associated with metal overload in the organism. Chelator agents have vital relevance for the treatment of these impairments. There are also numerous diseases with homeostatic imbalances in iron, copper and or zinc: neurodegenerative diseases, cardiovascular diseases, cancer and diabetes mellitus. Different chelating compounds have been examined for the treatment of these impairments.

The aim of this doctoral thesis was to perform a screening of metal chelating properties of different compounds, at (patho)physiologically relevant pH, with the objective of their potential therapeutic use or detection of toxicity toward removal of non-targeted essential metals (e.g. mentioned zinc), and characterization of the formed complexes. In order to fulfil these aims, two major approaches: the competitive (the hematoxylin and bathocuproine methods) and non-competitive approach (the Job and our complementary methods) were applied, and also novel methodologies were developed (zinc chelation and very sensitive method for hydroxyl radical detection). Different compounds were tested: flavonol isoquercitrin, flavonoid derivatives flavonolignans from silymarin, metabolites of isoflavonoids formed by human microflora and coumarins.

Within the screening, following novel finding were published:

1. Detailed analysis of isoquercitrin complex with iron and copper at 4 (patho)physiological pH conditions (7.5, 6.8, 5.5 and 4.5);
2. Screening of copper chelation and reduction properties of 4-methylcoumarins;
3. Detection of interactions of microbial metabolites of isoflavonoids with copper and zinc.

Concerning methodologies, the following outcomes were reached:

1. Antioxidants can profoundly interfere with the hematoxylin assay aimed at initial cupric chelation screening. This was documented by use of a set of different antioxidants with no chelating sites.
2. The research of metal chelators would not be sufficient without testing their effect on the Fenton reaction since even the potent chelator can behave as pro-oxidants (e.g. EDTA). For this purpose a HPLC method coupled with coulometric detection has been developed.
3. Another important objective of this work was to develop a method for zinc chelation. This was accomplished using zinc detection by a spectrophotometric indicator dithizone.

It can be concluded that this research opened novel possibilities not only for our research group but possibly also for the other investigators interested in pharmacology of trace metals.

ABSTRAKT V ČEŠTINĚ

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Název dizertace: Screening nových chelátorů mikrobiogenních kovů

Železo, měď a zinek jsou mikrobiogenními prvky majícími zásadní úlohy v řadě fyziologických procesů v lidském organismu. Homeostáza těchto přechodných kovů je velmi pečlivě regulována, protože, mimo jiné

    a) volné nebo nepevně vázané ionty železa a mědi mohou katalyzovat produkci hydroxylového radikálu;

    b) nedostatek zinku, ale i ostatních dvou jmenovaných kovů, je spojen s významnými poruchami.

Dědičná hemochromatóza, sekundární transfúzní přetížení železem a Wilsonova choroba jsou známými případy patologických stavů spojených s nadbytkem kovů v organismu. Podání chelátorů má nepostradatelnou úlohu při léčbě těchto chorob. Existuje také řada nemocí s poruchou homeostázy železa, mědi a zinku jako jsou neurodegenerativní nemoci, kardiovaskulární choroby, nádory a diabetes mellitus. Různé chelátory byly zkoušeny pro léčbu těchto stavů.

Cílem této doktorské práce bylo uskutečnit screening chelatačních vlastností různých chelátoru za různých (pato)fyziologických pH podmínek s výhledem na jejich potenciální terapeutické využití nebo zjištění toxicity ve vztahu k necílovým esenciálním kovům (např. zmíněný zinek), a charakterizace vytvořených komplexů. Aby byly tyto cíle splněny, byly použity dva hlavní přístupy: kompetetivní (hematoxylinová a bathokuproinová metoda) a nekompetetivní přístup (Jobova a naše doplňková metoda). Byly také vyvinuty nové metodiky pro chelataci zinku a velmi citlivá metoda pro detekci hydroxylového radikálu. Byly otestovány různé sloučeniny od flavonolu isokvercitrinu přes flavonoidní deriváty flavonolignany ze silymarinu, metabolity isoflavonoidů tvořené lidskou mikroflórou až ke kumarinům.

Ve vztahu ke screening byly publikovány tyto nové výsledky:

1. Detailní analýza tvorby komplexu isokvercitrinu s železem a mědí za 4 (pato)fyziologických pH (7,5; 6,8; 5,5 a 4,5);
2. Screening měď chelatačních a redukčních vlastností 4-methylkumarinů;
3. Zjištění interakcí mikrobiálních metabolitů isoflavonoidů s mědí a železem.

Ve vztahu k metodologii bylo dosaženo následujících cílů:

1. Antioxidanty mohou výrazně ovlivnit hematoxylinovou metodiku pro úvodní screening chelatace měďnatých iontů. Tato interference byla prokázána na řadě antioxidantů neobsahujících ve své struktuře chelatační skupiny.
2. Výzkum chelátorů by nebyl úplný, kdyby nebyl otestován jejich vliv na Fentonovu reakci, protože i velmi silných chelátor se může chovat jako pro-oxidant (např. EDTA). Z tohoto důvodu byla vyvinuta HPLC metodika spojená a tzv. „coulometrickou" detekcí.
3. Jiným důležitým cílem práce byl vývin metodiky pro chelataci zinku. Toto bylo dosaženo za použití spektrofotometrického indikátoru dithizonu.

Závěrem může být řečeno, že tento výzkum otevřel nové možnosti nejen pro naši výzkumnou skupinu ale potenciálně i pro jiné výzkumníky se zájmem o farmakologii stopových kovů.

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1. INTRODUCTION

Transition metals iron, copper and zinc are trace elements with invaluable importance for the human body as they are part of a variety of enzymes and other functional proteins. Homeostasis of iron, copper and zinc is carefully regulated in the human body by sophisticated mechanisms. Its disruption by both genetic factors and environmental influences result in the unwanted effects of these metals. Loose or unstable iron and copper are efficient catalysts of the Fenton reactions leading to the formation of highly toxic free radicals. This is relevant in systemic metal overload conditions (such as in hemochromatosis and Wilson's disease), but can also follow local metal dysbalance (e.g. in the case of ischaemia, tumors). Zinc is considered to have very low toxicity, but its removal by metal chelators, which are mostly non-selective, is of important clinical relevance. It should be also emphasized that all these three metals are also playing roles in other diseases not directly related to metal overload, In particular neurodegenerative diseases, such as Alzheimer's and Parkinson's, cardiovascular disease, cancer or diabetes mellitus.

In cases of intoxications or other metal excess conditions, *chelating agents* are used in the treatment. Chelators are organic polyvalent compounds forming complexes with metals. These complexes should be redox inactive and excreted from the body by the liver or the kidney.

The most know clinically used chelators are deferoxamine for iron and trientine for copper. There are no approved clinically used zinc chelators.

Due to the relatively narrow clinical use in current practice and on the other hand, great therapeutic potential in various diseases is desirable to find new substances capable of chelate these transition, ideally selectively, metals in order to treat this imbalance in the human body and prevent toxicity. Also the potential toxicity ensuing from excessive chelation should be considered.

2. THEORETICAL BACKGROUND

2.1 Elements in the human body

The human body contains elements that can be classified as abundant elements and trace elements (**Figure 1**). Abundant elements can be divided in major elements (96% of the total body weight) and semi-major (accounting for 3 to 4% of the total body weight). Trace elements form the rest, which is about 0.2%.

Shortly, major elements are involved in the formation of covalent bonds and are important constituents of tissues (oxygen, carbon, hydrogen, nitrogen); semi-major elements, which exist in the ionic state and are involved in functions of the living body through maintenance of osmotic pressure and membrane potentials (potassium, sodium and calcium). Deficiency of major elements can lead to nutritional disorders, and their presence in excess can cause obesity [1-3]. Deficiencies or excess states of semi-major elements often result in water and electrolyte abnormalities.

While trace elements, also known as micro-nutrients or in the case of microbiogenic metals, are elements that are contained in low or even tiny quantities in the human body. Their excess can, on the contrary, cause noxious effect on the living organism. Although they are present in low concentrations, they are involved in many different physiological processes due to their presence as active centers of enzymes or other trace bioactive substances such as vitamins. Less is known about the regulation of their levels and effect of different drugs on their body content [4].

Since some trace elements are forming parts of different biomolecules, a shortage of a single trace element is often associated with non-specific clinical manifestations, e.g. it manifests as a combination of various symptoms. In combination with the absence of specific clinical markers associated with their deficiency, it is often difficult for clinicians, at the beginning, to consider the symptoms to be associated with lack of a trace element [5].

The effect of trace elements *in vivo* is quite specific and therefore a deficiency of one element can be prevented or remedied only by that, and not by another element. This specificity of trace elements action *in vivo* contrasts with the much less specific behavior of trace elements *in vitro*. The high degree of specificity *in vivo* is brought by carriers with specific sites that a) recognize a certain element when it enters the organism and b) distribute it for the delivery to the sites of need. Among the large carrier molecules are plasma specific proteins such as transferrin and ceruloplasmin, but on the other hand as well the non-specific albumin. The binding capacity

of specific protein carriers is under physiological conditions undersaturated. Under these normal conditions, for example, transferrin carries only one third of its maximum iron load. The reserve binding capacity can be considered an effective buffer against excessive exposures; toxicity from non-specific organ distribution of trace elements usually results only after this buffering capacity is exceeded [6].

Also, the action of a trace element is specific and is dependent on several properties such as valence state, redox potential, ionic radius, coordination number, coordination geometry, spin state and rate of ligand exchange. These properties, in their entirety, distinguish one element from another. There is not a general consensus about the classification of trace elements. They have been classified according to the WHO classification [7], the Frieden's classification of elements [8] or the Frieden's Categorical Classification of Elements [9]:

According to WHO classification, nineteen trace elements have been divided into three groups:

1. Essential elements: zinc (Zn), copper (Cu), iron (Fe), selenium (Se), chromium (Cr), cobalt (Co), iodine (I), and molybdenum (Mo).
2. Probably essential elements: manganese (Mn), silicon (Si), nickel (Ni), boron (B), vanadium (V).
3. Potentially toxic elements: fluorine (F), lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As), aluminum (Al), lithium (Li).

Frieden's classification is a biological classification of trace elements based on their amount in tissues:

1. Essential trace elements: boron, cobalt, copper, iodine, iron, manganese, molybdenum, and zinc.
2. Probably essential trace elements: chromium, fluorine, nickel, selenium, and vanadium.
3. Physically promotive trace elements: bromine, lithium, silicon, tin, and titanium.

While according to the Frieden's categorical classification, which however includes not only trace elements, twenty-nine types of elements present in the human body are arranged into five major groups as follows:

1. Group I: basic components of macromolecules such as carbohydrates, proteins, and lipids (carbon, hydrogen, oxygen, and nitrogen);

2. Group II: nutritionally important minerals also referred to as principal or macro elements (daily dose per adult is $\geq$ 10 0mg/ include sodium, potassium, chlorine, calcium, phosphorus, magnesium, and sulfur);

3. Group III: essential trace elements. The trace elements are also called minor elements (daily dose per adult is $\leq$ 100 mg/day include copper, iron, zinc, chromium, cobalt, iodine, molybdenum, and selenium);

4. Group IV: additional trace elements. Their role is yet unclear and they may be essential. Examples include cadmium, nickel, silicon, tin, vanadium, and aluminum.

5. Group V: these metals are not essential and their functions are not known. They may produce toxicity in excess amounts. Examples include gold, mercury, and lead.

This dissertation project could not include all these metals and it is based mainly on 3 microbiogenic elements: iron, copper and zinc.

Figure 1: Elements found in the human body [10]. The green boxes are the abundant elements, the yellow are the trace elements and the orange ones are the remaining elements

2.2 Metal toxicity

Metal ions are common part of human diet and hence their exposure is physiological, however, under certain conditions, they can be absorbed in high doses and hence be also toxic to humans. Toxic effects can have different symptoms, which depend on the metal, dose, type of compound and other factors including health conditions of the affected person. The toxicity can be both acute (high-dose exposure) intoxication and chronic metal overload. Acute ingestion of toxic metal ions can be accidental or voluntary. Chronic one may depend on environmental, occupational or iatrogenic causes or can follow genetic abnormalities. In determining the toxicity of an element it is important to consider the concentration (or dose) range, chemical form including oxidation states in order to better understand the behavior of that element in the human body. Some trace elements can easily switch between oxidation states and this redox cycling can be associated with generation of reactive oxygen species (ROS). In particular copper and iron are able to trigger the Fenton reaction (equation 1). These metals are acting as a catalyst in this reaction.

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + + OH^- + {}^{\bullet}OH \qquad \text{(equation 1)}$$

Indeed copper in excess, especially in its free hydrated form (i.e., Cu^{2+}), can be potentially toxic to both plant and animal organisms as can was documented by altering membrane permeability and by affecting chromatin structure, protein synthesis, and various enzyme activities [11]. Such effect can be mediated by ROS. The Fenton reaction is leading to highly reactive ROS: the hydroxyl radicals ($HO^{\bullet}$) which results from the reaction of the redox-active metal with hydrogen peroxide (H_2O_2) which is ubiquitous in the cells of organisms as a by-product of oxygen metabolism [12]. Generally, H_2O_2 is relatively harmless as it reacts with biomolecules at relatively low rates and specific enzymes such as catalase and glutathione peroxidase facilitate its removal. However, in the presence of certain metals, the presence of H_2O_2 may lead by the described process to the formation of the highly reactive and damaging $HO^{\bullet}$.

Similarly, iron can redox cycle, which in fact is one of its main biological features. This ability to donate or receive an electron, i.e., to convert between its ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms a useful and essential property for many enzymatic redox reactions, but it can be, on the other hand, harmful when free iron participates on the mentioned Fenton reaction. It need not to be emphasized that hydroxyl radical reacts with most biological molecules, leading to the degradation of these compounds themselves through a series of chain reactions [13, 14].

2.3 Metals

2.3.1 Iron

Iron, is a chemical element with symbol Fe and atomic number 26. It is a brittle, hard substance, classified as a metal in group 8 of the periodic table of the elements. Iron is an abundant element on earth [15] and is essential component of almost every living organism [16, 17] with the exception of a few species such as *Borrelia burgdorferi* [18]. Iron has the possibility to be present in various oxidation states (+2, +3, +4, +6), but forms compounds mainly in the oxidation states +2 (iron (II), "ferrous") and +3 (iron (III), "ferric"). In the human body, iron mainly exists in complex forms bound to different molecules either very tightly or reversibly-exchangeable iron. The former examples are iron inside the porphyrin structure (heme iron such as hemoglobin or myoglobin, cytochromes P450) or non-heme tightly bound iron such hemosiderin. The reversible forms represent Fe-sulfur clusters or iron transporters or storage molecules (transferrin and ferritin). The function of iron is very large, it encompasses i.a. oxygen transport, energy formation, xenobiotic metabolism and DNA-synthesis [19]. Body iron content is approximately 4.0 g in men and 3.5 g in women [20]. About 70% of iron in the body is found in the red blood cells (hemoglobin) and in muscle (myoglobin). About 6% of body iron is a component of certain proteins, essential for respiration and energy metabolism, involved in the synthesis of collagen and some neurotransmitters. About 25% of the iron in the body is stored as ferritin which is iron storage protein which is found both intracellularly and circulating physiologically in low concentrations as well in plasma [21] .

Iron also is needed for proper immune function [22]. In particular the role of Fe2+ in immunity is essential for the proliferation of immune cells and its maturation, especially lymphocytes, relate to the generation of a specific response to infection. The body has the ability to decrease the iron availability to be exhausted by infectious elements by proteins such as transferrin and lactoferrin. Furthermore, iron is necessary for the proliferation of bacteria, parasites, and neoplastic cells. Iron surplus could possibly promote the growth of infections and the invasion of tumoral cells. The immune system possesses bacteriostatic mechanisms that diminish the availability of the metal, interfering with bacterial development. Moreover, the system uses iron as the intermediary in the production of bacteriostatic cells [23].

2.3.1.1 Fate of iron in the organism

Absorption of dietary iron occurs in the duodenum and upper jejunum and it is carefully regulated to maintain equilibrium between absorption and loss of body iron [24]. Most dietary non-heme iron is in the ferric (Fe^{3+}) form, which must be reduced to ferrous (Fe^{2+}) iron for efficient uptake. This conversion is realized on the apical luminal membrane by ferrireductase - duodenal cytochrome B or via reductants presented in the diet such as vitamin C [25, 26]. After the reduction, ferrous ions are transported by divalent metal transporter 1 (DMT1) through apical membrane. Most of the iron is delivered from enterocyte to the circulating plasma protein transferrin through the basolateral iron transporter ferroportin [16, 27]. Oxidation of iron precedes its loading onto transferrin [28]. Extracellular iron circulates in the plasma in a non-reactive form bound to transferrin, which can be taken up by cells following binding to transferrin receptor 1 (Tfr1), presented on the cell surface. Almost all circulating iron is bound to transferrin under physiological condition, non-transferrin-bound iron can increase in iron overload and it contributes to pathological processes associated with these conditions. The major site of iron utilization is the bone marrow, where it is used for the synthesis of haemoglobin. Conservation and recycling of iron is essential to replenish the iron need for haemoglobin synthesis. Systemic iron homeostasis depends on the coordinate control of intestinal iron absorption, iron recycling and iron storage (**Table 1, Figure 2**). In cells, the primary function of ferritin is the sequestration of iron in a non-toxic form, which minimizes the ability of iron to catalyze the above-mentioned formation of ROS.

Once iron is absorbed there is no physiologic mechanism of excretion of excess iron from the body. The only passive excretion ways are cell desquamation and blood loss including menstruation or bleeding [29].

Table 1: Distribution of iron [30]

Protein	Tissues	Iron (mg)
Haemoglobin	Erythrocytes	2600
Myoglobin	Muscles	400
Enzymes	Liver	25
Transferrin	Plasma and ECF	8
Ferritin and Hemosiderin	Liver,	410
	Spleen,	48
	Bone marrow	300

ECF: extracellular fluid
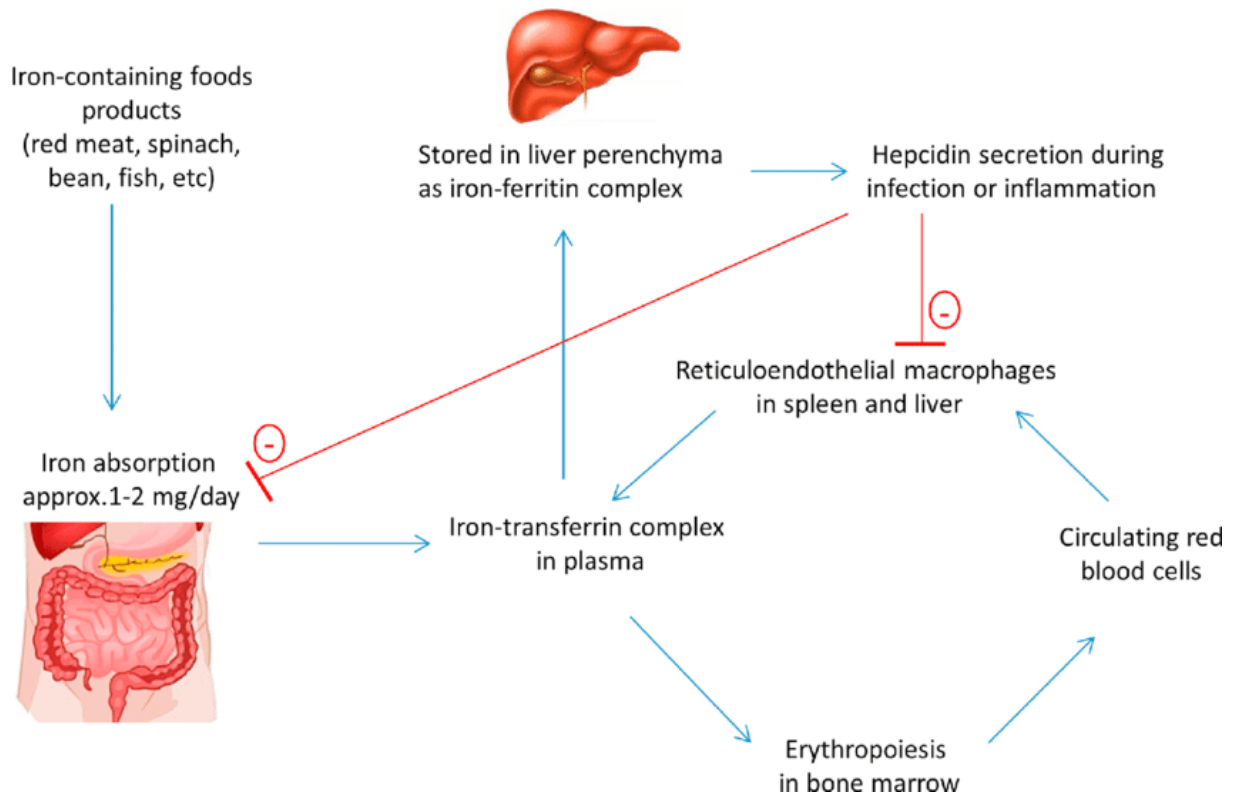
Figure 2: Iron kinetics in human organism and its regulation be hepcidin. Absorption of iron from food products requires appropriate reduced form of the element or heme iron. Daily iron absorption is approx. 1-2 mg. Within the cell, iron is exported by the protein ferroportin and transported via the blood by transferrin. Iron inside the cells can be either stored or used, e.g. in particular for the erythropoiesis. Within ferritin molecules, the element is stored in the ferric form associated with hydroxide and phosphate anions. Senescent red blood cells are phagocytized by macrophages and iron provided by the catabolism of hemoglobin is recycled. Hepcidin inhibits iron absorption from duodenum and iron recycling from macrophages by blocking ferroportin activity [31].

2.3.1.2 Pathophysiology of iron: deficiency and iron overload

Since mammals lack of a regulatory pathway for iron excretion, iron concentration in body tissue must be tightly regulated because both excessive iron and lack of iron are associated with human pathologies.

Iron deficiency

Iron deficiency occurs when there is insufficient absorption of this metal. The reason may not only be based on insufficient iron intake but it can be caused by inhibition of iron absorption which is known in chronic inflammatory disorders [32]. Insufficient absorption leads to abnormally low levels of red blood cells (RBCs), which have also smaller size.

When there is a decrease in the total amount of RBCs or hemoglobin in the blood, or a lowered ability of the blood to carry oxygen, anaemia can occur. Although there are different types of anemia, iron-deficiency anemia is the most common [33].

Concrete common causes of iron deficiency include inadequate iron intake due to poor diet or restrictive diets, inflammatory bowel disease or other chronic inflammatory disorders, increased requirements during pregnancy and blood loss through heavy menstrual bleeding or internal bleeding. Most of symptoms and signs of iron deficiency anemia are non-specific like fatigue, headache, dizziness, impaired physical capacity, increased susceptibility to infections, retardation of growth and impaired cognitive performance seen mainly both in infants and children.

Treatment of iron deficiency anemia is rather simple and inexpensive in most subjects and entails mainly oral treatment with ferrous salts or alternatively ferric polymaltose complex [34] [35]. Despite lower absorption of ferric iron, there is renewed interest in therapeutic use of ferric maltose, which likely mimic heme absorption. The reason is not only better absorption of heme ferric iron but also suggestion that a large influx of Fe^{2+} even from therapeutic doses may cause oxidative damage [36]. Although treatment failure mostly results from an inadequate dose, persistent blood loss, which exceeds iron absorption, and poor compliance, failure, may also be the result of malabsorption. The cause may be mentioned bowel inflammation in the duodenal region, coeliac disease or a genetic defect in one of the proteins involved in intestinal iron absorption. If the iron transport defect is (also) localized in the erythrocytes, any form of iron therapy will remain ineffective.

Iron overload

Iron overload indicates accumulation of iron in the body from any cause. It can be accidental, but the most important causes are hereditary haemochromatosis (HHC) and through the treatment of diseases such as thalassemia, incorrect hemoglobin formation, with extensive blood transfusions (secondary iron overload) [37, 38]. Another form of iron overload is a so called dysmetabolic iron overload syndrome (DIOS) that corresponds to an increase in both liver and body iron stores associated with various components of metabolic syndrome in the absence of any identifiable cause of iron excess [39]. The excessive iron accumulates and is not easily excreted. Once the iron-storage mechanisms are saturated, excess amounts of the metal are released into the circulation, where they can catalyze the formation of ROS with subsequent tissue damage [40-42]. To avoid these effects, iron chelators such as deferoxamine are given to chelate the iron and facilitate its excretion and hence attenuating the symptoms of iron overload. In hereditary iron overload, also flebotomy is used. In some types of HHC, iron chelators are also needed [43].

2.3.2 Copper

Copper occupies a position between nickel and zinc in the group IB of the periodic table and it is a noble metal, like gold and silver. It has useful physical properties, such as visual aspect, alloying ability, low corrosion, malleability, and high thermal and electrical conductivity. Copper does not dissolve in acidic environments unless an oxidizing agent is present and the formation of a green layer of copper carbonate over the metal protects it from further oxidation. Copper in the oxidation state +2 is the most common by far; other known oxidation states include +1, +3 and +4, and of these copper$^+$ is the most common.

In the human body, copper is required for the functioning of many enzymes, such as cytochrome c oxidase, which is complex IV in the mitochondrial electron transport chain, ceruloplasmin, Cu/Zn superoxide dismutase, and in amine oxidases [44, 45]. These enzymes catalyze reactions of oxidative phosphorylation, free radical scavenging and neutralization, and neurotransmitter synthesis. Copper is found in all body tissues and plays a role in the formation of red blood cells and needed for correct function of nerve cells and immune. Most copper in the body is found in the liver, brain, heart, kidneys, and skeletal muscle. Both too much and too little copper can affect different organs and in particular the brain physiology. In addition to known copper genetic diseases (Menkes and Wilson's diseases), copper CNS imbalance is also linked to the Alzheimer's disease. Other than genetic deficiency is rare, but the lack of copper can lead to cardiovascular disease [46] and hematological problems (neutropenia, thrombocytopenia and anemia) [47].

2.3.2.1 Kinetics of copper

The whole process of copper kinetics is shown shortly in the **Figure 3**. The absorption of copper occurs mainly in the stomach and depends on different factors including chemical form of copper, dietary components, interaction with other metals, such as zinc, selenium, and cadmium [48]. Elevated levels of zinc promote the formation of intestinal metallothionein, which blocks copper absorption by the formation of complexes with copper in the intestinal cells [49].

The absorption of copper from the diet is about 65–70% which represents approximately 1 mg daily [50]. Metallic copper is insoluble and therefore, the absorption of metallic copper from the gastrointestinal tract is probably small. Little data are available on the dermal absorption of copper compounds and for the absorption of copper from the lungs. After absorption from the stomach

and from the duodenum, copper is transported in the blood by binding to albumin and transcuprein [51-54]. In this initial distribution, most of the copper was first deposited into the liver and the kidney. This binds about 95% of the serum copper while the remainder of the copper in serum forms complexes with albumin here transcuprein, may complex copper. Majority of copper which enters the liver is complexed with c ceruloplasmin, which, in turn, is released to the circulation and was found to be a major source of copper for other tissues.

The liver is also the major site of deposition of copper, it can release large quantities of copper in the blood at concentrations exceeding 50 μg/mL when liver necrosis occurs [55]. This release causes the rapid accumulation of copper in erythrocytes associated with the subsequent oxidative damage to the them.

The major route of elimination for copper is biliary excretion. In studies of volunteers, the urinary excretion of copper accounts only for approximately 3% of the absorbed dose of copper from normal diets [56]. The biological half-life of copper in the blood after the ingestion of 0.29 mg ^{67}Cu ranged from 13 to 33 days as measured by flame atomic absorption spectrophotometry [50].
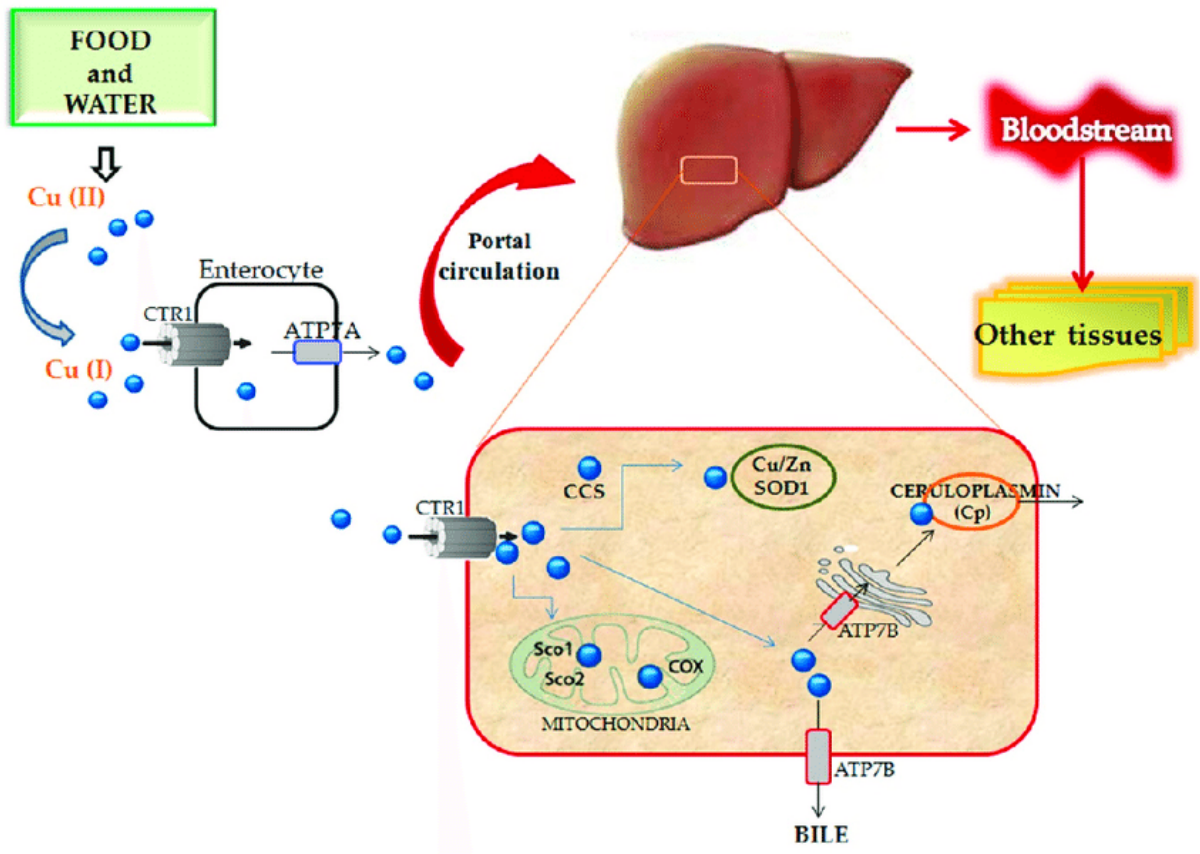
Figure 3: Copper absorption, distribution and metabolism [57].

2.3.2.2 Copper deficiency and excess

Copper plays an essential role in human physiology and, consequently, both excess and lack of this metal are associated with pathological states [58].

Copper deficiency is rare compared to iron deficiency. Anyway known causes of copper deficiency are bariatric surgery [45, 59], hereditary disorders [60, 61] and other diseases such as celiac disease probably due to malabsorption in the intestines. Bariatric surgery, such as gastric bypass surgery, is used for weight control but the disruption of the intestines and stomach due the surgery can cause impair the absorption of copper, but also of iron, vitamin B_{12} and many other nutrients [45]. Increased consumption of zinc can also cause copper deficiency and in fact this approach is also used for the treatment of copper overload. Some works shown that prolonged use of denture adhesive creams containing high quantities of zinc can cause copper deficiency with myeloneuropathy [62, 63]. Menkes disease is a genetic disorder associated with copper deficiency caused by a defective gene involved in the kinetics of copper in the body. Copper deficiency can have hematological consequences, such as anaemia, myelodysplasia, low white blood cell count [44]. It need not to be emphasized that myelodysplasia is an indicator of possible future leukemia development. Copper deficiency is associated with neurological manifestations like sensory ataxia (irregular coordination due to proprioceptive loss), spasticity, muscle weakness, and more rarely visual loss due to damage in the peripheral nerves, myelopathy [45] and rarely optic neuropathy [64]. Copper deficiency can be treated with either oral copper supplementation or intravenous copper [61]. On the other hand, excess of copper causes gastrointestinal disorders such as diarrhea, abdominal pain, nausea, and vomiting. Probably the responsible for its toxicity comes from its ability to accept and donate single electrons as it changes oxidation state. At high concentrations copper is known to produce oxidative damage to biological systems, including peroxidation of lipids or other macromolecules [65]. This catalyzes the production of very reactive radical ions, such as hydroxyl radical by triggering the copper based Fenton reaction (equation 1, [66]). As in the case of iron, copper ability to easily convert between cuprous and cupric ions renders the copper toxic when there is un-sequestered copper in the organism.

Copper overload is associated with Wilson disease (WD), a rare genetic disorder characterized by levels of serum copper markedly elevated due to the sudden release of the metal from liver tissue stores [67]. For the treatment of WD, it is possible to use different copper chelators such as penicillamine, trientine, dimercaprol, dimercaptopropane sulfonate. Possible the future of the copper chelation treatment towards WD will be in the most modern approach based on the use of tetrathiomolybdate (TTM) [68]. Unfortunately, TTM has not yet been approved by FDA. Its

mechanism of action is based on the formation of zinc-tetrathiomolybdate-protein complex both in the circulation and in the gastrointestinal tract [69]. All currently available WD treatments are associated with adverse effects (such as neurological worsening) in a subset of patients, which can require adjustment, substitution, or even discontinuation of treatment. TTM has been demonstrated to be safer as it preserve neurologic function [70].

2.3.3 Zinc

Zinc is also considered an essential transition metal ion. It is present endogenously in human tissues and it is involved in many biological process [71-74]. A clear proof of importance of zinc ensues from the fact, that at least 3% of the genes encode zinc proteins [75]. Zinc was found to be essential also for its presence in a row of enzymes (e.g. carbonic anhydrase, Cu/Zn-superoxide dismutase, oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases) [76]. Zinc has primarily a structural role in the "zinc finger" motif [75]. Summarizing its function, zinc play an important role in various signaling pathways, including neurotransmission, cell division, development and differentiation [77, 78]. Zinc is relevant in many processes, its removal from enzymes or transcription factors by chelators is rendering them inactive.

2.3.3.1 Zinc kinetics

The gastrointestinal tract absorption occurs mainly in two steps. The first one concerns initial uptake across the luminal brush border into the enterocytes, the second one is the transfer from these mucosal cells to the blood. Zinc is not only absorbed and but also secreted from the gastrointestinal tract. In relation to absorption, it is taken up most rapidly from the duodenum but there are also other results indicating that the absorption occurs largely in the small intestine (**Figure 4**) [79]. Zinc administered in aqueous solutions to fasting subjects is absorbed efficiently (60-70 percent), whereas absorption from solid diets is less efficient and varies depending on zinc content and diet composition [80]. The endogenous intestinal losses can vary from 0.5 mg/day (7 mmol/day) to more than 3 mg/day (45 mmol/day), depending on zinc intake [81]. Urinary and skin losses are of the order of 0.5-0.7 mg/day (7-10 mmol/day) each and depend less on normal variations in zinc intake [81]. Starvation and muscle catabolism increase zinc losses in urine. The body has no zinc stores in the conventional sense. In conditions of bone resorption and tissue catabolism, zinc is released and may be reutilized to some extent. Human experimental studies with low-zinc diets 2.6-3.6 mg/day (40-55 mmol/day) have shown that circulating zinc levels and activities of zinc-containing enzymes can be maintained within normal range over several months [82], which highlights the efficiency of the zinc homeostasis mechanisms. Zinc kinetics is tissue and organ specific. The intracellular concentration of unbound "free" Zn^{2+} is extremely low. An intricate homeostatic system of proteins regulates cellular Zn^{2+} distribution and perhaps controls a hierarchy of zinc-dependent functions. Eukaryotic zinc transporters have a major, but still only partly defined, regulatory role [73]. These transporters are encoded by ancient gene families that

span over all phylogenetic levels. The ZIP family (SLC39A family) is involved in cellular Zn^{2+} uptake [83]. One member of this family, Zrt3, is known to transport stored Zn^{2+} out of a vacuolar intracellular compartment during adaptation to zinc deficiency. The cation diffusion facilitator family of transporters (SLC30A family) mediates efflux of Zn^{2+} across the cell membrane or into storage vesicles. These transporters are regulated by transcriptional and posttranscriptional mechanisms to maintain zinc homeostasis via up- or down-regulation at a cellular and organ level [84]. Expression is tissue specific. Zinc transporter 1 (ZnT-1) and zinc transporter 4 (ZnT-4) are expressed ubiquitously, whereas zinc transporter 2 (ZnT-2) expression is limited to the small intestine, kidney, placenta, and liver. When zinc intake is restricted, intestinal and kidney ZnT-2 expression is extremely low, and the levels of both ZnT-1 and ZnT-2 messenger RNAs are increased in the intestine, kidney, and liver with high zinc intake [85]. Although zinc transporter expression is responsive to variations in dietary zinc intake, many details of the molecular regulation of zinc metabolism and homeostasis still await clarification. Zinc is excreted from the body through the kidneys, skin, and intestine [86].
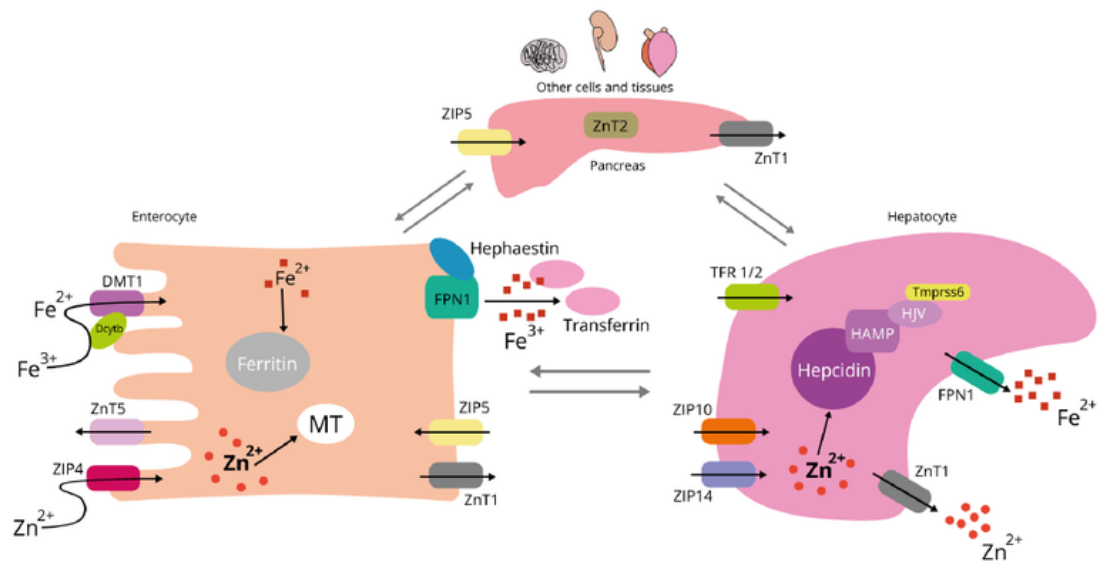
Figure 4: The mechanism of Zn absorption in human hepatocytes [87].

2.3.2.2 Pathophysiology of zinc: deficiency and zinc overload

Both zinc deficiency and excess can occur in human. Zinc deficiency is much more frequent and can be related to inadequate dietary intake, increased requirements and/or excretion. Increased excretion can, for example, take place inadvertently due to therapeutic administration of metal chelators since they are mostly non-selective. Indeed, zinc depletion caused by two iron chelators, deferoxamine and deferiprone, was observed in a mice model [88]. Similarly, D-penicillamine, as mentioned, a drug used to remove excessive copper in Wilson's disease, can cause severe zinc depletion [89]. Symptoms of zinc deficiency include chronic diarrhea associated with malabsorption, regional enteritis, coeliac sprue, and cystic fibrosis [90, 91]. Studies in pregnant experimental animals have demonstrated as well a higher occurrence of fetal malformations [92, 93].

Zinc overload it is more seldom since the zinc homeostatic regulation in the human body is extremely efficient. Zinc toxicity is not only rather a rare but probably also mostly reversible condition. Normally zinc is considered to be a non-toxic micronutrient at supplementation levels ≤ 100 mg/day. However, intoxication can occur at higher doses. Poisoning can occur also in the workplaces, e.g. from inhalation of $ZnCl_2$ fumes, and in persons with excessive oral exposure to zinc dietary supplements or in patients hemodialyzed with water stored in galvanized steel tanks. Severity and symptoms of intoxication depend on the route of zinc entry into the organism. There are more, not fully elucidated mechanisms which can participate in zinc toxicity [94]. These include direct pro-oxidation and induction of inflammation associated with higher zinc levels and also at least partly the fact that zinc excess decreases expression of copper-dependent enzymes, such as superoxide dismutase and ceruloplasmin, which are important endogenous antioxidants [73, 86, 95-97]. There is also a genetic disorder associated with very high concentrations of zinc in plasma. This phenomenon is called hyperzincaemia and it represents a rare inborn error of zinc [98].

A summary comparing basic characteristics of all three metals of interest is shown in the **Table 2**.

Table 2: Comparison of metals content in human body and related proprieties

	Total body content (g)	Total concentration in blood (μM)	Metal in circulation	Free metal in circulation	Excretion route
Copper [99]	0.05–0.12	10^1	95 % is bound to ceruloplasmin	2–5%	70-95% excreted in bile
Iron [100]	3-4	13-27	70 % RBCs, 25 % ferritin 5-10 % is bound to myoglobin	1–2%	no physiological regulatory mechanism for excretion 1-2 % excretion only by exfoliation of mucosal surfaces (1 mg, + 0,5 mg in menstruating women)
Zinc [99]	2	10^1	2-3 % is bound to albumin	1–2%	85% fecal excretion

^{1} Total concentration in plasma\serum

2.4 Pharmacological treatments for metal intoxication in humans

2.4.1 Chelation therapy

Usually in order to treat metal toxicity, compounds forming complexes with metal ions in order to attenuate the metal toxicity by transforming them in a less toxic excretable complexes are used. These compounds are called therapeutic chelating agents. This treatment can have also potential pitfalls since the complex formation can lead to adverse effects in some cases:

a) an increase in the metal intestinal absorption when the complex is formed in the gut;

b) dislocation of complexed metal ions to more vulnerable sites: e.g. complexation can permit the transit of the metal ion through the blood-brain-barrier leading to unexpected neurological damages.

The stability of a complex is the basic requisite for a chelating agent. Chelators can be classified as bidentate, tridentate and hexadentate according to the number of functional site on the molecule able to bind the target metal ion at the same time. The denticity of the ligand determines the number and the stoichiometries of the formed complexes. As a general rule, hexadentate chelators are preferred because they form only one kind of complex. On the contrary, ligands with lower denticity can form multiple complexes, whose speciation pattern depends both on the total ligand concentration and on metal/ligand ratio. The formation of lower stoichiometry complexes is sometimes unsuitable because these complexes can be themselves more toxic than the parent metal ion, or they may not protect the organism adequately from deleterious reactions. Anyway the denticity is not the only decisive parameter, since hexadentate chelator EDTA is known to potentiate the Fenton reaction [101].

Selectivity toward the target metal ion is another requisite for an ideal chelator, but in real situations it can be likely satisfied only for certain metal ions. Selectivity is the capacity of a chelator to form complexes with the target metal ion characterized by stability constants several order greater than those for other metal ions. In particular the clinically used chelators should exhibit selectivity for the target metal ion and should not have high affinity, and hence affect, physiologically relevant metal ions whose levels should not be perturbed. For this reason, besides the differences between the stability constants, also the relative amounts of the target and the

physiological metal ions have to be considered. Besides the unwanted action of the chelator toward the essential metal ions, also a high concentration of a physiological metal ion can perturb the complex formation between the chelator and the targeted toxic metal ions.

The behavior of a chelator depends *in vivo*, besides the above mentioned parameters, also on kinetic factors, connected to: a) degradation of the chelating agent, b) velocity of complex formation between the chelator and the free metal ion in the plasma, and c) exchange reaction between the metal bound to endogenous molecules and the chelating agent. Many chelating agents are metabolized in the body to species which lose the chelating properties of the parent molecule. These reactions can be very different, from the glucuronidation of hydroxypyrydinones, through the acetylation of trientine, to the formation of –S-S- bonds between dimercaprol and SH-containing ligands. The correct choice of drug administration becomes of vital importance when this kind of metabolic transformation is rapid, as for example the subcutaneous infusion of deferoxamine. The formation of complexes between the free metal ion in plasma and the ligand is more complicated *in vivo* conditions, since the circulating toxic metal ion in plasma is generally bound to different endogenous molecules, ranging from large macromolecules such as transferrin and albumin to low molecular weight ligands as citrate.

The chelator must apparently accelerate the kinetics of the metal elimination. In particular the excretion pattern is very slow for certain metals as aluminum, chromium and palladium. Also, the distribution should be considered. A chelator due to its physicochemical properties can act only on the circulating (not necessarily free) metal ion. Once the circulating amount of the metal is chelated and excreted, a slow equilibrium will be reconstituted between the intracellularly-bound and circulating toxic metal ion: the kinetic of this equilibrium will decide the periodicity of chelation treatment. The kinetics of the exchange reaction between the metal ion bound to endogenous molecules and the chelator depends on a variety of factors, among which the structure and the affinity of the chelator toward the metal. The knowledge of the different kinetic behaviors of the chelators in use for a given metal ion has conducted to improved therapeutic schemes. As far as the chemical requisites regarding the absorption and the bioavailability are concerned, three aspects have crucial importance [102]: molecular size, lipophilicity and net charge [103, 104].

Lipophilicity is generally estimated by the water–octanol partition coefficient (P). These general properties have been used by Lipinski et al. [105], adopting a four parameter analysis, to predict membrane permeability. Their guidelines state that a poor oral absorption is likely when:

- ✓ molecular weight > 500 g/mol;
- ✓ log P below 5;
- ✓ more than 10 hydrogen bond donors are present in the molecule (expressed as a sum of OH and NH groups);
- ✓ more than 10 hydrogen bond acceptors are present in the molecule (expressed as a sum of O and N atoms).

These parameters are also relevant for the penetration of the chelating agent into cells, and are valid as well for formed metal complex excretion. In this last situation the formation of a neutral complex is of paramount importance: in the case of Fe^{3+} complexes it is more appropriate to use a chelating agent bearing coordinating groups as –CO-COH or – CO-NOH (like in hydroxypyridinones or in hydroxamates) than – COH-COH or COH- COOH (as in catechols or in salicylates), since the first ones lead to uncharged easily excretable complexes, and the second ones to negatively charged complexes [106].

2.5 Chelating agents

2.5.1 Deferoxamine (DFOA)

DFOA (**Figure 5**) is the most important and well know iron chelating agent [107]. It is a trihydroxamic acid with three residues of 1-amino-5-N–hydroxyaminopentane, two of succinic acid and one of acetic acid organized in a linear array. The free amino group explains its very high water solubility. Initially used only in the therapy of acute iron poisoning [108], DFOA was later introduced in transfusional iron overload in thalassemia treatment. Since DFOA given orally is poorly absorbed, it must be administered subcutaneously, intramuscularly or by intravenous infusion with a small portable syringe pump, ideally for 9–12 h each day to be effective [109-111]. On entering blood by intravenous injection, DFOA plasma clearance [112] is rapid with a half-life of 5 to 10 min. The metabolism of DFOA is by oxidative deamination of the N terminus, DFOA - B and DFOA -C are the principal metabolites that are formed in the liver. Their amount is inversely proportional to the availability of chelatable iron [113]. Consequently, when there is abundant chelatable plasma iron with augmented levels of ferrioxamine, lower amount of DFOA is available for metabolism because ferrioxamine is not taken into the liver [114].
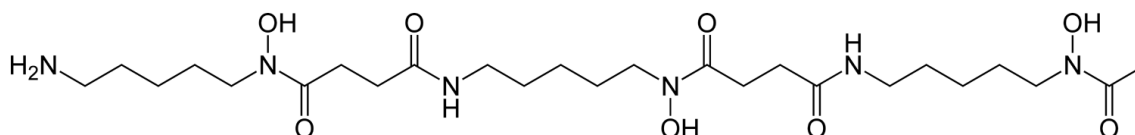

Figure 5: Chemical structure of DFOA

2.5.2 Deferiprone

Deferiprone (**Figure 6**) is given by mouth two or three times per day. It is given frequently in a dose of 75 mg/kg/day considering its partial transformation to the non-chelating deferiprone-glucuronide form [115]. It is well absorbed from the human intestinal tract [116]. It forms 5-membered chelate rings in which iron is bound by two oxygen atoms. Little is known about mobilization of iron stored in hepatocytes and in Kupffer cells by deferiprone. Deferiprone, in common with other low molecular weight iron chelators, likely removes Fe^{3+} from ferritin by penetration through the protein shell and may mobilize iron even from hemosiderin, lactoferrin and transferrin [117]. The major metabolite of deferiprone in human is the glucuronide, which, as a result of conjugation of the 3-hydroxyl function, is unable to bind iron. The majority of deferiprone–iron complex is excreted in the urine (70%), while only little iron is excreted in the feces [118]. Deferiprone, such as other bidentate ligands, has a clear advantage over DFOA with respect to oral viability and distribution. By virtue of its relatively low molecular weight, it may easily penetrate most cell membranes encompassing blood–brain barrier and placental barrier. The efficacy of deferiprone in inducing and maintaining a negative iron balance in iron loaded transfused patients has been demonstrated by the first long-term trials [115]. Iron chelation therapy with deferiprone in a large series of iron-overloaded subjects affected by thalassemia intermedia showed a reduction of liver iron stores and a normalization of serum ferritin levels [119].
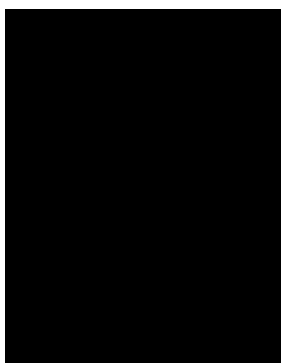
Figure 6: Chemical structure of deferiprone

2.5.3 British anti Lewisite (BAL)

BAL, chemically 2,3 dimercaptopropan-1-ol and known also as dimercaprol (**Figure** 7) **was** used for copper toxicity. In fact, it was the first antidote for metal intoxication. Being strongly susceptible to oxidation, it is unstable, and difficult to store. Due to its high toxicity, BAL was just used for acute intoxications. BAL is a parenterally administered and its treatment is accompanied by a variety of adverse effects including nausea and vomiting, tachycardia and hypertension, and fever. BAL administration is contraindicated in patients affected by glucose-6-phosphate dehydrogenase deficiency due to risk of hemolysis [120]. It has been used for the treatment of arsenic, mercury, lead, gold and other toxic metal ions until recent years [121].
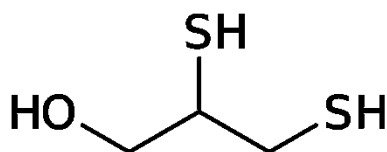
Figure 7: Chemical structure of British anti-Lewisite

2.5.4 D-penicillamine

D-penicillamine (**Figure 8**) is largely used for copper excretion in Wilson's disease. However, it is not a classical chelator and its activity has been explained by reduction of cupric ions which results in mobilization of copper and its urinary elimination. In fact, the chelation potency of D-penicillamine toward copper is week [122]. There is also a plenty of adverse reactions which are frequently associated with its administration such as rash, proteinuria, neutropenia, mouth ulcers, vomiting and diarrhea [123].
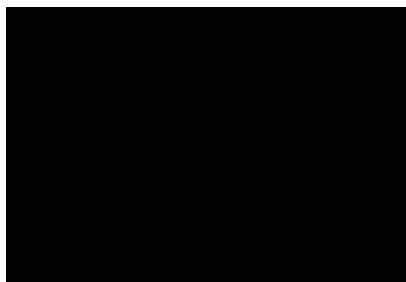
Figure 8: Chemical structure of D-penicillamine

2.5.5 Ethylenediaminetetraacetic acid (EDTA)

EDTA (**Figure 9**) is the most used polyaminocarboxylic acids in metal chelation. It has low intestinal absorption (<5%), and hence it is effective when administered by i.v. infusion. Due to potential toxicity, it is given in the form of a calcium salt. This chelator is rapidly excreted in the urine without undergoing a significant metabolism. Basically, EDTA forms stable complexes with almost all metal ions. A continued exposure to EDTA can cause depletion of essential metals, especially Zn, Cu, and Mn. Zinc binding is surely implied in some cases of acute toxicity presented by $CaNa_2EDTA$ [124].
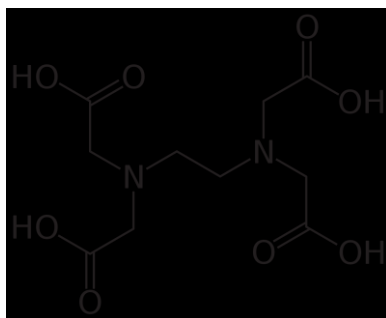
Figure 9: Chemical structure of EDTA

2.5.6 Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN)

TPEN (**Figure 10**) chelates several metal ions such as Cu^{2+}, Ag^+ and Fe^{2+} [125-127] but in particular has a high affinity for Zn^{2+} [128-130].

It is a membrane-permeable zinc chelator; decreases the intracellular level of zinc and induces apoptosis (i.e., as documented by cell shrinkage and formation of apoptotic bodies with DNA fragmentation and formation of a typical DNA ladder pattern) [131].
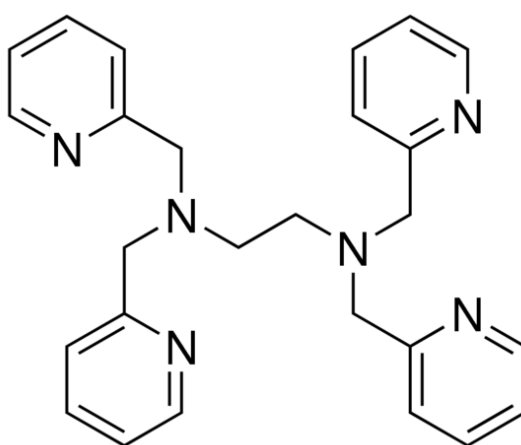
Figure 10: Chemical structure of TPEN

2.5.7 Triethylene tetramine dihydrochloride

Trientine (**Figure 11**) is a copper-chelating agent with similar indication to penicillamine. The difference between D-penicillamine and trientine consist that the latter

competes for copper bound to albumin and does not enter the liver [132]. Trientine is clearly a potent copper chelator in contrast to D-penicillamine [122]. In contrast to BAL and D-penicillamine, trientine does not have sulfhydryl groups. Compared to D-penicillamine, trientine is relatively free of adverse effects, but neurological worsening may occur infrequently [133]. Trientine is one of the most effective and potent copper chelator used for the treatment of WD.
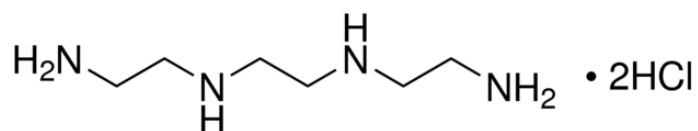
Figure 11: Chemical structure of trientine

3. ABBREVIATIONS

BAL: British anti-Lewisite

BCS: bathocuproinedisulphonate

CNS: central nervous system

DFOA: deferoxamine

DHS: dehydrosylibin

DIOS: dysmetabolic iron overload syndrome

DMT1: divalent metal transporter 1

DOPAC: 3,4-dihydroxyphenylacetic acid

EDTA: ethylenediaminetetraacetic acid

FDA: Food and drug administration

GSH: reduced glutathione

GSSG: oxidized glutathione

HHC: haemochromatosis

HPLC: high performance liquid chromatography

HVA: homovanillic acid

O-DMA: O-desmethylangolensin

·HO: hydroxyl radical

RBCs: red blood cells

ROS: reactive oxygen species

Tfr1: transferrin receptor 1

TPEN: N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine

TTM: tetrathiomolybdate

WD: Wilson's disease

WHO: World Health Organization

ZIP: zinc importer protein

ZnT-1: zinc transporter 1

ZnT-2: zinc transporter 2

ZnT-4: zinc transporter 4

4. AIM OF THE WORK

The aims of this work were:

a) screening of metal chelating properties of different compounds with the objective of their potential therapeutic use or deciphering of their physiological/pharmacological role and characterization of the formed complexes;

b) development of novel methods for screening of metal chelation and properties of formed complexes.

5. ARTICLES PUBLISHED IN JOURNALS WITH IMPACT FACTOR ASSOCIATED WITH A TOPIC OF DOCTORAL DISSERTATION

1. *"The Stoichiometry of isoquercitrin complex with iron or copper is highly dependent on experimental conditions"*

CATAPANO MC, TVRDÝ V, KARLÍČKOVÁ J, MIGKOS T, VALENTOVÁ K, KŘEN V, MLADĚNKA P, *Nutrients*, 2017, 9(11):1193.

Impact Factor: 4.171 (2018)

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5707665/

2. *"Mono and dihydroxy coumarin derivatives: Copper chelation and reduction ability"*

CATAPANO MC, KARLÍČKOVÁ J, TVRDÝ V, SHARMA S, PRASAD AK, SASO L, CHHILLAR AK, KUNEŠ J, POUR M, PARMAR VS, MLADĚNKA P., *J Trace Elem Med Biol*, 2018, 46:88-95.

Impact Factor: 2.895 (2018)

https://www.sciencedirect.com/science/article/pii/S0946672X17307137?via%3Dihub

3. *"A simple, cheap but reliable method for evaluation of zinc chelating properties"*

CATAPANO MC, TVRDÝ V, KARLÍČKOVÁ J, MERCOLINI L, MLADĚNKA P, *Bioorg Chem*, 2018, 77:287-292.

Impact Factor: 3.926 (2018)

https://www.sciencedirect.com/science/article/pii/S0045206817308246?via%3Dihub

4. *"An original HPLC method coupled to coulometric electrochemical detection for the monitoring of hydroxyl radical generation via Fenton chemistry"*

CATAPANO MC, PROTTI M, FONTANA T, MANDRIOLI R, MLADĚNKA P, MERCOLINI L *Molecules,* 2019, 24(17): E3066.

Impact Factor: 3.060 (2018)

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6749383/

5. *"Hematoxylin assay of cupric chelation can give false positive results"*

MACAKOVA K, **CATAPANO MC,** TVRDÝ V, KLIMKOVA K, KARLÍČKOVÁ J, MLADĚNKA P, *J Trace Elem Med Biol*, 2019;52:29-36.

<div align="right">Impact Factor: 2.895 (2018)</div>

https://www.sciencedirect.com/science/article/pii/S0946672X18304140?via%3Dihub

6. *"Interaction of isolated silymarin flavonolignans with iron and copper"*

TVRDÝ V, **CATAPANO MC**, RAWLIK T, KARLÍČKOVÁ J, BIEDERMANN D, KŘEN V, VALENTOVÁ K., MLADĚNKA P, *J Inorg Biochem*, 2018, 189:115-123.

<div align="right">Impact Factor: 3.224 (2018)</div>

https://www.sciencedirect.com/science/article/pii/S016201341830285X?via%3Dihub

7. *"The influence of microbial isoflavonoid specific metabolites on platelets and transition metals iron and copper"*

MIGKOS T, APPLOVA L, HORKY P, TVRDÝ V, KARLÍČKOVÁ J, **CATAPANO MC**, TOMANEK M, POUR M, MLADĚNKA P, *Phytomedicine*, 2019, 62:152974.

<div align="right">Impact Factor: 4.180 (2018)</div>

https://www.sciencedirect.com/science/article/pii/S0944711319301400

5.2 A PUBLICATION NOT RELATED TO THIS DOCTORAL DISSERTATION

8. *"Determination of oxycodone and its major metabolites in haematic and urinary matrices: Comparison of traditional and miniaturized sampling approaches"*

PROTTI M, **CATAPANO MC**, SAMOLSKY DEKEL BG, RUDGE J, GERRA G, SOMAINI L, MANDRIOLI R, MERCOLINI L, *J Pharm Biomed Anal,* 2018.

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6. AUTHOR´S CONTRIBUTION

This doctoral dissertation is based on 7 papers referred above with numbers 1-7. The candidate is a first author of first four publications.

1. **The Stoichiometry of isoquercitrin complex with iron or copper is highly dependent on experimental conditions**

   ➢ performing all stochiometric experiments and analysis of the results
   ➢ literature search on the topic of flavonoids and metal chelation
   ➢ writing the initial draft of the article and participation in the finishing and revision of the manuscript

2. **Mono and dihydroxy coumarin derivatives: Copper chelation and reduction ability**

   ➢ participation in the design of experiments
   ➢ performing and analyzing the experiments
   ➢ writing a draft and participation in the finishing of all manuscript

3. **Simple, cheap but reliable method for evaluation of zinc chelating properties**

   ➢ participation in the design of experiments
   ➢ literature search
   ➢ performing and analyzing the experiments
   ➢ writing a draft and participation in the finishing of all manuscript

4. **An original HPLC method coupled to coulometric electrochemical detection for the monitoring of hydroxyl radical generation via Fenton chemistry**

   ➢ participation in the design of experiments
   ➢ performing all initial experiments and analyzed experiments
   ➢ writing the first draft of the article and participation in the finishing of the whole manuscript

5. **Hematoxylin assay of cupric chelation can give false positive results**

   ➢ performing UV-Vis non-competitive experiments
   ➢ literature research
   ➢ writing the initial version of the article and participation in the completion of the whole manuscript

6. Interaction of isolated silymarin flavonolignans with iron and copper

  ➢ participation in the experiments
  ➢ preparation of the figures
  ➢ participation in the manuscript correction

7. The influence of microbial isoflavonoid specific metabolites on platelets and transition metals iron and copper

  ➢ performing and analyzing experiments regarding chelation of transition metals iron and copper
  ➢ preparation of the graphical abstract
  ➢ involvement in manuscript preparation including the preparation and adjustment of the references

7. DISCUSSION

The aim of this work was to characterize the interactions of pure substance with the transition metals (iron, copper and zinc) at patho-physiologically relevant pH. The selection of pH ranging from 4.5 to 7.5 assumed that: a) physiologically the most relevant interactions can take place in the gastrointestinal tract where the pH raises from the duodenum to jejunum; b) there are important differences in pH also in pathological conditions which can be represented by a low pH in cancer cells or after ischaemia [134, 135].

Basically, two major approaches were employed: the competitive and non-competitive approach. For the later, two methods have been used: the Job's method and the complementary approach. The first one is also known as the method of continuous variation, it is a simple analytical approach which is used to the determination of stoichiometry of two interacting components [136]. In this method, the total molar concentration of two reactants is kept constant while their molar concentration ratios are continuously varied throughout the series of samples. Complementary methods are based on mathematical calculations of the stoichiometry. Compared to the Job's method, the total molar concentration of the tested substance was continuously varied, while the molar concentration of metal was kept constant throughout the series of samples. Since these non-competitive methods are giving unambiguous results only in the case of strong or moderate chelators [137], they cannot be applied in the case of chelators with low affinity to metals. In our case, we characterized with them in detail the stoichiometries of isoquercitrin (**Figure 12B**) [138] and those of oxidized and reduced glutathione [139].

In general, the research of future chelators followed in a logical manner experiments performed in the past in the research group of cardiovascular pharmacology and toxicology. Previous research on was performed on a row of flavonoids and isoflavonoids [140, 141]. This research was supplemented by experiments with flavonol isoquercitrin and flavonoid derivatives flavolignans from silymarin and metabolites of quercetin formed by human microflora (**Figure 12**). The selection of isoquercetrin was largely supported by the need of completing information of iron chelation. It was previously shown that its isomer rutin (**Figure 12C**) is able to form different complexes based on the excess of rutin over iron [137]. A similar phenomenon was observed in isoquercitrin. The study with isoquercitrin confirmed that it is a moderately-active ferrous, ferric, and cupric chelator, in particular under neutral or slightly acidic pH conditions, which is i.a. relevant for resorption of these metals in the digestive tract. In line with this, the most plausible chelation site is the catechol moiety. Stoichiometry of respective metal complexes is typically 1:1 under ideal conditions, but changes in excess of isoquercitrin or under competitive

conditions. This study also brought a secondary outcome—the necessity to use multiple methodologies in order to better establish the chelation behavior of moderately-active metal chelators.

The research of silymarin flavonolignans were stimulated by three facts:

1) there were some studies which showed that silybin decreased iron overload in hereditary hemochromatosis patients [142],

2) the structure of silymarin flavonolignans is derived from flavonol quercetin (Figure 12), which is a relatively potent iron chelator,

3) the extract is frequently used both as an approved drug and a popular food supplement, the general population's exposure it is hence substantial.

The possible interaction in the gastrointestinal tract is also clinically important, since there is a discussion if really the silymarin itself due to low bioavailability can affect systemic metals. The systemic bioavailability of all flavonolignans from silymarin is considered to below or around 1% [143, 144]. Anyway, its interaction in the gastrointestinal tract with metals presented in the diet is another issue which is undisputable. Even in theoretical absence of chelating properties of silymarins, the interaction is considered to be relevant because there are many studies reporting antioxidant activity of flavonolignans from silymarin and antioxidant property means in most cases also reducing properties. As was written above in the chapter 2.3.2, iron is absorbed mainly in the duodenum in the form of ferrous ions and reductants significantly improve its absorption. Indeed, we observed that some of the tested flavonolignans, in particular silychristin A, can reduce ferric ions.

Before we performed this study, there existed, as mentioned only little data supporting the potential clinical use of silybin against iron toxicity. No data on the interaction of pure silymarin components, in particular pure flavonolignans, with transition metals were available. There has only been a single focused study, which demonstrates the complexation of silybin (mixture of diastereomers A and B (**Figure 12D and 12E**) with ferric ions under non-competitive conditions. This study brought rather unexpected results which were questioned in our study [145]. Based on our complex study, we are of the opinion that silymarin is a very week chelator and cannot form stable complexes with iron. In the contrast, its dehydroform, 2,3-dehydrosilybin, was shown to be a relatively potent copper chelator. However, it is not present in silymarin in particularly high concentration, so its contribution on the possible metal chelating effect of silymarin *in vivo* would be rather low [146].

For copper, the data are less conclusive, but again its reduction to cuprous ions before absorption is highly plausible. In general, the presence of the 2,3-double bond markedly enhanced also copper chelation potential and also increased metal reduction.

In terms of stereoisomerism, it apparently does not play a role in dehydrosilybin, probably because the optical center is far from the chelation site. Silychristin A is in contrast a compound with low chelation potential but with strong reducing properties, and hence the behavior of both silymarin components can be very different. These *in vitro* results can be used as a basis for future testing of the effect of silymarin flavonolignans on iron/copper absorption in cell cultures and *in vivo*.
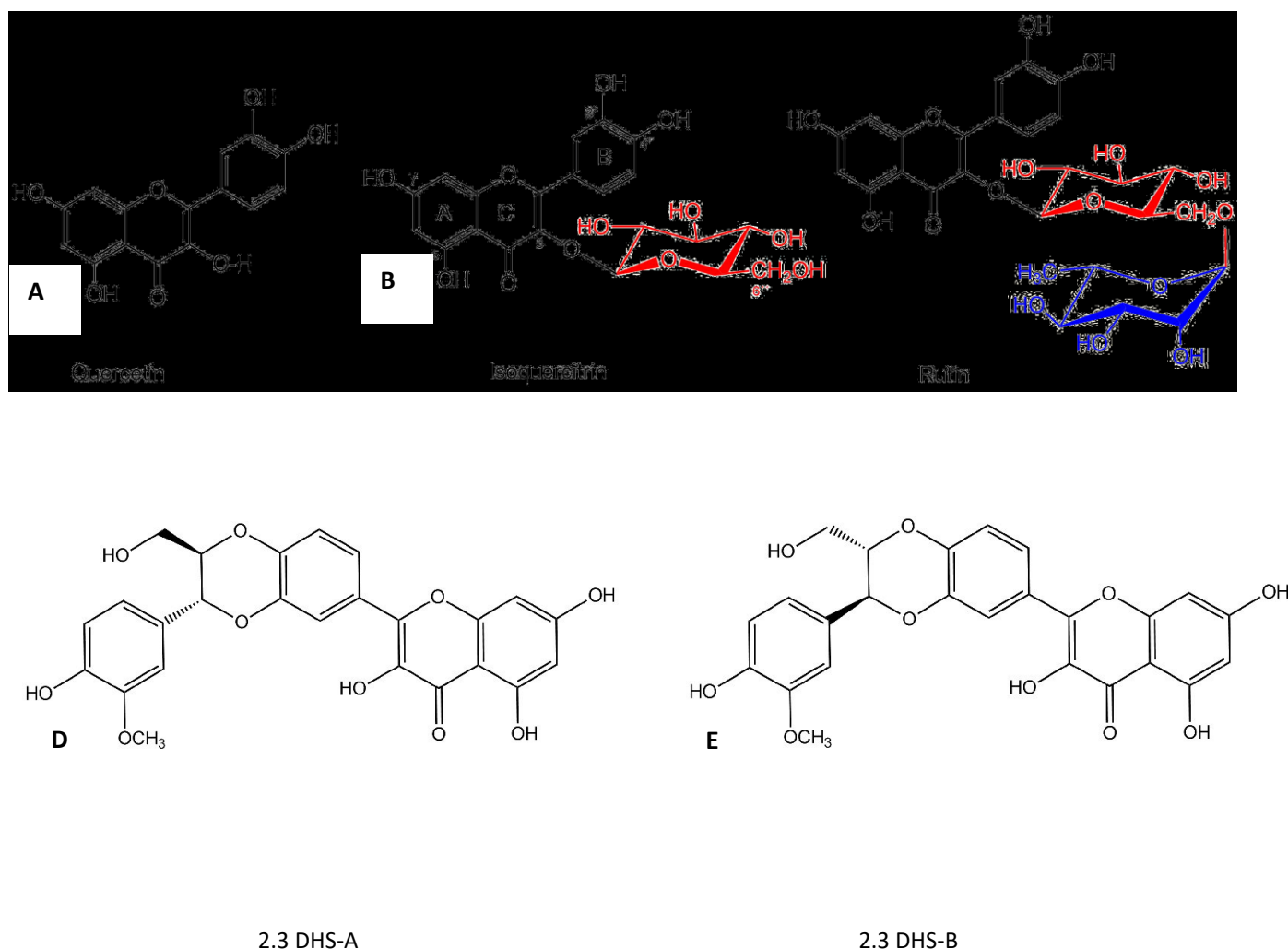

2.3 DHS-A

2.3 DHS-B

Figure 12: chemical structures of (**A**) quercetin, (**B**) isoquercitrin, (**C**) rutin, (**D**) 2,3-dehydrosilybin A and (**E**) 2,3-dehydrosilybin B.

Interaction of isoflavoids with iron and copper was also previously covered by our group [141], but only very limited data existed on the interaction of their small phenolic metabolites. Iron chelation was tested first by the ferrozine method; but the potential of all tested compounds to chelate iron was low or negligible. Only O-DMA was able to chelate iron at high compound:Fe^{2+} ratios. This is most likely due to presence of a keto group in the proximity of a phenolic group. Apparently, the other tested substances have no chelating sites. A similar situation was observed in the case of Cu chelation by these metabolites. In general, there is a need for a development of a novel copper chelator since the approved treatment modalities for copper excess are limited. There is only one potent clinically used copper chelator (trientine). D-penicillamine should also be mentioned, but this substance seems to be a weak copper chelator (chapter 2.5.4) regardless of its profound effect on copper excretion. The currently examined tetrathiomolybdate which forms Cu-tetrathiomolybdate-protein complexes is of interest, however, it has not been yet approved by FDA likely due to unresolved safety concerns. As also stated above, research of copper or metal chelators has been directed towards their potential impact on some neurodegenerative disorders, in particular on Alzheimer disease. Mostly the drugs derived from 8-hydroxyquinoline have been tested. But, surprisingly, clioquinol was shown to act rather as a metal redistributor than a copper chelator [147]. Thus, given the very limited amount of the current knowledge, research of novel copper chelators is highly desirable also from this point of view. Another potential indication is cancer [148]. However, considering the huge but highly divergent therapeutic potential, different properties are clearly needed for various indications. For example, to increase metal elimination under metal overload conditions, strong chelators are needed, while complex-forming compounds with redox-cycling properties are likely to be more suitable for tumor treatment. Hence, both copper chelation and reduction have to be always analyzed. Again this research followed the line of the previous investigation of the research group in particular in terms of coumarins, which were showed in a previous study to be pH-dependent iron chelators and reductants [149]. This novel study [150] showed that *o*-dihydroxycoumarins are moderately active cupric ions chelators without affinity to cuprous ions. The 6,7- and 7,8-dihydroxycoumarins studied in this work are able to form complexes with cupric ions in the stoichiometric ratios ranging from 1:1 to 2:1 depending on pH and coumarin: Cu^{2+} concentration ratio. These coumarins are simultaneously able to reduce cupric ions, and are therefore unsuitable as therapeutics under copper excess conditions. For this reason, some of this active 4-methylderivatives, may have a potential in cancer treatment. In comparison the interactions of coumarins with iron shown that 7,8-dihydroxy-4-

methylcoumarin is more active iron chelator than 6,7-dihydroxy-4-methylcoumarin, the latter seems to be superior iron chelator, in particular in acidic conditions.

During these years we used also a competitive method for screening of copper chelation based on two consecutive tests – the hematoxylin and the bathocuproine assays (introduced in our groups in 2013 [122]). The first is mildly competitive since the indicator reacts with cupric ions in a ratio of 1:1 and since it absorbs in the visible area. The second is highly competitive. It uses sodium bathocuproinedisulphonate (BCS), which is a selective indicator for cuprous ions, in a 20× times excess over cuprous ions. Over the 5 years of our use of the former assay, we have noticed that some antioxidant compounds with no clear chelation site were partially active in the hematoxylin assay. Since these compounds were direct antioxidants, they may possess metal reducing activity and hence reduce the cupric ions into the cuprous ones and so render them "invisible" for the indicator hematoxylin. To confirm the hypothesis that the hematoxylin assay can give false positive results with antioxidants, we selected 3 known reducing but not chelating agents with physiological and/or experimental importance: hydroxylamine, vitamin C and a water-soluble form of vitamin E – trolox. Reduced (GSH) and oxidized (GSSG) glutathione were also included. The hypothesis was confirmed, since vitamin C, hydroxylamine and trolox "chelated" copper according to the hematoxylin method but neither BCS method or non-competitive assessment showed chelation. This agrees with the theoretical assumption, since they do not possess chelation sites in their chemical structures.

In addition, this study [139] showed that GSSG chelates both cupric and cuprous ions but has no potential to reduce cupric ions. In contrast, GSH forms unstable complexes with cuprous and cupric ions which is likely the base for its physiological role in the intracellular copper shuttle [151]. Oxidation of GSH to GSSG seems to be really important for cell protection since GSSG can chelate free metals. Although, it seems that low ratios (GSH to Cu ions) enable metal based oxidative damage, while in greater excess of GSH over Cu ions, protective antioxidant effects are observed [152, 153]. This appears to correspond to our data suggesting that copper chelation is not involved and copper reduction may be responsible for the phenomenon in low ratios. In higher ratios, direct ROS, in particular OH· scavenging can mediate the antioxidant effects.

Also for the second aim of this dissertation thesis, the work went on the previous group results which included the preparation of two methods for chelation of copper and iron and use of HPLC method with spectrophotometric detection of hydroxyl radical [154].

The topic of zinc is of high interest since as mentioned above, the current treatment of Zn poisoning is supportive. The used chelating agents for Zn, including EDTA, BAL, D-penicillamine and N-acetylcysteine, were never thoroughly investigated in relation to this metal and all these

formerly clinically used Zn chelating agents suffers from different disadvantages. The first two compounds are usually preferred in patients with significant Zn toxicity. But their clinical use in this condition is far from being ideal: there are still problems regarding the toxicity and the activity appears to be insufficient. The latter is even more likely for the third and fourth compound. Moreover, screening of zinc chelation activity can lead to both, the development of novel and selective zinc chelators for zinc intoxication treatment and to check if current metal (e.g. iron and copper) chelator cannot influence Zn homeostasis and thus have potential side effects. In that study [155], we developed step by step a novel zinc chelation screening method using a spectrophotometric indicator dithizone. The sensitivity of this method was always below 1 μM of zinc. The usefulness of this method was also checked by two know chelators EDTA and TPEN.

The research of metal chelators would not be sufficient if the researchers will not be in possession of a method for detection of their effect on the Fenton reaction. This is largely driven by the fact, that neither the most powerful of chelators need not be the inhibitor of that reaction. Indeed, this was shown in the case of EDTA [156]. HPLC method with spectrophotometric detection was not very sensitive since it required relatively high concentration of the metal and therefore for the reason of low solubility of some chelators did not enable to test high chelator excess to metal ratios. This was overcome by the coulometric detection. The first published study [157] was focused on studying the effect of different known or possible antioxidants on the interaction between iron and copper ions and ·OH. The developed method was then used to evaluate the antioxidant and/or pro-oxidant activity of various bioactive molecules, by comparing hydroxylation products (and thus ·OH production rates) in the Fenton's reaction mixtures with and without bioactive molecules. These compounds interact differently with the metal present in the reaction mixture and may exhibit chelating, reducing, no activity or even oxidative activity. The tested substances were: 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-chloro-7-iodo-8-hydroxyquinoline, catechin, deferoxamine, EDTA, homovanillic acid (HVA), quercetin, phloroglucinol and trientine. This methodological paper will be in a close future supplemented with other papers analyzing different ratios of metal chelators over the metals itself. Also, the impact of different pH conditions will be included.

In conclusion, it can be stated that notwithstanding some results were rather disappointing form the scientific point of view, e.g. relatively low chelation activity of coumarins, the results from this dissertation presented novel data, some of which can facilitate the research in this area. In particular the novel methodologies for screening of zinc chelation, notification on pitfalls in hematoxylin methodology and detection of hydroxyl radical formed by the Fenton reaction seems to be of interest for other research groups. It must be admitted that this research is not complete,

in particular, in relation to testing of the effect of chelators in more biologically relevant conditions. These short coming are currently under investigation in our laboratory and the research group will likely in the future possess methodologies targeted for the testing of the effect of chelators on tumor cells, metal containing enzymes and on metal absorption in a cell model.

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9. LIST OF FIGURES AND TABLES

10. CONGRESS CONTRIBUTIONS

10.1 Oral Presentations

➤ **8th postgradual and 6th postdoctoral conference, Faculty of Pharmacy, Charles University, 2018**

"Assessment of the stoichiometry of iron and copper with dehydrosilybin A and B"

➤ **10th postgradual and 8th postdoctoral conference, Faculty of Pharmacy, Charles University, 2020 (planned)**

"Probing the structure and function of the cytosolic domain ZnT8 with Ni^{2+}"

10.2 Conference Posters

➤ FONTANA T, **CATAPANO MC**, MLADĚNKA P, MERCOLINI L, "Fenton reaction coupled to liquid chromatography with electrochemical detector for hydroxyl radical monitoring", *International Conference*, *Young Chemists Symposium* (MYCS), Rimini, October 27-29, 2017 (Italy)

➤ TVRDÝ V, **CATAPANO MC**, KARLÍČKOVÁ J, MLADĚNKA P, "Introduction of a simple, cheap but precise method for evaluation of the zinc chelating properties", *Redox 2018 - 20th International Conference on Antioxidants*, Paris, June 30, 2018 (France)

➤ **CATAPANO MC** & MLADENKA P "The formation of complexes between glutathione reduced-oxidized and copper ions", *12th International symposium Pharmaceutical sciences,* Ankara, June 26-29, 2018 (Turkey)