DISSERTATION

Possible links between iron and tryptophan metabolism in individuals with iron deficiency and anaemia

submitted by

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Statutory Declaration

"I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis.

Due acknowledgement has been made in the text to all other material used.

Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on Good Scientific Practice ".

Julian Wenninger
Graz, March 2020

Disclosures

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Abbreviations and Definitions

AAPCC	American Association of Poison Control Centres
ABCB10	ATP-binding cassette subfamily B member 10
ACD	anaemia of chronic disease
ADP	adenosine-di-phosphate
AIDS	Acquired Immunodeficiency Syndrome
ALAS2	delta-aminolevulinate synthase 2
APP	acute phase protein
ApoF	apoferritin
APR	acute-phase response
ARLD	alcohol related liver disease
ATP	adenosine-tri-phosphate
BMP	bone morphogenetic pathway
CDC	Centres for Disease Control
CFU	colony forming unit
CHr	reticulocyte haemoglobin content
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CNS	central nervous system
CO	carbon monoxide
CRA	cancer-related anaemia
CRP	C-reactive protein
CYPP450	cytochrome P450
Dcytb	duodenal cytochrome B
DMT1	divalent metal transporter 1

eGFR	estimated glomerular filtration rate
EPO	erythropoietin
ERFE	erythroferrone
Fe	ferrum
FFP	fresh-frozen-plasma
fL	femtolitre
FPN1	Ferroportin-1
GABA	γ-aminobutyric acid
GBD	Global Burden of Disease
GI	intestinal tract
GVHD	graft versus host disease
HAMP	a gene that regulates hepcidin expression
HAV	hepatis A
HBV	hepatitis B
HCP1	heme carrier protein 1
HEPC	hepcidin
HFE	human homeostatic iron regulator protein (high $Fe2^{+}$)
HIV	Human Immunodeficiency Virus
HLM	hemosiderin laden macrophages
i.v.	intravenous
IBD	inflammatory bowel disease
ICU	intensive care unit
IDA	iron deficient anaemia
IDO	indoleamine 2,3-dioxygenase;
IL-6	interleukin-6

IONP	iron oxide nanoparticles
INF-γ	interferon-γ
IRE/IREP	iron-responsive element/iron-regulatory protein
Ireg1	iron-regulated transporter 1
kDa	kilodalton
LMICs	low- and middle-income countries
LNAA carrier	large neutral amino acid carrier
MCV	mean cell volume
MCV	mean cell volume
Mfrn	Mitoferrin
MOF	multi-organ-failure
mRNa	messenger ribonucleic acid
MTP1	metal tolerance protein 1
NAD	Nicotinamide adenine dinucleotide
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
NASH	non-alcoholic steatohepatitis
NMDA	N-methyl-D-aspartate
NSAID	non-steroidal anti-inflammatory drug
NRAMP1	natural resistance-associated macrophages protein 1
OTC	over-the-counter
Pg	picogram
PH	porotic hyperostosis
RBC	red blood cell count
RDW	red cell distribution width
RES	reticuloendothelial system

ROS	reactive oxygen species
SLC25A38	mitochondrial glycine transporter gene
SMAD	a group of proteins that transduce TGF-ß signals into the cell’s core
sTFR	serum transferrin receptor
TDO	Trp 2,3-dioxygenase
TfR	transferrin receptor
TGF	transforming growth factor
TIBC	total iron-binding capacity
TNF-α	tumour necrosis factor α
Trp	tryptophan
VAD	vitamin-A-deficiency
WHO	World Health Organisation
WRA	women of reproductive age
QUIN/QA	Quinolinic acid
mL	millilitre
μg	microgram
μL	microliter

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Abstract in German

In unserer Studie haben wir mögliche Zusammenhänge zwischen Tryptophan, Kynurenin und Kynureninsäure mit Markern des Eisenstoffwechsels (zB. mittleres Erythrozyteneinzelvolumen, mittleres korpuskuläres Hämoglobin, Ferritin, Ferritinsättigung, Serumeisen, Transferrin, löslichen Transferrinrezeptor, Retikulozytenhämoglobin) und Hämoglobin von 430 Personen evaluiert, die Gruppen mit und ohne Eisenmangel oder Anämie unterteilt wurden.

Insgesamt 430 PatientInnen wurden zu einem Check-up ihres Eisenstatus‘ zugewiesen und in diese Querschnittsstudie inkludiert. Alle TeilnehmerInnen haben schriftlich in die Studie eingewilligt. Sie hatten venöse Nüchtern-Blutabnahmen zwischen 8 und 10 Uhr in der Früh. Diese Proben wurden untersucht: der Eisenstatus (zB. mittleres Erythrozyteneinzelvolumen, mittleres korpuskuläres Hämoglobin, Ferritin, Transferrinsättigung, Serumeisen, Transferrin, löslichen Transferrinrezeptor, Retikulozytenhämoglobin), der Tryptophanstoffwechsel (Tryptophan, Kynurenin, Kynureninsäure, das Verhältnis Kynurenin zu Tryptophan sowie der Kynureninsäure- Kynureninindex), die Nierenfunktion (Kreatinin, geschätzte GFR) und das CRP.

Biomarker des Tryptophanstoffwechsels korrelierten positiv mit Hämoglobin sowie Eisenstoffwechselmarkern (p-Werte: <0.001 – 0.038; r-Werte: 0.100 – 0.305). Die stärkste Korrelation wurde zwischen Tryptophan und Hämoglobin ($p < 0.001$, $r = 0.305$) beobachtet. Die Regressionsanalyse ergab die höchsten R^2-Werte zwischen Hämoglobin und Tryptophanmarkern. Insgesamt 115 PatientInnen mit Eisenmangel zeigten niedrigere Tryptophan sowie Kynureninsäurewerte verglichen mit 315 PatientInnen ohne Eisenmangel. Sechs PatientInnen mit einer Anämie bei chronischer Krankheit hatten die niedrigsten Tryptophanspiegel und das größte Verhältnis von Kynurenin zu Tryptophan im Vergleich zu 11 PatientInnen ohne Eisenmangel und 413 Personen ohne Anämie.

Unsere Untersuchung zeigte einen geringen bis mittelgroßen Zusammenhang zwischen Hämoglobin, Eisenstoffwechsel – und Tryptophanstoffwechselmarkern. Weitere Studien sind notwendig, um diesen ursächlich genauer zu untersuchen.

Abstract in English

In our study, we evaluated potential associations of tryptophan, kynurenine, and kynurenic acid with indicators of iron metabolism (i.e., mean corpuscular volume, mean corpuscular haemoglobin, ferritin, transferrin saturation, serum iron, transferrin, soluble transferrin receptor, reticulocyte haemoglobin) and haemoglobin in 430 individuals grouped by the presence or absence of iron deficiency or anaemia.

All study participants, which were included in this cross-sectional study provided their written informed consent. They were admitted for a medical check-up of their actual iron status and underwent venous blood sampling after an overnight fasting state in the morning (between 8.00 and 10.00 a.m.). The samples were used to investigate the iron metabolism (i.e., haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, ferritin, transferrin saturation, serum iron, transferrin, soluble transferrin receptor, reticulocyte haemoglobin), the tryptophan metabolism (tryptophan, kynurenine, kynurenic acid, kynurenine/tryptophan ratio, kynurenic acid/kynurenine index), the renal function (creatinine, estimated glomerular filtration rate [eGFR]), and the C-reactive protein.

Indicators of tryptophan metabolism were positively correlated with haemoglobin and markers of iron metabolism (p-values: <0.001 – 0.038; r-values: 0.100 – 0.305). The strongest correlation was observed between tryptophan and haemoglobin ($p < 0.001$, $r = 0.305$). The cubic regression model yielded the highest R-square values between haemoglobin and tryptophan markers. Overall, 115 patients with iron deficiency showed lower tryptophan and kynurenic acid concentrations compared to 315 individuals without iron deficiency. Six patients with anaemia of chronic disease were observed with the lowest serum tryptophan levels and the highest kynurenine/tryptophan ratio compared to 11 individuals with iron deficiency anaemia and 413 non-anaemic patients.

This study showed little to moderate associations between haemoglobin, biomarkers of iron metabolism and tryptophan markers. Further studies are needed to get better insight in the causality of these findings.

1. Introduction

In the last few years, the clinical interest in evaluating the iron and tryptophan metabolisms have increased as questions about their relation and importance for various diseases were raised.

Iron (Latin *ferrum*, atomic number 26, d-block of the periodic table of elements) as a transition metal is never found as a free but with a plethora of organic and inorganic ligands. The extent to which the iron reacts with other elements is highly dependent on its surroundings and environment such as the pH, the temperature the chemical properties of possible ligands. Ferrous sulphate (Fe(II)) is often found in anoxic surroundings and easily oxidised to ferric chloride (Fe(III)) when exposed to oxygen, this is of great interest in terms of bioavailability (Schwertman 1991, Ilbert & Bonnefoy 2013).

Tryptophan is an essential amino acid, which is utilized for protein biosynthesis and metabolized via the kynurenine pathway. It is also known as a precursor of the serotonin synthesis in the central nervous system, thus the possible importance for development and progression of psychiatric disease is more and more discussed.

The enzyme indoleamine 2,3-dioxygenase, which is induced by proinflammatory cytokines (i.e., interferon-γ, tumour necrosis factor-α), converts tryptophan into kynurenine (Gabbay et al. 2010). This degradation of tryptophan is expressed by the kynurenine/tryptophan ratio, which was first introduced by Fuchs et al. 1990 and which is able to assess the activity of the indoleamine 2,3-dioxygenase in the situation of an active immune system (Myint 2007).

Kynurenine is further metabolized into the neuroprotective kynuric acid and several neurotoxins (Gabbay 2010). The ratio between kynurenic acid and kynurenine enables the assessment of the neuroprotective index (Myint 2007). Since kynurenic acid hardly crosses the blood brain barrier, serum concentrations may not reflect the actual situation in the brain.

The kynurenine pathway as the primary route for tryptophan catabolism has received increasing attention as its dysregulation is considered to be associated with inflammation, tumour proliferation, neurodegenerative diseases and psychiatric diseases such as depression (Davis & Liu 2015). The tryptophan availability and metabolism have been reported to be associated with malabsorption conditions (i.e., fructose malabsorption) and mood disorders (Ledochowski et al. 2001, Enko et al. 2018).

An increased indoleamine 2,3-dioxygenase activity with an enhanced degradation of tryptophan is also considered to be involved in the drop of blood levels of haemoglobin and the development of anaemia (Schroecksnadel et al. 2003, Weiss et al. 2004, Pawlak et al. 2001, Eleftheriadis et al. 2016).

Furthermore, the question raises if the tryptophan metabolism has a potential link to iron deficiency or iron deficiency anaemia (IDA). This issue has been addressed by Weiss et al. 2004 but did not allow a convincing final conclusion at that time.

The heightened awareness of potential adverse effects (i.e. changes in cognitive development, immune function, energy metabolism or temperature regulation) of iron deficiency has renewed efforts to reduce the prevalence of this micronutrient deficiency (Ferrari et al. 2011).

Currently, there is no full international consensus on disease markers to be used for assessing the human iron status.

Plasma ferritin levels (iron deficiency: < 30 ng/mL) and transferrin saturation values (iron deficiency: < 20%) remain the widespread biomarkers in defining iron deficiency in clinical practice (Muñoz et al. 2009, Goodnough et al. 2011).

Nevertheless, there is still a lack of study designs investigating the serum levels of tryptophan and tryptophan metabolites in large cohorts of patients with iron deficiency or anaemia.

The underlying study of this thesis was conducted to investigate possible associations between the parameters of tryptophan and iron metabolism and haemoglobin levels in a large cohort of patients grouped by the presence or absence of iron deficiency or anaemia.

Starting with relevant information on the metabolic processes and data of iron, tryptophan and related diseases and biochemical pathways in chapter 1, the hypothesis is outlined in chapter 2, the study conducted is portrayed in detail with special focus on repeatability and reproducibility in chapter 3. Following these brief outlines, the results and findings are presented in chapter 4 and, finally, discussed and contextualised appropriately in chapter 5. Concluding, chapter 6 will list limitations & recommendations. In the last chapter, all sources cited or otherwise mentioned in this thesis are listed.

1.1 Historical remarks

Anaemia or sideropenia as a chronic state of iron deficiency has long been a ‘scourge for mankind’ and proved difficult to both diagnosis and treatment; even though already in classical antiquity and even the time of the Egyptians first cures and treatments were tested.

Early skeletal findings of humans often display structural alterations known as porotic hyperostosis (PH) but were found in regions in which inherited anaemias were uncommon. PH may lead to thinner bone layers. When the prehistoric humans started to settle down, transferring from a society of hunters to one of gatherers, dietary deficiencies became more obvious - they consumed less meat (such as deer, rabbits etc.) and shifted more to produce containing less iron. This theory was further backed when in the 18th and 19th centuries skeletons of people who dwelled in poor and underprivileged areas of London portrayed characteristic bone thinning (Poskitt 2003).

When the prevalence of iron deficiency anaemia (IDA) was found to rise as bone variances did, a connection to the change of humans transferring from - hunters to gatherers became more obvious. The famous Ebers Papyrus, Roman encyclopedist Celsus, whose opus De Medicina was used in practical medicine and teaching until the middle ages as well as his Greek colleague Galen of Pergamon who shaped medicine for a thousand years to come, endorsed the use of iron for splenic enlargement and menorrhagia (Saunders et al 1958).

It was not until the 1660s when Thomas Sydenham (1624-1689), the ‘father of English Medicine’ and teacher of John Locke, advocated the use of iron for chlorosis, nowadays known as hypochromic anaemia (Guggenheim 1995, Pearce 2016). Jean Varandal, a professor of medicine in Montpellier, coined this term in 1615 coming from the Greek word χλωρός (chlōrós) meaning a yellowish green; which is nowadays still used in describing a mineraldeficiency of green plants (Duden 2020).

Already some years later in 1713, French chemists Etienne-Francois Lémery und Nicholas Geoffroy assumed a connection between blood and rust as they share a similar spectrum of colouration – iron was found in the ashes of blood, thus provoking the observation they may be connected. In 1745, iron was first found in red blood cells. Physicians of that time attributed this disorder to young women and adolescent girls with blood loss from menses or inadequate dietary uptake (Brumberg 1982). Some argued that symptoms such as pallor, shortness of breath and heart conditions were due to wearing tight-lacing corsets, resulting in intensified reflux oesophagitis (Lee 1998) with loss of blood or deficient dietary intake (Hudson 1977).

Sydenham had a great impact on other scientists; he laid the foundation for them to further his findings. In 1831 Foedisch demonstrated that anaemic blood lacked iron and in 1840 Hoefer pointed out that blood of afflicted patients' reddish colour was less intense (MacDowell 2017). In 1843 the chemist Justus Liebig viewed haemoglobin as the most important (and sole) physiological iron compound in the human organism and as the "ferment of breathing" (Euler 1934) and in 1866 the German physician Felix Hoppe was the first to discover the reversible oxygenation of haemoglobin (Hoppe-Seyler 1866).

Figure 1. Gabriel Metsu "Doctor's Visit" and Jan Steen „Doctor's Visit", both painted around 1660, depicting symptoms of a young woman suffering from chlorosis such as paleness and exhaustion. Courtesy of the State Hermitage Museum, St. Petersburg, Russia.

Physicians prescribed beverages containing iron fillings, iron-rich spring water and recommended taking cures in renowned sanatoriums; some even advised drinking animal blood to reduce symptoms of chlorosis (Brueschke 1971). First pills however were first prescribed by Blaud in 1832. He theorised that the disease "arises from - a faulty formation of blood as a result of which blood is an imperfect fluid or the colouring matter is defective so it is no longer suitable for stimulating the organism and maintaining the regular exercise of its functions".

The pills he had successfully tested on 32 patients suffering from chlorosis contained 1,39g of ferrous sulphate (containing 64mg of iron) and 0.1g of potassium carbonate.

A Bohemian physician, pharmacist and doctor to the famous Schwarzenberg family, Albert Popper, advocated the use of sulfuric acid and iron (Vitriolum martis) and potassium carbonate (Sal tartari) as treatment for chlorosis (Raimann 1841).

These remedies were in use for many decades to follow, sometimes refined, later even combined with arsenic, which was widely believed to facilitate the bodily uptake and used in many pharmaceutical products and even tapestry, effectively causing many poisonings (MacDowell 2017). Even at that time, some doctors and scientists speculated that iron may not be intestinally resorbed to a satisfactory amount but egested without further interaction. This was seen in connection with the administering of organic and inorganic iron; with only the first being recommended for treatment and the latter being responsible for flatulency (Brueschke 1971).

In 1872, French internist Armand Trousseau, the first physician to perform a tracheotomy and intubation, coined the term aphasia and the Trousseau's syndrome (thrombophlebitis migrans as a paraneoplastic symptom) recommended treatment of chlorosis with iron, even though he believed it to be a psychiatric condition linked to hysteria (Guggenheim 1995).

In 1896, Honigmann proved that iron can in fact be well-absorbed by the human body, refuting earlier hypotheses that stated otherwise; and in 1909 Schirokauter tried to verify that the iron ingested was substantially altered in the stomach, thus stating that the chemical condition the iron was in when given, didn´t have any impact on the resorption – although the opposite had already been shown in the 1850s (Brueschke 1971).

The effect of parenteral application was strongly disputed, with some scientists advocating for and others against it; Meyer and Williams displayed the lethality of sodium-tratrato-ferrate (III) by killing animals through i.v. application.

Later, this view changed within the scientific community and scientists such as Heilmeyer demonstrated positive effects of parenteral injection (Brueschke 1971). More and more differential diagnoses of anaemia were described such as the “achlorhydratic anaemia”, the “simple achylic anaemia”, the “simple gastrogenic anaemia”, stressing the possible connection to oesophagitis and many more.

At that time, physicians - believed chlorosis to be - a disease of younger women and hypochromic anaemia - a disease of older women (40+). It was not until 1932, that Heath and his colleagues finally identified and clearly demonstrated iron deficiency to be the main reason of hypochromic anaemia and also of chlorosis, at the same time proving that these result from the same deficiency (Heath 1932).

Apparently, anaemia and chlorosis were common themes of everyday life, widely known and familiar enough to be worked into in pieces of fine arts (cf. figure 1). Apart from pictorial arts as mentioned earlier, they were addressed also in writing. English author William Shakespeare (1564-1616) depicted symptoms of hypochromic anaemia at least twice:

1) "Pericles, Prince of Tyre", approx. 1607, act IV scene VI

Pandar *Now, the pox upon her green-sickness for me!*

Bawd *'Faith, there's no way to be rid on't but by the way to the pox.*

Here comes the Lord Lysimachus disguised.

The “green-sickness” refers to paleness and hypochromic anaemia. The remedy – sexual intercourse.

2) “Romeo and Juliet”, first published 1597, act 2 scene 2

Romeo *Arise, fair sun, and kill the envious moon,*
Who is already sick and pale with grief
That thou her maid art far more fair than she.
Be not her maid, since she is envious;
Her vestal livery is but sick and green,
And none but fools do wear it. Cast it off.

Romeo wants to take Juliet’s virginity, deceitfully claiming that goddess Diana, who watches over virgins, is envious of her beauty. Juliet is a follower of the goddess of the hunt, and in this scene compared to the moon “who is already sick and pale”. The followers of Diana, the “vestals” are virgins and mostly young women and her uniform, her “livery” is “but sick and green”.

Since especially adolescent women are afflicted by anaemia, some referred to it as the *morbus virgineus* “sickness of the virgins” or *febris amatorial*, “lover’s fever”; some doctors advocated sexual intercourse as remedial, working effectively against the state of virginity (Mabillard 2000).

From a medical point of view, this therapy seems unlikely to work.

1.2 Iron, iron homeostasis and markers of deficiency

1.2.1 Physical and chemical properties

Iron still plays many crucial roles for humankind, so there is even an era of human history named after it – the Iron Age. -The spread of new ways of producing it caused the collapse of the Bronze Age. Historians date this event to the 12th century B.C. The end of the Iron Age is marked by the beginning of historiography, dating in some regions to 500 B.C., in others to 800 A.D. Earliest iron was found in ancient China (Fernandez-Goetz & Ralston 2017).

Iron as a heavy metal is widespread in the earth's crust and is the planets´ most common element. In its crust however, it is rarer and surpassed by aluminium, oxygen and silicon and believed to be abundant in its core (Morgan & Enders 1980, Williams 2012). Here, iron forms many different iron minerals with other elements, such as haematite, limonite, magnetite, pyrite and siderite (Klein & Hurlbut 1985, Biswas 2005). Despite this abundance -, iron anaemia is the most common reason for anaemia as a whole, as will be demonstrated in the following.

Iron as a transition metal is never found as a free state but with a plethora of organic and inorganic ligands. Its Latin name, *ferrum*, lead to the chemical symbol of Fe in the d-block of the periodic table of elements – its atomic number is 26. Iron begins to melt at around 1500°C (1800K) and to boil at around 2860°C (3130K) and its density is 7.87 g cm^{-3} (Beutl et al 1994, Ilbert & Bonnefoy 2013).

While different oxidation states can be observed under laboratory conditions (cf. table 1), only 0, +2 and +3 are rather stable, they react readily with

-2 (d^{10})	Disodium tetracarbonylferrate, $C_4FeNa_2O_4$
-1 (d^9)	$Fe_2(CO)^{2-}$
0 (d^8)	Iron pentacarbonyl, Fe(CO)5
1 (d^7)	Cyclopentadienyliron dicarbonyl dimer, C14H10Fe2O4
2 (d^6)	**Ferrous sulphate, iron(II) sulphate, FeSO4**
3 (d^5)	**Ferric chloride, iron(III) chloride, FeCl3**
4 (d^4)	$Fe(diars)_2Cl^{2+}$
5 (d^3)	$FeO^{3-}{}_4$
6 (d^2)	Potassium ferrate, K_2FeO_4
7 (d^1)	$[FeO_4]^-$

Table 2. Iron oxidation states and compounds. For this thesis most important compounds are highlighted in bold (modified after Crichton 2001 and Talaiekhozani 2016).

oxygen and sulphur (Kirk-Othmer 1995). For living organisms, the ferrous and ferric states are most important. A neutral iron atom consists of four unpaired electrons in its 3d orbital and two paired ones in the 4s orbital.

In order to change it into ferrous sulphate, the 4s electrons need to be removed; to change it further into ferric chloride, one of the 3d electrons has to be extracted (Crichton 2001).

The extent to which the iron reacts with other elements is highly dependent on its surroundings and environment such as the pH, the temperature, and the chemical properties of possible ligands. Ferrous sulphate (Fe(II)) is often found in anoxic surroundings and easily oxidised to ferric chloride (Fe(III)) when exposed to oxygen.

The solubility and reactivity of iron depend greatly on the pH – at acidic levels abiotic oxidation takes place slowly but increases as the pH approximates to a neutral pH. Also the bioavailability differs as it connects with solubility; while Fe(III) has a low solubility (10^{-17} M) whereas Fe(II) is very soluble in water; in general atomic iron is insole in water, alkali, alcohol and ether but soluble in acids (Weast 1976, Schwertman 1991, Ilbert & Bonnefoy 2013).

In water or moist atmosphere, iron reacts with oxygen, creating various insoluble iron oxides, which are commonly known as rust (Cornell & Schwertmann 2003). As mentioned above, several ligands can bind to iron, thus forming different compounds. They also influence the redox potential; in acidic surroundings, only oxygen can be used as an e- acceptor, whereas in higher up to neutral pH also nitrate may be used by several microorganisms as an e- donor (Piasecki et al 2019).

This has a great impact on the binding capacity of enzymes and proteins in all living organisms to run different pathways with the redox potentials of iron co-factors ranging from about -0,5 V to + 0,6 V (Ilbert & Bonnefoy 2013).

1.2.2. Iron and its role in biology

Nearly all species and forms of life need iron to process biochemical reactions. It appears that only *Lactobacillus plantarum* and *Borrelia burgdorferi, the pathogen causing Lyme Disease,* do not need iron, with the latter using manganese instead of iron as an essential enzyme (Archibald 1983, Aguirre et al. 2013).

For most other living organisms, iron is - vital for many proteins and enzymes, a lack of iron causes symptoms of deficiency, diseases and, eventually, death. Ferrous sulphate and ferric chloride are the main agents of biochemical reactions in biology. As a cofactor, iron

1) is needed for storing, moving and activating of molecular oxygen,
2) is used for activating and disintegrating -peroxides,
3) is utilised for reduction of ribonucleotides and dinitrogen (N_2),
4) enables electron transfer via electron carriers of differing redox potentials (Outten & Theil 2009, Ilbert & Bonnefoy 2013).

Miller (2013) differentiates between four types of iron-containing proteins; mononuclear iron proteins (e.g. superoxide dismutase), diiron-carboxylate proteins (e.g. ferritin), iron-sulphur proteins (e.g. aconitase) and haem proteins (e.g. haemoglobin) with the first three, while being vital, the heme proteins are of even greater importance for humans as roughly half of the whole iron of our body is to be found in the haem.

Life appears to be centred around iron and its chemistry and unique abilities and characteristics. With the first emergence of the earliest cells, membranes developed and allowed to hinder and facilitate chemical reactions in differing environments. The redox or reduction potential, a measured variable of the tendency of a chemical species to be reduced or oxidised of the cytoplasm of the first cells is believed to be around -0,2 V while haemoglobin has a potential of +0,1 V. Fe/S clusters are widely seen to be the first carriers of oxygen, but the early biochemical reactions did not yield much ATP. Some sulphur microorganisms then used sulphide ions as electron donors to perform anoxygenic photosynthesis, while others developed into photoautotrophic organisms (Williams 2012).

With the increase of O_2 in the atmosphere over the course of billions of years, more and more Fe(II) was oxidised and precipitated as Fe(III); yet still many organisms kept iron for their biochemical reactions. As iron has a low solubility at pH around seven, neutrophilic forms of life need transporters or other means of solubilisation.

Prokaryotes developed siderophores; these secondary metabolites have a low molecular weight and high affinity for ferric iron, transporting them into the cells (Albelda-Berenguer et al. 2019) with some fungi being able to remove iron directly from a protein (Hissen et al. 2004).

This ability enables organisms to gain iron while infecting e.g. a human, this however triggers a heavy immune response in mammals (Ganz 2009, Weinberg 2009).

Therefore, these are important markers of virulence and pathogenicity (Sherrington et al. 2018). The second possibility to acquire iron is reducing it first and then channel Fe(II) transport into the cell (Dancis et al. 1992).

Mammalian cells acquire iron with transferrin, a means of transport similar to siderophores. With every ferritin being able to carry about 4500 iron atoms, storage of iron within the cell is possible not only in a soluble, but also non-toxic form (Ilbert & Bonnefoy 2013).

A too high number of free iron leads to risk of dangerous and potential damaging hydroxyl radicals, emphasising the importance of a regulated iron homoeostasis (Gutteridge 1989).

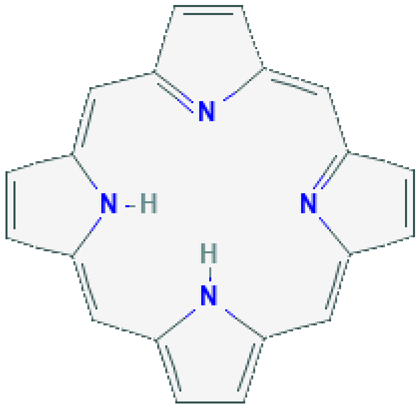

Figure 2. Chemical 2d structure or porphyrin; consisting of four pyrroles and four methine groups.

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Porphyrins consist of four altered pyrrole subunits connected at their carbon atoms via methine groups; since they absorb light, they appear coloured to humans. complexes of iron (haemoglobin), copper (haemocyanins), nickel and magnesium (chlorophyll) devolved gradually over time.

Haemoglobin formed with an iron porphyrin while haemerythrin needs two iron atoms to function; together with CYPP450 and said iron porphyrin they serve as carriers (Williams 2012).

1.2.3 Pharmacology and toxicology / toxicity

Effects on the human organism can be expected either through inhaling of fumes and dust, or i.v. injection (e.g. treatment of haematological cancers, thalassaemia, sickle cell disease) or ingestion of iron-containing preparations (Ho-Wang & Becker 2019). Nevertheless acute iron poisoning based on meat and produce is very unlikely, but some cases of OTC-multivitamin-poisoning were described; and while children are in danger of accidental ingestion of (high-dose) preparations for adults, poisoning of adolescents and adults are mostly linked to suicidal acts (Abhilash et al. 2013, Madiwale et al. 2006, Sane et al. 2018).

In the United States, the Annual Report of the American Association of Poison Control Centers (AAPCC) lists one death, 4072 exposures to iron, 3211 were unintentional ingestions, 2036 cases of children up to five years and 1161 hospital treatments (Mowry et al 2016).

Ingestion of elemental iron	
Up to 20mg per kg	Non-toxic
20 – 60 mg per kg	Moderate, mostly GI-symptoms
More than 60 mg per kg	Very toxic, high morbidity and mortality

Table 2. Ingestion of elemental iron in milligrams per kilogram and its effect on the human body after Bingham et al. 2001 and Ho-Wang & Becker 2019.

Iron ingestion leads to direct damage of the GI-mucosa, causing symptoms such as nausea, pain, vomiting and diarrhoea, but also haemorrhagic necrosis with blood loss and risk of infection and affecting all internal organs, especially the liver, causing multi-organ-failure (MOF). On a cell-level, it enters the mitochondria and interferes with all biochemical processes such as oxidative phosphorylation, lipid peroxidation, building of free radicals, all resulting in cell death, as discussed later in greater detail (Tenenbein 2001, Baranwal & Singhi 2003, Robertson & Tenenbein 2005).

Treatment of acute iron poisoning is necessary only if patients become symptomatic (including mild GI problems but no further worsening) after a surveillance period of up to 6 h after ingestion. Otherwise, they need prompt intensive care unit (ICU) treatment, including countering hemodynamic instability (using crystalloid infusion), whole-bowel-irrigation, gastric lavage if needed, vitamin K and fresh-frozen-plasma (FFP) against coagulopathy as well as deferoxamine as a chelating agent to purge iron from plasma and tissue; charcoal has little to no effect (Tenenbein et al. 1992, Ho-Wang & Becker 2019). Administering deferoxamine can colour the urine pinkish-red ("vin rose"), a typical sign of the chelation therapy progress (Williams & Erickson 1998).

In case a person is exposed to iron dust and fumes for a longer period of time, but in doses too low to cause an acute iron poisoning, iron overload may be the result. Quite often iron overload is caused by hereditary haemochromatosis or if too many blood transfusions were performed; these conditions will be discussed later in greater detail (Hider & Kong 2013).

As stated above, iron is abundant in nature, but specific events of poisonings are rare; they are sometimes connected with industrial safety and exposure while working. Therefore, workers in mining, welding and production of steel are in danger of inhaling iron dusts and oxides, such as iron oxide nanoparticles (IONP) with a diameter less than 100 nm, even though they are in use as pharmaceutical vectors (Kornberg et al. 2017).

It appears that the main path for harmful interaction is IONP-induced oxidative stress in cells; by inflammation they can cause tissue injury and damage, ultimately leading to higher rate of mitochondrial damage, pulmonary dysfunction, lung infections, fibrosis, cancer and genotoxicity (Ghio 2009, Li et al. 2015, Schumacker et al. 2015, Kornberg et al. 2017). Extra-pulmonary effects of these small particles are still not entirely clear, when IONP were labelled radioactively, they entered the blood stream in under ten minutes and accumulated in the spleen, liver and kidney, leading to damage there (Singhi & Baranwal 2005, Zhu et al. 2009).

The lung relies on the Divalent Metal Transporter 1 (DMT1) for iron transport into the cell and ferritin for storage with their expression elevated when exposed to higher levels of iron (Wang et al. 2002, Giorgi et al. 2015). This oxidative stress seems to be caused by an overload of free iron, which is released, when phagosomes try to degrade the nano particles. Subsequently the iron carriers cannot dispose of it quickly enough, essentially disturbing the cell's carefully balanced iron homeostasis (Kornberg et al. 2017).

1.2.3 Dietary absorption of iron

Similar to other organisms, the mammals have found two possibilities of maintaining their iron levels; first by intake and digestion, second by re-cycling of "used" iron within the body itself. Daily loss of iron in various forms (mostly through faeces) amounts up to one milligram. To attain a stable homeostasis, the amount of dietary absorption and recycled cells must meet the iron needs. Certain groups such as pregnant women and women suffering from hypermenorrhoea are at risk to lose up to three milligrams per day, effectively causing a constant danger of iron deficiency which occurs in up to 10% of all menstruating women (Goddard 2000).

As a whole, the human body consists of three to five grams of iron in different molecular and chemical combinations. This amount varies greatly depending on the gender, body mass, age and diet. The by far largest amount of it, about two-thirds, is seen to be contained in erythrocytes in form of their haemoglobin. About 10% are stored in cytochromes and muscular iron as well as enzymes that need iron for proper function. Up to one-third is believed to be in cells of the reticuloendothelial system and hepatocytes (Ponka 1997, Conrad et al. 1999).

Iron as a central atom in porphyrins is vital for the transport of oxygen in many animals, while other living organisms use copper; haemocyanin as used by mollusca and arthropoda needs two copper atoms that bind one oxygen molecule or magnesium, as in chlorophylls that may be found in plants, algae and bacteria (Burmester 2002). Many organisms use iron for redox reactions for electron transfer as they change easily from bivalent to trivalent state (Schwertman 1991, Ilbert & Bonnefoy 2013).

The qualitative amount of uptake depends heavily on its chemical structure – the rates of intake vary; absorption of iron as haem iron or proteins, e.g. from red blood cells and mitochondria, is greater than in form of iron salts and may reach up to 35% (McKie et al. 2001). This has an impact on dietary recommendations, as lifestyle-based iron deficiency occurs more often in individuals who refrain from eating meat and related products (Truswell 2010).

As with many other vitamins and dietary elements, iron uptake is predominantly organised via the gastrointestinal tract and the duodenum. There, crypt cells facilitate these reactions and transfer iron into the blood stream. These are located chiefly in the proximal small intestine.

Most of the iron that is not organic is absorbed at the brush boarder of duodenal enterocytes using the divalent metal transporter DMT1 (Gunshine et al. 1997, Conrad et al. 1999).

Most of the iron absorbed occurs as either ferric iron or haem iron. Since haem is soluble at the intestinal alkaline pH, it is separated from myo- and haemoglobin by pancreatic enzymes (Conrad et al. 1967). Hence, the biggest part of our body iron is haem (Conrad & Umbreit 2000). However, the human body cannot use ferric iron, Fe(III), which therefore has to be chelated via carbohydrates or amino acids, reduced via duodenal cytochrome B (Dcytb) or ascorbate ("vitamin C") ferrireductase, a cytochrome that most eukaryotes possess and rely on for iron bio-availability. It appears not to be the only one though, as knock-out mice have a regular iron status (McKie 2008). Understandably, the pH number of the surrounding liquid influence the chemical disposition and it appears that ferrous iron salts are more efficiently absorbed in regular conditions than ferric iron (Conrad et al. 1966). The exact pathway of haem absorption remains unknown (Qiu et al. 2006); within the cell, it is released by the inducible haem-oxygenase 1 (Ferris et al. 1999).

The divalent metal transporter 1 (DMT1) facilitates the shift into the cytoplasm, where re-mineralisation must be prevented. In case of haem ingestion, especially in form of meat and like cellular structures, the haem carrier protein 1 (HCP1) can be used for transport. Unlike other cells, enterocytes bear no receptor for transferrin (Pietrangelo et al. 1992). Further transport and export out of the cell are then facilitated by ferroportin. This protein is also called 'solute carrier family 40 member 1' (SLC40A1) and is the only protein known to-date that can transport iron out of the cell. Ferroportin is not only found on the membranes of the intestinal enterocytes but also on adipocytes, hepatocytes and macrophages (Donovan 2015). Its activity is inhibited by hepcidin (Nemeth 2004; Ward & Kaplan 2012). For this transport processes however, hephaestin is needed. This is an oxidase similar to ceruloplasmin and consists of copper as their metal agent, lack of hephaestin leads to mucosal iron retention (Hentze et al. 2010).

Iron absorption has to be controlled and monitored closely, since there is no means of excretion any overload iron, while iron uptake can be increased when there is higher requirement or consumption (e.g. pregnancy or higher rate of erythropoiesis). Commonly, the usual daily dietary uptake is up to fifteen milligrams per day, but the human body takes up only up to two milligrams of iron each day; these represent the loss by sloughing of cells, blood loss and sweat

- although data reported varies greatly. Iron overload originates from this incapability of iron excretion (Hentze et al. 2010, Fraser et al. 2011, Collings et al. 2013, Miller 2013).

1.2.3 Utilization

Most of the iron that is re-used in the body supplies the erythropoiesis and production of haemoglobin. This amounts to up to 25 milligrams per day and is heavily influenced by transferrin receptor R1 which influences iron loading of the erythrocytes (Hentze et al. 2010) but aided to a smaller extent by erythroblast iron acquisition (Leimberg et al 2008). Said erythroblasts also seem to transport iron in a straightforward manner from endosomes into mitochondria by close contact without relying on further means of transportation. (Sheftel et al. 2007). The transport into the mitochondria is facilitated by a protein of the inner membrane called mitoferrin 1 (Mfrn1/SLC25A37); Mfrn1 interacts with ABCB10, a protein (Chen et al. 2009). To avoid massive iron or haem overloading, it is either implemented into ferritin or transported elsewhere using ferroportin. A special ferroportin messenger RNA isoform is expressed for this cause.

When the mitochondrial iron uptake is not in equilibrium with the sink by haem or Fe/S cluster biogenesis, clustering of iron may a consequence. If this is the case, a special form of sideroblasts may be observed. They also can be of diagnostic value for diseases such as X-linked sideroblastic anaemia because of ALAS2 deficiency or autosomal-recessive deficiency if SLC25A38 (Hentze et al. 2010).

1.2.4 Iron recycling

Since the intestinal resorption of iron accrues for only about 10% of the iron needed, recycling is the key factor. About 1% of all erythrocytes are recycled on a daily basis with their iron being used in building new cells, accounting for up to 20 milligrams of iron from senescent erythrocytes (Miller 2013). Here, macrophages are needed to process this. They use phagocytosis of damaged or aged cells such as erythrocytes and use haem-oxygenase to free the haem. In order to transport the recycled goods, a protein called NRAMP1 (natural resistance-associated macrophages protein 1) is expressed. By this, a bivalent metal transporter similar to DMT1 can work the moving of iron from phagolysosomal membranes and phagocytic vesicles.

1.2.5. Iron overload

Iron overload originates from an incapability of iron excretion, as the dietary uptake is too low to create an overload; in other cases, however, this state is produced artificially, e.g. because of intensive blood transfusion (Hentze et al. 2010).

The human iron metabolism is, as mentioned above, greatly influenced by the erythropoiesis. As a healthy adult needs about 6-7g of haemoglobin every day, adequate uptake of iron is necessary to prevent iron deficiency and anaemia. Most of this is recycled through macrophage activity. Up to 1 g of the total amount of iron in the body is stored in the reticuloendothelial system and hepatocytes. Surplus apoferritin (also known as ferritin that is not bound to iron) is a relevant diagnostic mark for stored iron; 1 µg/L ferritin serum represents about 10 milligrams of stored iron (Ponka et al 1998). Levels below 10-30 µg/L indicate a low storage of iron and lowered total body iron altogether.

1.2.6. Iron homeostasis

Keeping a balanced iron homeostasis is of vital importance for the human body. As mentioned above, dietary absorption, recycled and stored iron are the sources of this element and hepcidin is the central regulatory protein of iron uptake (Nemeth et al. 2004, Sangkhae & Nemeth 2017). In case of chronic failure to provide the levels needed, the iron storage of the liver is the first to reduce or empty.

With the reduction of available iron, the serum ferritin level is a diagnostic marker of iron deficiency, albeit false-high numbers can be expected in presence of inflammation processes even when bone marrow stores are nearly empty (Rocha et al. 2009). Also, serum iron levels are decreased and the expression of transferrin is elevated to cater the higher need (Miller 2013). Cleary, erythropoiesis and erythroid cell are affected as well, resulting in a decrease of their average size and level of haemoglobin. In individuals with iron deficiency, the haem production is lowered while more zinc protoporphyrin is built (Crowell et al. 2006). In terms of blood smear and staining, a heightened level of red cell distribution width (RDW), lowered red blood cell count (RBC), as well as lowered RBC haemoglobin and mean cell volume (MCV) can be expected (Miller 2013).

In the following, several of the relevant molecules, proteins and receptors for the human iron metabolism and iron homeostasis are described.

Transferrin

Transferrin is a glycoprotein; in the body, different types of transferrin (apotransferrin that is not loaded with iron at the moment, and monoferric and differic transferrin) offer two binding spots with a high affinity for Fe(III). This fact allows circulation within the body bound to this glycoprotein. By this, iron is still soluble, but is yet easily transferred into a cell in need of iron by using the transferrin receptor, TfR1. Also, with this binding the toxic capacity of iron is greatly limited. In the human body about a third of all plasma transferrin is saturated with iron, in excess of 45% saturation, an iron overload could be consequential; whereas saturation levels below 15% may suggest iron deficiency. If this bound reaches more than 60%, the iron that is not bound starts to accumulate and harm parenchymal cells (Hentze et al. 2010). This indicates that the importance of iron homeostasis, the upkeep of a steady balance between usage, excretion and uptake in the human body is vital with ferroportin and hepcidin playing major parts (Lieu et al. 2001).

Transferrin receptor (TfR, sTFR)

The TfR is of vital importance for the human iron metabolism. This receptor consists of a homodimeric membrane glycoprotein (two identical 95 kDa subunits), capable of binding two transferrin molecules (Richardson & Ponka 1997). Its main function is to bind iron and move it to the transferrin receptor of a cell, and through binding and endocytosis translocate it into the cell. The synthesis of TfR is managed at a post-transcriptional level depending on the iron level of the cell (Feelders et al. 1999). Due to the fact that the pH is lower in the endosome (acidosome), the iron is detached from the transferrin, enabling its chelating and consequential usage in the cell. This lowering to about 5,5 pH is achieved by ATP-dependent proton pumps (Yamashiro et al. 1983).

The now released ferritin, the so-called apoferritin (apoF), remains bound to the receptor however and is not freed until the neutral pH outside the cell allows dissociation (West et al. 2000). Two isoforms of the transferrin receptor are known to-date, TfR1 and TfR2, the latter typically located in hepatocytes, duodenal crypt cells and erythroid cells (Hentze at al. 2004). TfR1 displays a greater affinity for differic transferrin and is modified by the level of cellular iron (Kawabata 2000, Johnson & Enns 2004), while HFE appears to influence the expression of the TfR2 (Joshi et al. 2015).

Another pathway may act similar to these receptors; the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), that also uses transferrin has a low affinity to iron but high capacity (Kumar et al. 2012).

After the endocytic cycle is finished, the TfR in the serum, the sTFR, can be measured; it is suggested as an exact marker for possible early iron deficiency as portrayed as a lack of tissue iron, erythroid proliferation and erythroid TfR expression. Additionally, unlike ferritin, it is not an acute phase protein and therefore also valuable in anaemia of inflammation (Feelders et al. 1999). Most of the body iron is needed for erythropoiesis, which is why their progenitor cells display a large amount of TfR (Koulaouzidis et al. 2009).

In some diseases, the sTfR levels are:

- elevated (diseases with higher erythroid proliferation, e.g. haemolysis, autoimmune haemolytic anaemia, polycythaemia vera, sickle cell disease; but also, when tissue iron is low in iron deficiency anaemia)
- normal (anaemia of chronic disease, leukaemia, haemochromatosis, solid tumours)
- lowered (chronic kidney disease, aplastic anaemia, after bone-marrow-transplantation) (Cartwright 1966, Scherzenmeier 1994, Feelders et al. 1999).

In their review article, Koulaouzidis et al. (2009) propose adding ferritin to the measurements of the sTFR due to the fact that sTFR may have high sensitivity but low specificity.

Ferroportin (FPN1, Ireg1, MTP1)

Ferroportin serves as iron exporter and is found in the intestine and macrophages of the tissues (Donovan et al. 2000). Set in the basolateral membrane of duodenal enterocytes, hepatocytes, macrophages and syncytiotrophoblasts, it regulates the flow of iron into the blood in connection with ceruloplasmin/hephaestin; whereas in other cells, ceruloplasmin is needed for conversion of Fe (II) to Fe (III) and further transport (Donovan et al. 2000, McKie et al. 2000, Hentze et al. 2004). Difficulties of ceruloplasmin formation leads to an enrichment of iron in macrophages, hepatocytes and cells of the central nervous system (CNS), impairing erythropoiesis and causing nerval degeneration. There is no reliable test for ferroportin activity and their exact interaction is not fully understood yet. Ferroportin is found in cells of the RES, which gather iron from old erythrocytes (Lieu et al. 2001, Hentze et al. 2004). Expression of ferroportin is also regulated by hypoxia-inducible factor 2 (Drakesmith et al. 2015) the iron-responsive element/iron-regulatory protein (IRE/IREP) (Muckenthaler et al. 2008), and iron-responsive micro-RNA (Sangokoya et al. 2013),

Ferritin

Ferritin functions as a storage of iron that is not immediately needed, thus diminishing the risk of cellular interaction and building of ROS (detoxification) as mentioned in the chapter of iron toxicity. It consists of 24 light and heavy chain subunits and can carry up to 4500 iron atoms. In case it is not carrying iron, it is identified as apoferritin (Hentze et al. 2004, Ilbert & Bonnefoy 2013). Ferritin has also an enzymatic function and transforms Fe(II) to Fe(III) for storage. Although the exact path of ferritin degradation is not well described, it is known that its degradation allows quick release of a high number of iron atoms if needed in a cell (Hentze et al. 2004). Ferritin also acts as an acute phase protein and can be compared to levels of CRP in order to verify infection (Collis 2011).

Hepcidin (HAMP, HEPC)

Hepcidin is found in many animals and has relevant antibiotic (e.g. *Escherichia coli*, *Staphylococcus aureus* & *epidermidis*) and antifungal properties (e.g. *Candida albicans*, *Aspergillus fumigatus* & *niger*). The molecule is rather small with 2-3kDa in size (20-25 amino acids) with an acid charge of +3 pH. Some insects have homologue called drosomycin, and it is built by their fat body (their equivalent to a liver) while plants use cysteine-rich defensive bodies. Hepcidin was first found in urine, and is built mainly in the liver, hence its name. Nevertheless, it is also expressed in the heart and spinal cord, albeit in lower amounts (Park et al. 2001).

The production of hepcidin is regulated by transcription, its levels are influenced by plasma and liver iron stores, inflammation and erythropoiesis (Ganz & Nemeth 2011), with a half-life of only a couple of minutes and a high daily production rate (Xiao et al. 2010).

Hepcidin is a key player in the iron metabolism. It hinders the iron release and therefore the uptake by influencing ferroportin, thus keeping iron balance. Further, if deficient, hepcidin causes iron overload diseases such as haemochromatosis and ß-thalassaemia (Roetto et al. 2003, Ganz & Nemeth 2011); if it is overproduced, it can lead to iron restricted anaemias in CKD, IDA and cancers (Ganz & Nemeth 2011, Fung & Nemeth 2013) and is found in inflammation processes and also acute hypoferraemia (Sangkhae & Nemeth 2017).

Apparently, TF, TfR1, TfR2 and HFE work as extracellular iron-sensors; Sangkhae and Nemeth propose that they stimulate the expression of hepcidin through the BMP pathway (bone morphogenetic proteins influencing growth of bones and tissues). Although a specific pathway seems to exist, further description is needed to identify the role of hepatocytes in intracellular iron finding (Ramos et al. 2011). Inflammation is suggested to influence hepcidin levels as well; the underlying mechanism is to deliberate hypoferraemia as a host defence against infection; IL-6 seems to have specific function here (Nemeth et al. 2003, Nemeth et al. 2004).

As mentioned earlier, hepcidin limits iron release and thus the available iron. However, in some situations when the demand for iron is greater, like in growth, hypoxia and pregnancy, hepcidin release must be suppressed to account for the need for iron. Erythropoiesis has a critical need of iron in form of haem. In contrast to earlier beliefs, EPO does not regulate hepcidin directly; HAMP expression is supressed. Erythroferrone (ERFE) was found to influence hepcidin, but the definitive role of these molecules in this pathway remains unknown to-date. Similarly, growth factors and sex hormones affect the expression of hepcidin, albeit details are yet to uncover (Sangkhae & Nemeth 2017).

Haem oxygenase (HO)

Three isoforms of haem oxygenase were described; they seem to influence inflammation processes as well as the development of atherosclerosis beneficially. The amount of haem produced is partly limited by HO. In order to cleave oxygen, it needs CYP450 reductase, NADPH and three molecules of molecular oxygen, producing equimolar amounts of biliverdin, Fe(II) and CO (Elbirt & Bonkovsky 1999; Araujo et al. 2012). Hence, HO-1 also protects the cell of ROS (as lysed RBC may yield Fe(III) for the Fenton chemistry) and of utmost importance in the iron homeostasis. Its vital importance of correct function becomes more understandable pondering that only two cases of babies with HO-deficiency were born so far (Kartikasari et al. 2009, Fraser et al. 2011).

Mitoferrin (Mfrn)

Mitoferrin exists in two homologue forms, mitoferrin 1 and 2, and is encoded by SLC25A37. They facilitate iron transport in mitochondria (mitochondrial carrier), haem synthesis and iron-cluster synthesis. In animal studies, deletion of relevant genes leads to problems in haemoglobinisation and a lack in mitochondrial iron and also difficulties in erythroblasts synthesis and assimilation. Although it is clear that these iron levels need to be controlled, the exact underlying mechanism is still unkonwn (Shaw et al. 2006, Paradkar et al. 2009).

Haemosiderin

Haemosiderin, consisting of Fe(III)hydroxid and sometimes parts of apoferritin, functions as a form of storage iron. About one third of it consists of iron. It typically appears in pathological processes in cells (e.g. haemosiderin laden macrophages, HLM can be found in idiopathic pulmonary fibrosis) and the body, albeit all cells can store iron in this form (Fairbanks & Beutler 1990, Fukihara et al. 2017).

Hephaestin

MCF such as hephaestin, caeruloplasmin (CP) and zyklopen (ZP) are suggested to play a key role in iron transport by oxidation of ferric to ferrous iron, thus enabling binding to transferrin.

Hephaestin is predominantly found in enterocytes of the small intestine and to a smaller extent in the kidneys, caeruloplasmin may be found in the liver, but also in astrocytes and the kidney (Vulpe et al. 1999, Kuo et al. 2004, Mohstad & Prohaska 2011, Bo et al. 2016). The exact mechanisms and interactions of most MCF but also kidney reabsorption is not clear (Zhang et al. 2007, Smith & Thévenod 2009).

1.3. Tryptophan and the kynurenine pathway

In our project, the kynurenine pathway and kynurenine (K) was of great importance. L-Tryptophan is a large neutral essential amino acid, found in all living organisms (Palego et al 2015). The required minimum daily intake of tryptophan is estimated with 175 mg for adult women and 250 mg for adult men (Peters 1991; Murray 2003). This metabolic pathway degrades nearly all of the tryptophan (Trp) to NAD+ and other crucial metabolites such as niacin and regulates the haem biosynthesis; most of these processes (three out of four, amounting to >90%) take place in the liver under non-inflammatory activity (Bender 1983). The first step of this pathway has a limiting function: Trp 2,3-dioxygenase (TDO) in the liver and indoleamine 2,3-dioxygenase (IDO) regulate further steps. TDO needs oxygen and haem for synthesis (Badawy 2017).

The full catalytic processes and metabolic pathway will not be discussed here, but relevant aspects are presented. TDO can be found in the liver, but also in the brain, while IDO is expressed in these organs but also in the kidneys (Meininger et al. 2011). Trp 2,3-dioxygenase is regulated in several ways as it is induced and activated by glucocorticoid, tryptophan (substrate), haem (cofactor), and by NAD(P)H (feedback inhibition). IDO however is inhibited by these in turn, only cytokines (increased transcription) promote IDO and limit TDO.

Trp is the precursor molecule of serotonin (5HT, 5-hydroxy-tryptamine), which has manifold functions in the CNS but also mediates immune and inflammatory activation and influences peripheral diseases such as fibromyalgia, IBS and chronic fatigue syndrome (Hudson & Pope 1996; Frazer & Hensler 1999; Berger et al. 2009). Furthermore, it is a precursor for melatonin ,which is known to modulate the circadian rhythm (Oxenkrug & Ratner 2012).

However, it is just a small fraction of L-Trp which is metabolised into serotonin, proposing that there is an underlying balance not fully understood yet (Bender 1983). Also, dietary and gut uptake seem to be influenced by the gut microbiome. Trp bound to albumin and free Trp are either transported into the brain through the LNAA carrier or metabolised via enterochromaffin cells and other cell types both in body and brain to proteins, kynurenines, serotonin and in brain CNS tissue to tryptamine and melatonin (Palego et al. 2016; Lanser et al. 2020).

In case the kynurenine pathway does not function correctly, several of its metabolites but also substrates and products accumulate or are too low. TDO and IDO were brought into connection with cancer, major depression, bipolar disease, bodyweight issues and porphyria (TDO), immune diseases, neurological and neurodegenerative disorders (IDO), therefore, posing a possible target for therapeutic intervention (Badawy 2013; Stone & Darlington 2013; Jayawickrama et al. 2015; Sheen & Soliman 2015; Lanser et al. 2020).

As displayed in figure 3, this toxicity seems to be caused due to quinolinic acid (QA) that binds to NMDA receptors as an agonist, which in turn reduce inhibitory GABA receptors' activity. Kynurenine enters the brain either through the BBB or is produced locally by astrocytes and microglial cells from Trp (Stone & Darlington 2002; Lugo-Huitron 2013). Moreover, also 3-HK, 3-hydroxykynurenine fosters programmed cell death of neuronal cells by producing ROS (Polyzos & Ketelhuth 2015), connecting it to diseases like Alzheimer's, Parkinson's and Huntington's (Valente-Silva & Ruas 2018, Sorgdrager 2019). Further dysfunctions of the kynurenine pathway will be portrayed in the discussion section of this thesis.

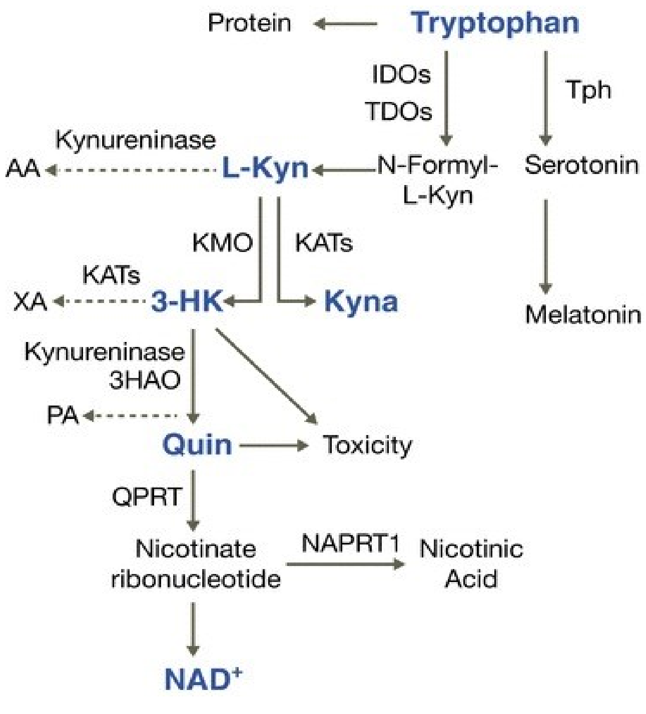

Figure 3. The kynurenine pathway (KP) and its metabolites.

Trp = Tph Tryptophan, IDO indoleamine 2,3-dioxygenase, Kyna kynurenine acid, Quin quinolinic acid, 3-HK 3-hydroxykynurenine, XA xanthurenic acid.

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1.4 Anaemia

1.4.1 Definition of Anaemia

With a disease as widespread and common through all peoples, sexes and ages, a binding definition of anaemia naturally is difficile. One possibility of outlining this condition is counting the red blood cells and/or haemoglobin (Hb) levels and compare them to a sample population. Another possibility of diagnosis is scanning for a low haematocrit, testing the RBC mean corpuscular volume, blood reticulocyte count, a simple blood film stain analysis or haemoglobin electrophoresis. Nevertheless, clinical signs of symptoms are not necessary for diagnosis of anaemia, as they are not visible in all patients (Balarajan et al. 2011, WHO 2011, Schreir 2018). In spite of these various techniques, measuring the haemoglobin is most customary in clinical daily routine and practice and therefore often used as the defining marker for anaemia (Chalco et al. 2005, Chaparro & Suchdev 2019).

Defining and establishing prevailing and universal thresholds for haemoglobin as the indicator for anaemia therefore is no trivial matter. The levels of haemoglobin vary from each individual to the next as influenced by

- age; as haemoglobin levels are highest in the new-born child, ranging from 17-21g/L which then sink in the next months after birth (Dewey & Chaparro 2007, Jopling et al. 2009), raise again in childhood and adulthood and decrease in older age (Nilsson-Ehle et al. 2000);
- pregnancy status, as the rise of the blood volume causes a decrease in haemoglobin levels in the beginning of the pregnancy (Tabrizi & Barjasteeh 2015, Kadry et al. 2018, Wang et al. 2018);
- sex, as difficult habits of diet (Beck et al. 2014) menstruation is associated with blood loss to a greater or lesser extent with women typically having a slightly lower haemoglobin level (Milman et al. 1998, Miller 2014);
- possibly race, as prevalence of anaemia differs between races, addressing the ongoing debate about nature vs. nurture (taking so-called “distal factors” into consideration) and if genetics or psychological factors are the main reason for this observation (Dong et al. 2008, Chaparro & Suchdev 2019);

- genetic and environmental factors, such as living on a higher level of altitude or using it for training of athletes and option to improve physical exercise as the ambient hypoxia leads to the production of erythrocytes, higher heart rate and hyperventilation (West 2004; Akunov 2018), while genetic conditions certainly can affect a person's haemoglobin level in one way or another (Barrera-Reyes & Tejero 2019).

In their 2011 report, the World Health Organisation recommended the following cut-offs for diagnosis of anaemias; they were first published in 1968 and remained relatively unchanged to date, they were, however, complemented to account for different groups of children and pregnant and non-pregnant women in 1989.

Moreover, the WHO also recommend adjustments with regard to the altitude, ranging from <1000 metres above sea level 0g/L, -2 g/L at 1000 metres, -5 at 1500 metres, -8 at 2000 metres, -13 at 2500 metres, -19 at 3000 metres, ending at -45 g/L haemoglobin at 4500 metres above sea level. Similarly, they propose adjustments for smokers with ranging from -0,3 g/L up to 1 packet a day to -0,5 for 1-2 packets a day and -0,7 for more two or more packets a day.

		Anaemia		
Population	**Nonanaemic**	**Mild**	**Moderate**	**Severe**
Children 6−59 months of age	≥110	100−109	70−99	<70
Children 5−11 years of age	≥115	110−114	80−109	<80
Children 12−14 years of age	≥120	110−119	80−109	<80
Nonpregnant women (15 years of age and above)	≥120	110−119	80−109	<80
Pregnant women	≥110	100−109	70−99	<70
Men (15 years of age and above)	≥130	110−129	80−109	<80

Table 3. Haemoglobin (g/L) concentrations to diagnose anaemia at sea level (WHO 2011, no changes were made).

In our study, we defined haemoglobin levels for males of 13-18 g/L and 12-16 g/L for females.

1.4.2 Signs and Symptoms of Anaemia

As described earlier, a huge number of people suffer from anaemia worldwide, impacting their quality of life and activities of daily life greatly. For all types of anaemias, the symptoms are much alike.

Anaemia is reported to influence mental performance, resulting in higher prevalence of both neurological and psychiatric diseases, weakness, headaches, tinnitus; and shortness of breath (Barragán-Ibañez et al. 2016). Not only restless-leg syndrome was attributed (Goodman et al. 1988), especially in pregnant women, to low levels of iron, but also Pica. Pica, distinct in the devouring of earth and soil (geophagia), appears to be very common in regions of Africa, where anaemia is widespread (Njiru et al. 2011). Low levels during pregnancy can account for delayed cognitive development and lasting issues in cognition and behaviour (Lozoff et al. 1991).

Anaemias can have various origins. The most important ones for this study will be portrayed in the following, only, since an in-depth review of all possible causes is not appropriate for achieving the set goal of this thesis.

1.4.3 Diagnosis, log sTFR and Thomas-Plot

To investigate a possible anaemia, reference haemoglobin levels (e.g. age, sex, gestation, altitude) should be considered combined with the clinical presentation, patient history as well as the serum ferritin, a complete blood cell count, a peripheral smear, and the reticulocyte count.

Feelders et al. (1999), Pasricha et al. (2010) and Johnson-Wimbley & Graham (2010) describe in their comprehensive reviews diagnostic procedures and evaluative markers that must always be seen with regard to the levels of the examining laboratory standards:

- Serum ferritin levels of <15–30 µg/L in adults and 10–12 µg/L in children indicate a high possibility of iron deficient anaemia.
- Mean cell volume and mean cell haemoglobin are normal or lowered (microcytic, hypochromic).
- Transferrin or total iron binding capacity is normal or high
- Transferrin saturation is low to (seldom) normal

- Soluble transferrin receptor is normal or high.
- Serum iron is low.

Soluble transferrin receptor-ferritin index (sTFR/log ferritin)

Oustamanolakis & Koutroubakis (2011) propose the soluble transferrin receptor-ferritin index as a means of characterisation and differentiation between different types of anaemia. According to Lee et al. (2002), it can be used as an additional diagnostic tool. Recent studies however found it to be a very useful parameter and recommend it (Skikne et al. 2011, Enko et al. 2015, Al-Rubaie et al. 2018, Krawiec & Kozuchowska 2019) .Unlike ferritin, the sTFR does not differ between sexes and is not influenced by inflammatory processes (APR) but its limits have not yet been standardised.

$$\mathrm{sTfR}/\log\ \mathrm{ferritin} = \frac{\mathrm{sTfR}\left[\frac{\mathrm{mg}}{\mathrm{L}}\right]}{\log_{10}\mathrm{ferritin}\left[\frac{\mathrm{ng}}{\mathrm{mL}}\right]}$$

Figure 4. Soluble transferrin receptor-ferritin index (sTFR/log ferritin)

Thomas Plot and anaemia

The Thomas-Plot categorises iron deficiency and enables to discriminate ACD and regular iron deficiency (IDA), also in a functional state. This diagnostic tool includes the reticulocyte haemoglobin content (CHr) and the soluble transferrin receptor-ferritin index and considers possible inflammation via the c-reactive protein (CRP).

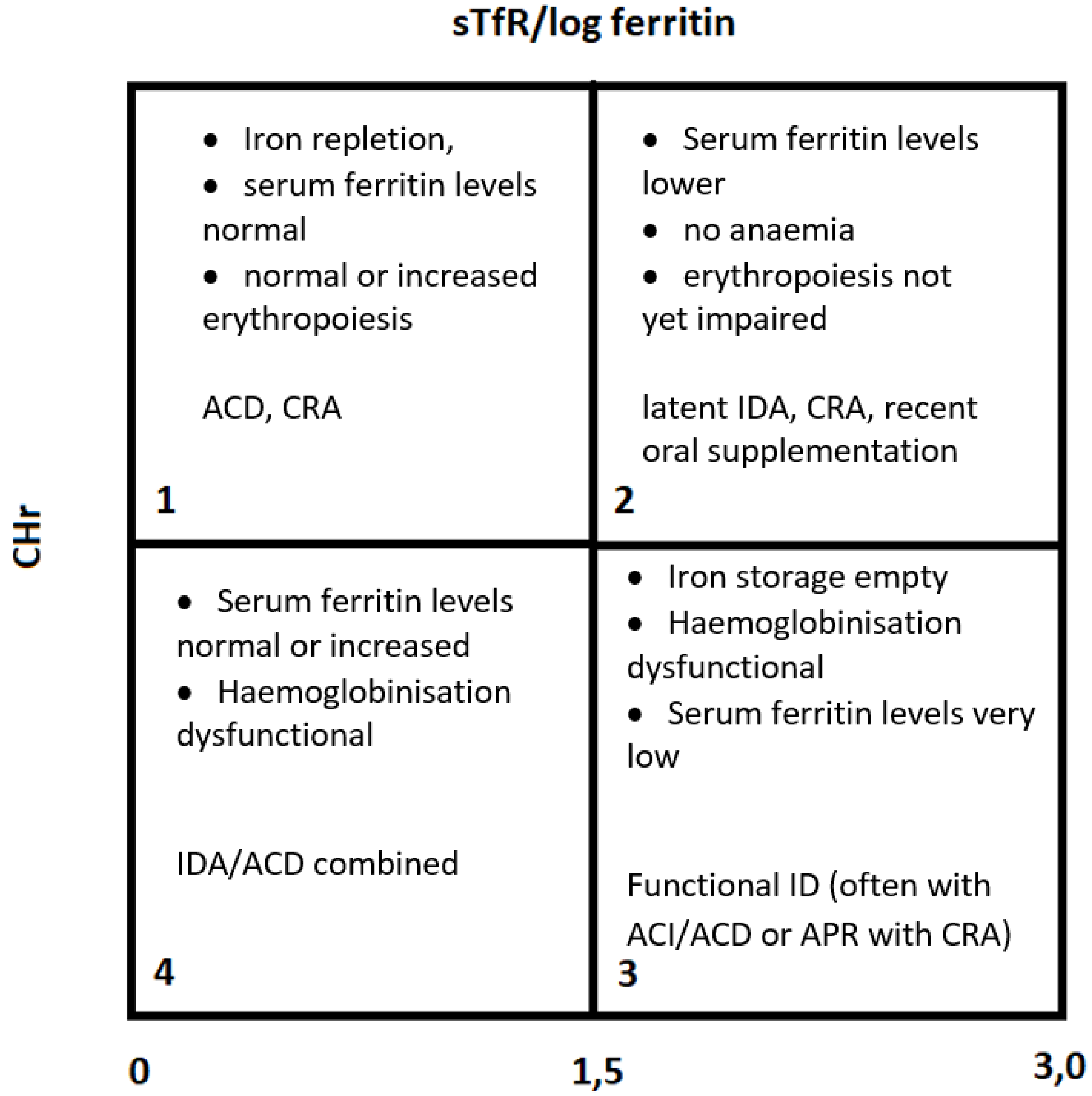

Figure 5. Thomas Plot according to Thomas & Thomas 2002 and Enko et al. 2015.

1.4.4 Management and treatment of Anaemia

Management and treatment of anaemias depend on the form (e.g. acute, chronic) and accompanying diseases and conditions. The underlying cause of the anaemia must be found as quickly as possible as severe diseases can be causal (Jimenez et al. 2015).

The first step is adjusting dietary uptake and finding appropriate diets to suit the iron requirements. Both vegetal or animal-based diets are possible and effective but combinations of dietary measures were found to be best. Vitamin C was found to be useful in studies that emphasised simultaneous intake of iron; possible calcium influence is not determined yet and high fibre diets appear to reduce iron intake. All in all, many dietary studies lack study duration and thorough planning (Patterson et al. 2001, Bach-Kristensen et al. 2005; Blanco-Rojo et al. 2011, Beck et al. 2014).

As intestinal uptake is limited, using this approach should be limited to mild forms of anaemia (11-11,9 g/L Hb in non-pregnant women and 11-12,9 g/L in men). Although higher doses seem to yield better outcomes, starting with 15-30 mg of elemental iron can already make a difference in terms of replenishing; it could however be increased to 100-200 mg. This oral approach is limited by tolerability and many patients experience side effects. Haemoglobin levels should rise by 2 g/dL within four to eight weeks, nevertheless, ferritin can take months to normalise (Rimon et al. 2005, Jimenez et al. 2015, Tolkien et al. 2015).

If this approach appears insufficient, i.v. injections of iron or even blood transfusions as a last resort should be considered. Dependent on the haemoglobin level, a dose adjusted to the body weight and sex is administered. Sometimes test doses are prudent to exercise and rule out severe side effects. In general, i.v. application leads to higher and faster repletion and lasting results (Lindgren et al. 2005; Kulnigg et al. 2008; Onken et al. 2014).

1.4.5 Iron deficiency anaemia (IDA)

Iron deficiency anaemia (IDA) is estimated to afflict up to 5% of the humans in the developed world and a common reason for hospital investigation and treatment (Calvey & Castleden 1987, Sayer & Long 1993), while others propose up to thirty percent of all people suffer from undiagnosed and under-managed forms of anaemia, especially IDA (Gasche et al. 2004, López-Sierra 2011, Kassebaum et al. 2014). It is one of the most prevalent forms of malnutrition, impacting both maternal and perinatal mortality as investigated in the Global Burden of Disease (GBD) project (Stoltzfus 2014).

IDA is suggested to influenced heavily by the socioeconomic status of a person, with lower status being associated with higher risk and prevalence, possible due to the economic influence on nutrition and accession to healthcare facilities as well as presumed lesser knowledge of healthy dishes (Artigas 1997).

Especially younger children and infants with an IDA are prone to psychiatric and behavioural disorders and a higher risk of infection in general (Bourre 2006a, Lozoff 2007, Walker 2007). Pregnant women may give birth to smaller children (low birth weight) and too early (pre-term) and their new-borns are at higher risk of sudden death. Those children are at elevated risk of suffering from iron deficiency, as well as adolescent girls and women in their premenopausal years, the so-called reproductive age (WRA) (Rasmussen 2001, Bourre 2006b, Scott 2011, Haider 2013).

Women mostly suffer of anaemia due to menstruation blood loss, while all other groups of patients have the highest risk of IDA because of gastrointestinal bleedings; other probable causes include malabsorption (often due to coeliac disease), colon and gastric carcinoma, gastrectomy, NSAID use and insufficient dietary uptake (Rockey & Cello 1993, Kepczyk & Kadakia 1995). Per every 2,5 millilitres of whole blood lost, up to 1 mg of iron is lost, this shows the crucial importance of looking for possible haemorrhage (Miller 2013).

Furthermore, the inflammatory bowel disease may be of a greater importance for the development of an iron deficiency anaemia as previously considered as numbers of patients with an inflammatory bowel disease (IBD) continue to rise, with some arguing to view IDA as a primarily gastrointestinal disease.

The abundance of reasons/causes of iron deficiency anaemia (IDA) is portrayed in fig. 6:

I. Inadequate iron supply

- A. Poor nutritional intake in children
- B. Malabsorption (also surgical/medical)
  - Celiac disease
  - Crohn's disease
  - Gastric bypass surgery/gastrectomy
  - Other miscellaneous disorders affecting the stomach and duodenum
  - Folate deficiency
  - B12 deficiency
- C. Abnormal transferrin function
  - Congenital atransferrinemia
  - Autoantibodies to transferrin receptor
  - Transferrin polymorphisms

II. Increased iron requirements

- A. Blood loss
  - A.1. Gastrointestinal Bleeding
    - Malignant neoplasms
    - Hookworm
    - Malaria
    - Peptic ulcer disease
    - Oesophageal varices
    - Hiatal hernia
    - Ulcerative colitis
    - Haemorrhoids
    - Gastritis
    - Angiodysplasia
    - Helicobacter pylori infection
    - Haemangioma
    - Amebiasis
    - Diverticulum
    - Polyps
    - Leiomyoma
    - Milk allergy in infants
    - Schistosomiasis
    - Hypergastrinemia
    - Trichuriasis
  - A.2. Excessive Menstrual Losses or Abnormal Uterine Bleeding
    - Menstrual bleeding
    - Uterine fibroids
    - Malignant neoplasms
  - A.3. Respiratory tract bleeding
    - Malignant neoplasms
    - Epistaxis
    - Infections
    - Telangiectasias
    - Idiopathic pulmonary hemosiderosis
    - Goodpasture's syndrome
  - A.4. Biliary Tract Bleeding
    - Malignant neoplasms
    - Cholelithiasis
    - Intrahepatic bleeding
    - Trauma
  - A.5. Urinary Tract Bleeding
    - Malignant neoplasms
    - Trauma
    - Urolithiasis
    - Inflammatory disorders
    - Glomerulonephritis
  - A.6. Blood Donation
  - A.7. Self-induced Bleeding (factitious anaemia)
  - A.8. Intravascular Haemolysis
    - Paroxysmal nocturnal haemoglobinuria
    - Mechanical erythrocyte trauma (as in valvular heart disease)
    - High-performance athletic activities
    - Other haemolytic disorders
- B. Chronic Renal Failure and Haemodialysis
- C. Disorders of Haemostasis
- D. Haematological Disorders
  - esp. sickle cell disorders and thalassaemia
- E. Increased Physiologic Requirements
  - Infancy
  - Pregnancy
  - Lactation
- F. Defective iron absorption
  - Aspirin
  - NSAID
  - H_2 antagonists
  - PPI
- G. Idiopathic

Figure 6. Possible reasons and causes leading to anaemia. After Lee 1998, Beutler 2006, Clark 2009, Chaparro & Suchdev 2019.

Apparently, not all gastroenterologists see the importance of treating IDA in the event that it is caused by a gastrointestinal disease. It is clear, however, that gastric and intestinal blood loss cannot be countered by intestinal re-absorption of the iron in the blood lost (Gasche et al. 2004).

1.4.6 Anaemia of chronic disease (ACD)

As mentioned earlier, hypoferraemia appears to be a way of defence against infection and infestation, as all organisms need iron for growth and survival. ACD is closely related to hepcidin, as it reduces the amount of iron released and thus the iron metabolism itself. IL-6 and other markers of inflammation trigger hepcidin excretion, reducing serum iron and transferrin (Nemeth et al. 2004).

After the iron deficiency anaemia, the anaemia of inflammation or chronic disease is the second most common form of anaemia, although exact prevalence rates can only be estimated (Dallman et al. 1984; Theurl et al. 2006). Even though it was fist described in the 1950s (Cartwright & Wintrobe 1952), the latest developments and insights gained about hepcidin brought new stimuli to this condition and understanding to its molecular pathways (Nemeth et al. 2003). These entities can be caused by a number of diseases such as infections, inflammatory diseases and tumours (Cartwright 1966); according to Weiss & Goodnough (2005), the prevalence ranges from

- 18-95% for infections both acute and chronic,
- 30-77% for cancer (solid and haematologic),
- 8-71% for autoimmune disorders,
- 8-70% in patients with GvHD (solid-organ-transplantation),
- 23-50% for chronic kidney diseases (CKD).

Several other disorders, such as autoimmune diseases (especially Rheumatoid Arthritis, Lupus erythematosus, Vasculitis, Sarcoidosis and IBD) and chronic heart failure are mentioned alongside the enumeration of Weiss & Goodnough (Cullis 2011).

Mostly patients, who are undergoing treatment or care in hospitals, suffer from ACD, possibly because their primary health condition requires this in the first place; it is less probable for anaemia of chronic disease to occur as the main disease entity (Means 2003; Culleton et al. 2006), a phenomenon also noticed in the risk for mortality and hospitalisation, which is five times higher than average in these groups (Riva et al. 2009).

This is further indicated as it is incidence and prevalence rise along with the age of the patients; men and women at and over the age of 85 years suffer from this condition to 26,1% and 20,1% respectively (Guralnik 2004). Of this group, roughly a third suffer from nutrient-deficiency anaemia, another third of unexplained anaemia and the smaller part of ACD (Woodmann 2005). Stadtman (2001) lists oxidative stress as crucial factor in genesis of this condition, along with GI-bleeding, renal insufficiency, sex hormone deficiency, bone marrow and metabolic diseases (Poggiali 2014). Oxidative stress and subthreshold inflammation processes are also discussed as causes or at least contributors to anaemia of ageing and anaemia of cardiac failure (Opasich et al. 2005, Ferruci & Balducci 2008). Apparently, they have an impact on the iron homeostasis to a greater extent than initially expected.

With respect to levels of haemoglobin, ACD ranges from 8-9,5 g/L and is characterised as a normochromic, normocytic anaemia with lower iron, transferrin levels low-normal and ferritin levels normal or increased and, normally, mild to moderate effects. Additionally, a low reticulocyte count can be found as well as lack of iron as it is consumed by the RES, eventually causing a reduced transferrin saturation (Weiss & Goodnough 2005; Cullis 2011; Poggiali 2014).

Nevertheless, as both IDA and ACD often appear together, there are fine differences to be found – most of times, transferrin is elevated in IDA while it is normal or lower in ACD. Ferritin as an acute phase protein is expected to be heightened in case of inflammation processes. Therefore it is an important information in chronic inflammatory diseases; the gold standard of a Perl’s stained bone marrow aspirate is not commonly performed as it is of little use in diagnosing an ACD (Koulaouzidis et al. 2009, Cullis 2011; Bableshawr 2013). Yet still, bone marrow aspirates seem to be inaccurate in about 30% of the cases and even when performed correctly (which is highly dependent on the operator), sometimes yield wrong results (Barron et al. 2001, Koulaouzidis et al. 2009).

The use of the serum transferrin receptor (sTFR) as a tool for measurement anaemias was proposed – it is part of the membrane receptor that is found on all cells in the body but in an increased number on erythroid progenitor cells, whereas in ACD they are not increased – but in the clinical setting this was discarded as too challenging and therefore was not standardised (Cook et al 1993; Mast et al. 1998; Fitzsimons 2002). Nonetheless, the ratio of the sTFR to the log of the serum ferritin was suggested to allow differentiation between ACD and IDA – is it < 1, anaemia of chronic disease is probable, while a ratio > 2 indicates low iron stores regardless of ACD (Malope 2001; Skikne 2008).

1.4.7 Other forms and causes of anaemia

Apart from IDA, other nutrition-based types of anaemia include a lack of vitamin A and B12, D, E as well as folate, riboflavin, copper and zinc (Wieringa et al. 2016). In most cases, these elements and vitamins serve as haematopoietic basis; shortage leads to defects in development and maturation of blood cells, while elements such as copper and zinc are needed in the core structure of enzymes of the iron metabolism, like ceruloplasmin (Hacibekiroglu 2015). Olivares (2007) suggests that the reduction of copper could also cause a diminished production of erythropoietin (EPO) and lower count of enzymes that reduce reactive oxygen species (ROS), thus decreasing the life span of red blood cells, ultimately inducing anaemia. Dahgman (1999) found that hypoxia and ROS modulate EPO gene expression, further influencing the disease and its progression.

Vitamin-A-deficiency (VAD)

The effect of Vitamin A on anaemia was established through animal and human studies. It appears that Vitamin A is involved in the erythropoiesis through retinoids (influencing e.g. apoptosis and erythropoietin), anaemia of infection and the iron metabolism itself (Semba & Bloem 2002). Low vitamin A levels also reduce the iron binding capacity and transferrin saturation (Bloem et al. 1988). A characteristic of VAD is that the iron storage in the liver, spleen as well as serum ferritin are elevated (Michelazzo et al. 2013). Less clarity can be expected from the blood smear, as it is open for debate if cells are hypochromic and/or hypochromic and microcytic (Semba & Bloem 2002). As already mentioned, VAD also influences infections, hence diagnosis is more difficult in patients with chronic inflammation or even ACD (Larson et al. 2017).

Vitamin-B-deficiencies

These vitamins have an important role in the iron metabolism and composition of haemoglobin; they include B2 - riboflavin, B6 - pyridoxine, B12 - cobalamin and folate (Chaparro & Suchdev 2019). Insufficient supply of these vitamins can induce macrocytic anaemia, with erythrocytes being larger, but carrying less haemoglobin. This can be verified by a peripheral blood smear, showing macroovalocytes and hypersegmented neutrophils although they can also appear in other forms of anaemia (Green & Dwyre 2015). Cobalamin deficiency is sometimes caused by gastric atrophy; but also the other forms of vitamin-B-deficiencies often derive from malabsorption (Chaparro & Suchdev 2019).

As mentioned earlier in table 7, several infestations, e.g. by worms and protozoa, can cause significant blood loss and anaemia, but also other aetiological factors for anaemia are known:

1) Schistosomiasis: Currently, 5 different varieties of the *Schistosoma* parasite are known, and most of them affect sub-Saharan Africa with up to a quarter milliard of individuals infected. Especially women suffer from these parasites and are proposed to be the most frequent cause of anaemia among them (Kassebaum et al. 2014). A high estimated number of unknown cases is to be expected. Apart from blood loss through infection, the exact biochemical iron-metabolism relationship between Schistosoma and humans is unknown (Friedman et al. 2005). In children, they also cause fatigue, weakness and impair cognitive function (Nelson 1996) and they are suggested to trigger anaemia of infection (Butler et al. 2012).

2) Malaria: *Plasmodium falciparum, P. tropicana,* and *P. vivax*, the most common species in terms of harmfulness for humans, can cause serious disruption to the human iron metabolism as the protozoon needs iron for growth and development. Most of all cases and fatalities (of these mostly children < 5 years) due to malaria can be found in Africa, being a burden to the development (WHO 2017). Malaria contributes to anaemia in several ways, e.g. through destruction of infected as well as non-infected red blood cells and quickening the RBC degradation and shortening their lifespan; blood loss does not occur (Skorokhod et al. 2011).

3) Hookworm: Those helminths (*Necator* (!) *americanus* and *Ancylostoma duodenale*) cling to the intestinal mucosa and suck blood. According to Smith and Brooker (2010), each hookworm needs 0,3-0,5 mL of blood per day. Depending on the density of individuals, hookworms therefore can cause severe anaemia, particular in otherwise sick persons. Hookworms are predominant in sub-Saharan Africa and southeast Asia, with over 700 million people estimated to be affected (Bungiro & Cappello 2011, CDC 2013).

4) Tuberculosis: Mostly due to haemoptysis and malnutrition because of reduced appetite, Tb is a relevant factor. As much as up to 82% of patients suffering from tuberculosis also are anaemic (Minchella et al. 2013). Especially with Tb, cross-infections are common and elevate the overall risk of this disease and poor outcome in patients (Papathakis & Piwoz 2008). According to Gil-Santana et al. (2019), Tb-anaemia shows a unique biochemical pattern of infection, that is closed linked to markers of inflammation.

5) HIV: Up to 70% of patients suffer from anaemia in course of their disease (Lim & Levine 2006). According to Redig and Berliner (2013), alterations in the blood cell count are frequent in patients with HIV or AIDS, often they portray a normochromic, normocytic anaemia with low reticulocytes and erythropoietin but regular iron storage. They also claim that the HIV causes a persistent acute phase reaction, increased hepcidin and that it is a marker for the progress of the HIV/AIDS. Similar to the progress of the anaemia itself, hepcidin is also in connection with higher mortality (Mocroft et al. 1999, Belperio & Rhew 2005).

6) Genetic diseases: Several genetic disorders, such as the sickle cell disorders or forms of thalassaemias affect the iron metabolism. Modell and Darlison (2008) estimate that about 330.000 children are born every year and suffer from an inherited haemoglobin disorder; they claim that 83% inherit a form of sickle cell anaemia, the others some types of thalassaemia. Most of those children are born in the so-called low- and middle-income countries (LMICs) (Weatherall 2008). Both sickle cell disorders and thalassaemias result in chronic haemolytic anaemia and disturbed erythropoiesis (Ansong et al. 2013; Muncie & Campbell 2009).

Infections with different parasites at the same time are possible and have an additive worsening effect, frequently leading to anaemia (Brooker et al. 2007, Ezeamama et al. 2008). They are responsible for poor outcome and critical health conditions especially in low- and middle-income countries, which sometimes do not allow the same utilisation of public healthcare facilities as other countries.

1.4.8. Forms of iron overload

As the body has no excretion pathway for iron, keeping the iron balance is crucial. Apart from acute iron poisoning, iron overload in a chronic form often derives from genetic and inherited diseases. These include

- Hereditary haemochromatosis, HFE and non HFE- haemochromatosis are by far the most important conditions in regards to iron overload as primary diseases;
- additionally, blood transfusions can cause transfusion overload,
- thalassaemias,
- haemolytic and sideroblastic anaemia
- dietary or parenteral iron overload
- as well as liver diseases such as ARLD (alcohol related liver disease), HAV (hepatis A), HBV (hepatitis B), non-alcoholic steatohepatitis (NASH)

which can lead to iron overload (Kohogo et al. 2008; Wood et al. 2009; Fleming & Ponka 2012).

Symptoms and consequences include cirrhosis and hepatocellular necrosis (HN) and cancer (HCC) due to inflammation, endocarditis, cardiac arrythmia, cardiac hypertrophy and cardiomyopathy (cardiac failure), endocrine dysfunction due to iron deposits (early or late puberty, sexual immaturity, thyroid dysfunction), pancreatic toxicity (glucose intolerance and diabetes); clearly, iron overload has a high burden of disease (Zurlo 1989; Adam et al 1997; Bacon et al. 1999; Olivieri 1999, Fung et al. 2006).

Management of genetic forms and acquired ones are similar - normal ferritin levels are aspirated - consisting of dietary adjustment (alcohol, iron uptake, vitamin C), iron chelation therapy, venesection (4-500ml) and liver transplantation. In cases of necessary blood donations as therapy (e.g. for thalassaemia), the transfusion is combined with chelation agents (Barton et. Al 1998; Kohogo et al. 2008; Al Qasem et al. 2018).

2. Hypothesis

Hypothesis: A possible link between tryptophan of the human metabolism and iron homeostasis exists.

Ho: The tryptophan serum level and tryptophan break-down index differ in patients with different forms of anaemia (ACD, IDA, healthy).

H1: The tryptophan serum level and tryptophan break-down index in patients suffering from different forms of anaemia (ACD, IDA, healthy) is the same.

To evaluate whether this suggested link can be proven, the following points were investigated:

1) Establishing a working-definition of relevant parameters for anaemia like ferritin and haemoglobin and tryptophan metabolism such as tryptophan, kynurenine and kynurenic acid

2) Measuring of serum levels of the above-mentioned parameters in a cohort of patients

3) Comparison of medians of the performed laboratory parameters

4) Evaluation of a possible link between different forms of anaemia, markers of anaemia like ferritin and haemoglobin and tryptophan metabolism such as tryptophan, kynurenine, and kynurenic acid

3. Material & Methods

The following data and information were already published in *Sci Rep* 9, 14548 (Wenninger et al. 2019) as part of my dissertation and are reproduced here accordingly and partially identically.

3.1 Study design and patients

In our cross-sectional study, a total of four hundred-thirty consecutive patients, who were admitted by general practitioners and specialists to the outpatient clinic for a medical check-up of their actual iron status, were included. All participants provided their written informed consent beforehand. They underwent venous blood sampling in the morning between 8.00 and 10.00 a.m. as described in the following after an overnight fasting state.

The samples were used to evaluate the iron metabolism (i.e., haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, ferritin, transferrin saturation, serum iron, transferrin, soluble transferrin receptor, reticulocyte haemoglobin), the tryptophan metabolism (tryptophan, kynurenine, kynurenic acid, kynurenine/tryptophan ratio, kynurenic acid/kynurenine index), the renal function (creatinine, estimated glomerular filtration rate [eGFR]), and the C-reactive protein.

The inclusion criteria for this study were defined as

- a minimum age of 15 years,
- referral to our outpatient clinic for assessment of the actual iron status and
- performance of laboratory analyses within 4 hours after blood sampling.

The exclusion criteria for this study were

- Patients with oral or intravenous iron supplementation within the last year before the beginning of the study,
- Patients with unavailability of all necessary study parameters were excluded from the study and

- Patients below the age of 15.

Approval for performing this study was obtained from the Ethical Committee of the Johannes Kepler University Linz (Linz, Austria). The study was carried out according to the latest version of the Declaration of Helsinki, ethical principles of medical research involving human subjects.

3.2 Blood collection and laboratory procedures

Here we describe the exact sample procession for means of reproduction and replicability. From all of our 430 study participants, one 2 mL VACUETTE® EDTA tube was collected for

- haemoglobin,
- mean corpuscular volume,
- mean corpuscular haemoglobin, and
- reticulocyte haemoglobin measurements.

One 4 mL VACUETTE® LH lithium tube for

- ferritin, transferrin,
- iron,
- soluble transferrin receptor, and
- C-reactive protein analyses.

One 4 mL VACUETTE® Z Serum Sep Clot Activator tube (all tubes were from Greiner Bio-one International GmbH) for

- tryptophan,
- kynurenine, and
- kynurenic acid measurements.

Plasma and serum samples were centrifuged at 2000 × g for 10 minutes at room temperature.

The determination of haemoglobin (anaemia: men: <13 g/dL; women: <12 g/dL), mean corpuscular volume (reference range: 80–99 fL), mean corpuscular haemoglobin (reference range: 27–36 pg) and reticulocyte haemoglobin (reference range: 28–35 pg) was performed on an ADVIA® 2120i Haematology Analyzer (Siemens, Vienna, Austria).

Ferritin (iron deficiency: <15 µg/L) was measured by chemiluminescent technology, serum iron (reference range: 65–175 µg/dL) by a bi-chromatic photometry method, transferrin (reference range: 2–3.6 g/L), soluble transferrin receptor (reference range: 0.76–1.76 mg/L) and C-reactive protein (0–3 mg/L) by nephelometry on a Dimension Vista® 1500 System (Siemens, Vienna, Austria).

Iron deficiency was defined as a plasma ferritin level <15 µg/L and/or a transferrin saturation <20% (Muñoz et al. 2009, WHO 2011, Enko et al. 2015).

The biomarkers of tryptophan metabolism (i.e., tryptophan, kynurenine and kynurenic acid) were determined by high-performance liquid chromatography with a simultaneous ultraviolet and fluorimetric detection system (Hervé et al. 1996).

In brief, 100 µL plasma sample was deproteinized by adding 100 µL of 5% (v/v) perchloric acid. After vortexing and 5 min centrifugation at 11,000 × g, 20 µL of the clear supernatant was injected into the chromatographic system.

Separations were achieved on a Chromolith RP18e column (100 × 4.6 mm, 5 µm, Merck Darmstadt, Germany) at 30 °C by isocratic elution with a mobile phase (pH 4.9), which consisted of 50 mmol/L ammonium acetate, 250 mol/L zinc acetate and 3% (v/v) acetonitrile, at a flow-rate of 0.8 mL/min.

Kynurenine and tryptophan were detected on an Agilent 1200 VWD detector (Agilent, Palo Alto, CA, U.S.A.) at 235 nm, kynurenic acid was detected fluorometrically on an Agilent 1260 FLD detector.

The acquisition and processing of the chromatograms were performed using an Agilent 1200 system equipped with a Chemstation software (Agilent, Palo Alto, CA, U.S.A.). All reagents were p.A. grade from Merck (Darmstadt, Germany).

The intra-assay coefficients of variations (CVs) for different concentrations varied between 0.7–2.9% for tryptophan, 1.7–4.3% for kynurenine, and 2.6–4.5% for kynurenic acid.

The inter-assay CVs ranged between 6.3–9%, 2.0 5.4%, and 8.4–11.6% for tryptophan, kynurenine, and kynurenic acid, respectively.

The reference ranges of the tryptophan metabolites and the kynurenine/tryptophan ratio and neuroprotective kynurenic acid/kynurenine index were calculated according to the literature (Myint et al. 2007, Myint et al. 2009, Midttun et al. 2009).

Creatinine (males: 0.7–1.3 mg/dL; females: 0.55–1.02 mg/dL) was measured using an enzymatic method applied on a Roche Cobas Mira (Roche Diagnostics, Vienna, Austria).

The eGFR (normal: >70 mL/min/1.73 m2) was calculated applying the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (Levey et al. 2009).

3.3 Statistics

All parameters were recorded in a descriptive statistical manner, tabulated and evaluated. Not normally distributed continuous variables were presented as medians with interquartile ranges (Q1–Q3). Categorical variables were expressed as percentages.

For the comparison between two groups for not normally distributed continuous variables (verification with the Kolmogorov-Smirnov test with Lilliefors correction at a type-I error-rate of 10%) the Mann-Whitney-U test was used.

For the comparison between three or more groups for not normally distributed continuous variables (verification with the Kolmogorov-Smirnov test with Lilliefors correction at a type-I error-rate of 10%) the Kruskal-Wallis-test was performed.

To assess potential correlation between two continuous variables the Spearman's rank correlation coefficient (not normally distributed data) was used. Furthermore, regression models (linear, log-linear, quadratic, cubic, exponential, fractional polynomial, cubic spline) were performed to assess the association between variables.

The type I error was set to 5% (two-sided) without adjustment for multiple testing, so all p-values are only descriptive.

The percentage of transferrin saturation was calculated based on the formula: transferrin saturation (%) = serum iron (µg/dL) × 70.9/transferrin (mg/dL). The differential diagnosis of IDA and ACD was based on Thomas-Plot analyses as described previously (Thomas & Thomas 2002; Enko et al. 2013; Enko et al. 2015).

In brief: for the discrimination of the IDA and ACD the cut-off for the soluble transferrin receptor/log ferritin ratio depended on the acute-phase reaction and was 1.5 for a C-reactive protein ≤5 mg/L and 0.8 for a C-reactive protein >5 mg/L, respectively.

For statistical analysis, the statistical computing software R Version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org) including the standard package splines respectively the optional package mfp (version 1.5.2) and the Analyse-it® software version 4.92 (Analyse-it Software, Ltd., Leeds, United Kingdom) were used.

4. Results

4.1 Study population characteristics

We set a ferritin <15 µg/L and/or a TSAT <20% as a cut-off, so 115/430 (26.7%) individuals (97 females and 18 males) were found with iron deficiency. All in all, 17/430 (4%) patients (15 females and 2 males) had anaemia. Of these persons, 11 (64.7%) individuals were identified with IDA and 6 (35.3%) patients (5 females and 1 male) with ACD, respectively.

The baseline characteristics of the study population are presented in Table 4. Of all 430 study participants, 290 (67.4%) were female, and 140 (32.6%) were male. The median age was 39 (range: 15 – 82) years.

Study population (n = 430)	*Min*	*Median*	*Max*	*Mean*	*SD**	*Reference ranges*
Age (y)	15	39	82	40.6	15.5	-
Height (cm)	139	169	198	170	9.4	-
Weight (kg)	20	70.5	149	72.9	17.1	-
Body mass index (kg/m^2)	7.3	24.2	49.1	25.1	5.1	18.5 – 25.0

Table 4a. Baseline characteristics of the study population (Wenninger et al. 2019).

4.2 Correlations between indicators of tryptophan and iron metabolism

The indicators of tryptophan metabolism correlated with iron metabolism. Serum tryptophan levels were positively correlated with levels of haemoglobin ($p < 0.001$, $r = 0.305$) and ferritin ($p = 0.038$, $r = 0.100$). Kynurenine and kynurenic acid showed positive correlations with haemoglobin (p-values <0.001, r = 0.176 and 0.296), soluble transferrin receptor (p = 0.007 and 0.038, r = 0.131 and 0.100), and ferritin (p-values < 0.001, r = 0.186 and 0.301), respectively.

Study population (n = 430)	*Min*	*Median*	*Max*	*Mean*	*SD**	*Reference ranges*
Mean corpuscular volume (fL)	66.9	86.2	98.1	86	4.3	80 – 99
Mean corpuscular haemoglobin (pg)	19.9	29.8	35.9	29.7	1.8	27 – 36
Ferritin (µg/L)	1	58	401	80.2	27 - 105	≥ 15
Transferrin saturation (%)	2	27	87	28.1	12.2	≥ 20
Iron (µg/dL)	6	101	264	103.5	40.2	65 – 175
Transferrin (g/L)	1.8	2.6	4.6	2.7	0.4	2 – 3,6
Soluble transferrin receptor (mg/L)	0.6	1.1	4.6	1.2	0.4	0.76 – 1.76
Reticulocyte haemoglobin (pg)	21.1	31.3	35.1	31.1	1.8	28 – 35
Tryptophan (µmol/L)	30.5	60.1	96.5	60.5	10.1	43 – 89
Kynurenine (µmol/L)	1.1	2.4	4.3	2.4	0.5	1.0 – 2.9
Kynurenic acid (nmol/L)	8.2	31.1	85.3	33.2	13.2	20 – 93
Kynurenine/tryptophan	0.02	0.04	0.08	0.05	0.01	-
Kynurenic acid/kynurenine	4	13.1	31.1	13.9	4.7	-
Creatinine (mg/dL)	0.3	0.7	1.2	0.7	0.1	Males: 0.7 – 1.3 Females: 0.55 – 1.02
eGFR (mL/min/1.73 m^2)	60.6	105.4	160	105.5	17.8	> 70
C-reactive protein (mg/L)	0	0	33	1	3.4	0 – 3

Table 4b. Baseline characteristics of the study population (cont.) (Wenninger et al. 2019).

Kynurenine/tryptophan = tryptophan break-down index; kynurenic acid/kynurenine = neuroprotective ratio; eGFR, estimated glomerular filtration rate; min, minimum; max, maximum; SD, standard deviation. *In case of a skewed data distribution the 25th and 75th centiles are presented instead of SD.

4.3 Assessment of the associations between soluble transferrin receptor and indicators of tryptophan metabolism

The univariate log-transformed linear regression model for the log-transformed soluble transferrin receptor is presented in Table 5. The tryptophan metabolism indicators kynurenine and kynurenine/tryptophan displayed a statistically relevant influence on soluble transferrin receptor.

LOG Soluble transferrin receptor (mg/L)	*ß-coefficient*	*p-value*	*95% CI*	*Percentage change*
LOG Tryptophan (µmol/L)	0.007	0.882	-0.088 – 0.102	0.11%
LOG Kynurenine (µmol/L)	0.174	<0.001	0.081 – 0.268	2.02%
LOG Kynurenic acid (nmol/L)	0.068	0.157	-0.026 – 0.163	0.44%
LOG Kynurenine/tryptophan	0.153	0.001	0.059 – 0.247	1.61%
LOG Kynurenic acid/kynurenine	-0.034	0.482	-0.129 – 0.061	-0.25%

Table 5. Univariate log-transformed linear regression model for the log-transformed soluble transferrin receptor. The column “percentage change“ portrays the increase/decrease of soluble transferrin receptor under the assumption of a 10% increase of the independent variables. CI, confidence interval (Wenninger et al. 2019).

LOG Ferritin (µg/L)	*ß-coefficient*	*p-value*	*95% CI*	*Percentage change*
Model 1: crude				
LOG Tryptophan (µmol/L)	0.105	0.029	0.011 – 0.200	6.45%
LOG Kynurenine (µmol/L)	0.144	0.003	0.050 – 0.238	6.66%
LOG Kynurenic acid (nmol/L)	0.272	<0.001	0.180 – 0.363	7.05%
LOG Kynurenine/tryptophan	0.059	0.221	-0.036 – 0.154	2.43%
LOG Kynurenic acid/kynurenine	0.221	<0.001	0.128 – 0.313	6.60%
LOG Ferritin (µg/L)	**ß-coefficient**	**p-value**	**95% CI**	**Percentage change**
Model 2: adjusted for C-reactive protein				
LOG Tryptophan (µmol/L)	0.110	0.026	0.013 – 0.207	6.76%
LOG Kynurenine (µmol/L)	0.147	0.003	0.052 – 0.242	6.77%
LOG Kynurenic acid (nmol/L)	0.274	<0.001	0.183 – 0.366	7.12%
LOG Kynurenine/tryptophan	0.063	0.210	-0.035 – 0.161	2.58%
LOG Kynurenic acid/kynurenine	0.230	<0.001	0.136 – 0.325	6.91%

Table 6. Univariate log-transformed linear regression models for log-transformed ferritin estimating crude effects (model 1) and adjusted for inflammation (C-reactive protein, model 2). The column “percentage change” shows the increase of ferritin under the assumption of a 10% increase of the independent variables. CI, confidence interval (Wenninger et al. 2019).

4.4 Assessment of the associations between ferritin and indicators of tryptophan metabolism

The univariate log-transformed linear regression model for log-transformed ferritin estimating crude effects (model 1) and adjusted for the C-reactive protein (model 2) is demonstrated in Table 7. The tryptophan metabolism indicators kynurenic acid and kynurenic acid/kynurenine showed a statistically relevant influence on ferritin. Adjusted for the C-reactive protein, the association between ferritin and the tryptophan markers remained similar.

LOG Haemoglobin (g/dL)	*ß-coefficient*	*p-value*	*95% CI*	*Percentage change*
LOG Tryptophan (µmol/L)	0.305	<0.001	0.214 – 0.395	1.59%
LOG Kynurenine (µmol/L)	0.107	0.027	0.012 – 0.201	0.42%
LOG Kynurenic acid (nmol/L)	0.261	<0.001	0.170 – 0.353	0.57%
LOG Kynurenine/tryptophan	-0.111	0.021	-0.206 – -0.017	-0.39%
LOG Kynurenic acid/kynurenine	0.233	<0.001	0.141 – 0.325	0.59%

Table 7. Univariate log-transformed linear regression model for log-transformed haemoglobin. The column “percentage change“ displays the increase/decrease of haemoglobin under the assumption of a 10% increase of the independent variables. CI, confidence interval (Wenninger et al. 2019).

4.5 Tryptophan metabolism measurements in individuals with iron deficiency

In total, 115 patients with iron deficiency had different tryptophan levels (57.8 [52.7 – 63.8] vs. 61.0 [54.6 – 66.5] µmol/L, $p = 0.005$) and kynurenic acid serum levels (27.9 [20.4 – 35.3] vs. 32.5 [24.7 – 42.4] µmol/L, $p < 0.001$), and also different kynurenic acid/kynurenine index levels (12.2 [9.2 – 15.4] vs. 13.5 [11.1 – 17.4], $p = < 0.001$) compared to 315 individuals without iron deficiency, respectively.

The box-and-whisker plots of tryptophan, kynurenic acid and kynurenic acid/kynurenine for patients with and without iron deficiency are illustrated in Fig. 7 and 8.

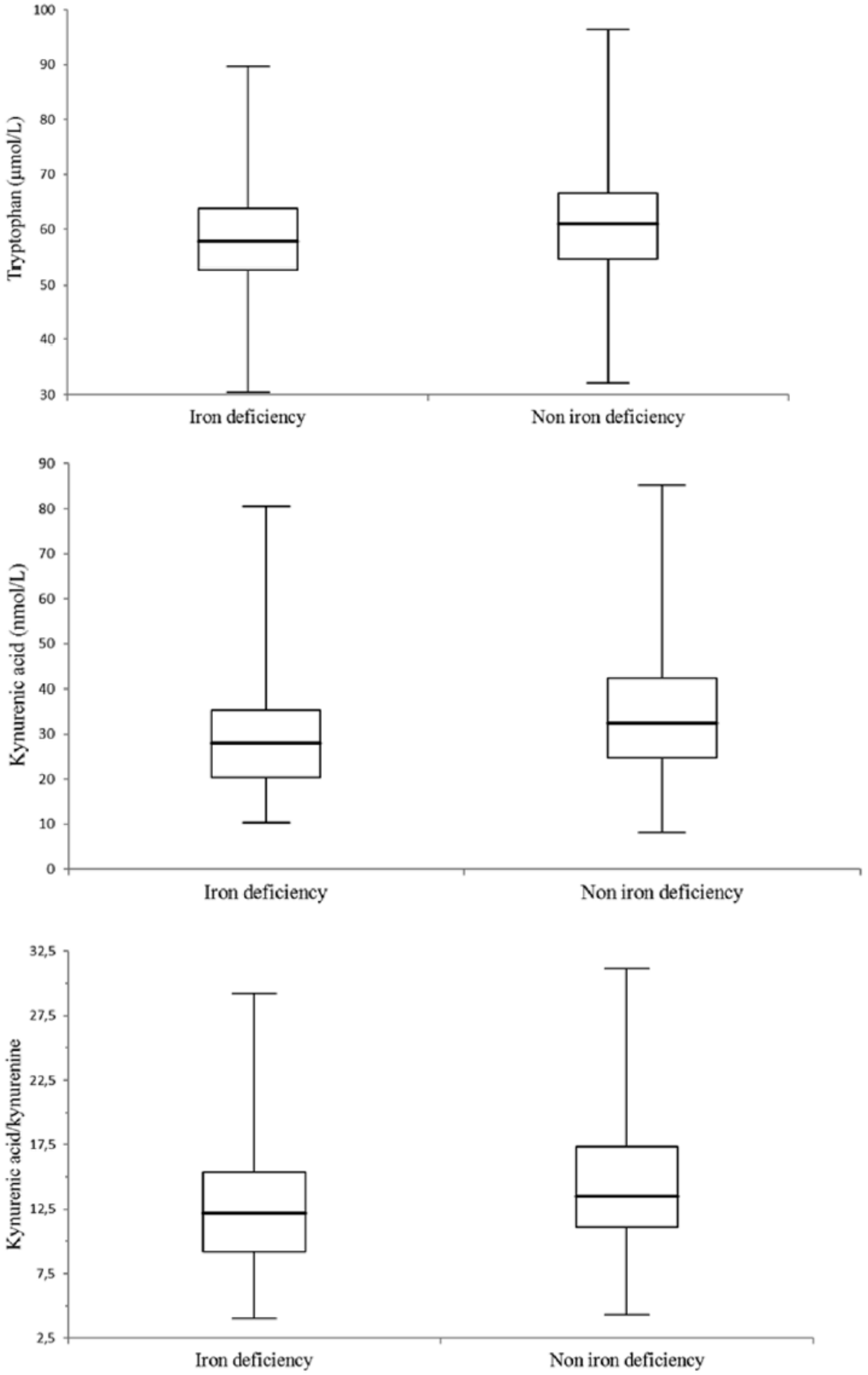

Figure 7. Tryptophan metabolism and iron deficiency. Tryptophan, kynuric acid, and neuroprotective ratio (kynurenic acid/kynurenine) comparisons between 115 and 315 individuals with and without iron deficiency (p-values were 0.005 for tryptophan and <0.001 for kynurenic acid and kynurenic acid/kynurenine). The central boxes represent the 25th to 75th percentile range.

The lines inside the boxes show the median value for each group. Minimum and maximum are indicated as whiskers with end caps (Wenninger et al. 2019).

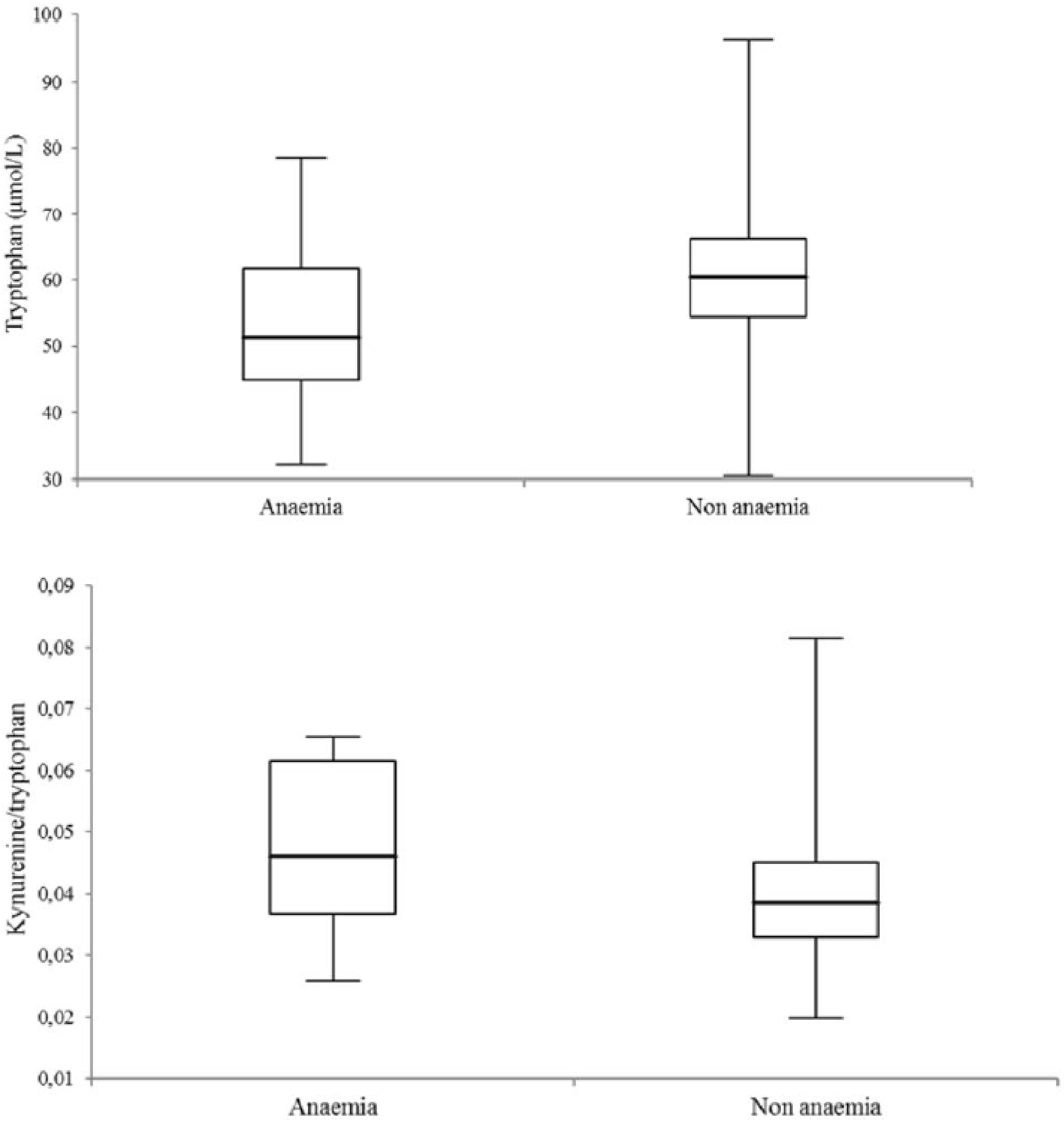

Figure 8. Tryptophan metabolism and anaemia. Box-and-whisker plots of tryptophan serum level and tryptophan break-down index (kynurenine/tryptophan) comparisons between 17 anaemic and 413 non-anaemic individuals (p-values were 0.003 and 0.012).

The central boxes represent the 25th to 75th percentile range. The lines inside the boxes show the median value for each group. Minimum and maximum are indicated as whiskers with end caps (Wenninger et al. 2019).

4.6 Tryptophan metabolism in anaemic patients

As shown in Fig. 9, patients with anaemia had lower median tryptophan serum levels (51.3 [44.9 – 61.7] vs. 60.5 [54.5 – 66.2] µmol/L, p = 0.003) and a higher kynurenine/tryptophan ratio (0.05 [0.04 – 0.06] vs. 0.04 [0.03 – 0.005], p = 0.012).

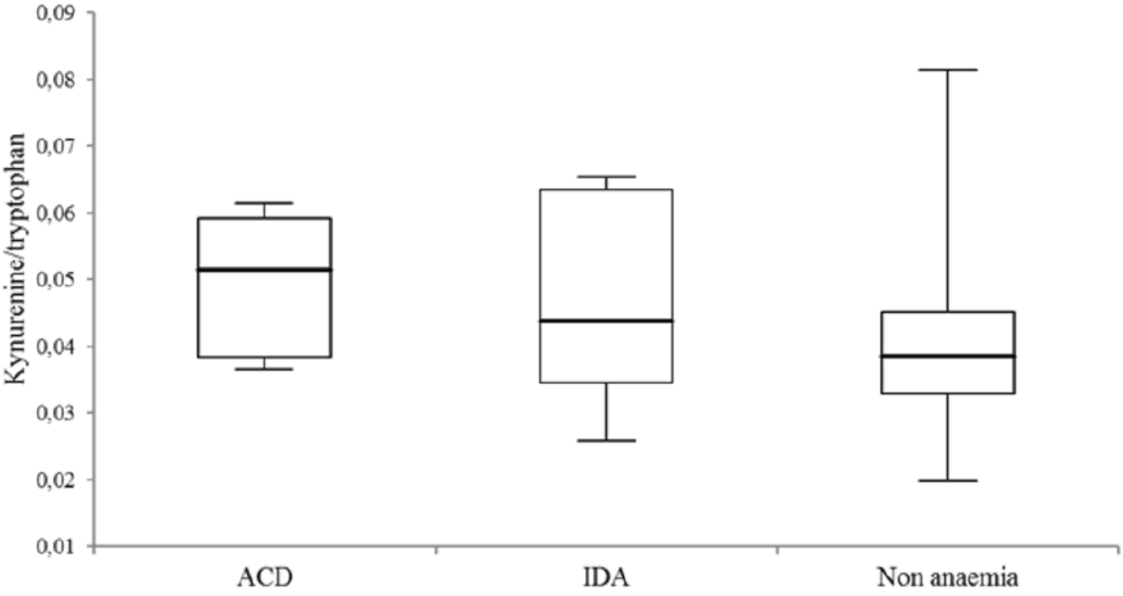

Figure 9. Tryptophan metabolism and categories of anaemia. Box-and-whisker plots of tryptophan break-down index (kynurenine/tryptophan) comparisons between 6 and 11 individuals with anaemia of chronic disease (ACD) and iron deficiency anaemia (IDA) and 413 non-anaemic subjects (p-values were 0.004 and 0.031).

The central boxes represent the 25th to 75th percentile range. The lines inside the boxes show the median value for each group. Minimum and maximum are indicated as whiskers with end caps (Wenninger et al. 2019).

The univariate log-transformed linear regression model for the assessment of associations between haemoglobin and indicators of tryptophan metabolism is demonstrated in table 8.

Furthermore, we fitted quadratic, cubic, exponential, fractional polynomial, and cubic spline regression models for possible non-linear relationships.

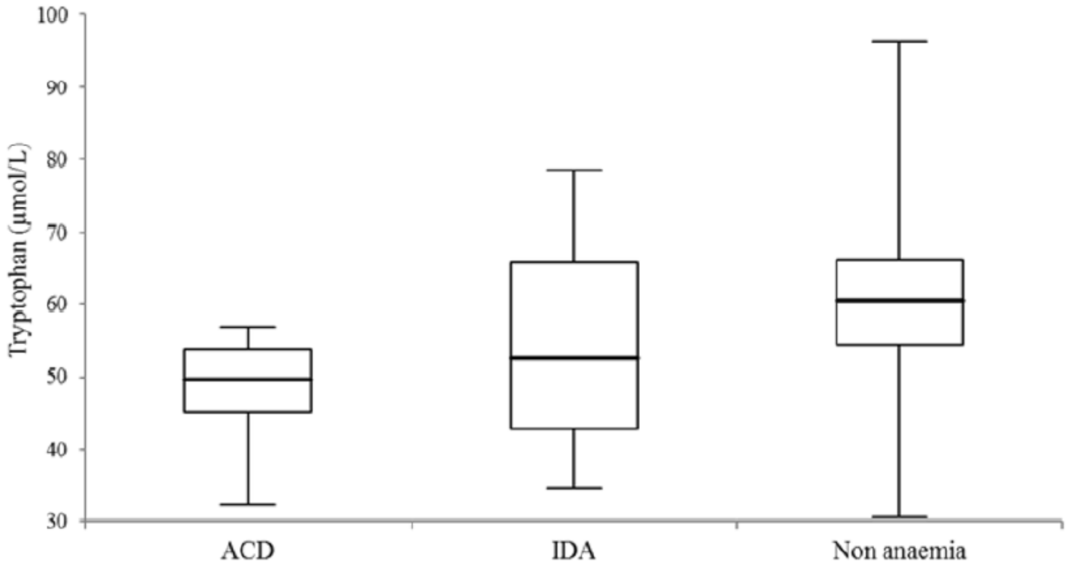

Figure 10. Tryptophan serum level and categories of anaemia (cont.). Box-and-whisker plots of tryptophan break-down index (kynurenine/tryptophan) comparisons between 6 and 11 individuals with anaemia of chronic disease (ACD) and iron deficiency anaemia (IDA) and 413 non-anaemic subjects (p-values were 0.004 and 0.031). The central boxes represent the 25th to 75th percentile range. The lines inside the boxes show the median value for each group. Minimum and maximum are indicated as whiskers with end caps (Wenninger et al. 2019).

The cubic regression model yielded the highest, yet relatively small, R-square values (R^2 = 0.101 for tryptophan, R^2 = 0.028 for kynurenine, R^2 = 0.080 for kynurenic acid, R^2 = 0.027 for kynurenine/tryptophan, and R^2 = 0.064 for kynurenic acid/kynurenine), indicating little to moderate associations between haemoglobin and tryptophan indicators despite the small p-values (p <0.001 for tryptophan, kynurenic acid and kynurenic acid/kynurenine, p = 0.007 for kynurenine, and p = 0.008 for kynurenine/tryptophan).

Markers of tryptophan metabolism categorized by types of anaemias (ACD and IDA) are illustrated in Fig. 3 (A and B). Six patients with ACD (n = 6) showed the lowest median tryptophan serum levels (49.6 [45.2 – 53.9] µmol/L) and the highest kynurenine/tryptophan ratio (0.05 [0.04 – 0.06]) compared to 11 individuals with IDA (tryptophan: 52.4 [42.7 – 65.9] µmol/L; kynurenine/tryptophan: 0.04 [0.03 – 0.06]) and 413 non-anaemic patients (tryptophan: 60.5 [54.5 – 66.2] µmol/L; kynurenine/tryptophan: 0.04 [0.03 – 0.05]) (p- values were 0.004 and 0.031), respectively.

5. Discussion

In our study, we found positive correlations between the serum tryptophan, kynurenine, kynurenic acid concentrations and haemoglobin and ferritin levels. The strongest association of these was observed between the tryptophan and haemoglobin concentrations ($p < 0.001$, $r = 0.305$). The cubic regression model showed the highest, yet relatively small, R^2-values, indicating little/moderate associations between haemoglobin and indicators of tryptophan metabolism.

5.1 Lowered serum tryptophan levels were found with lowered haemoglobin values

Lowered serum tryptophan levels were found in connection with lowered haemoglobin values. A 10% increase of tryptophan was shown to be associated with a 1.59% increase of haemoglobin. The tryptophan availability is a limiting factor for protein biosynthesis and cell growth and is also of importance for erythropoiesis (Weiss et al. 2004).

Tryptophan is a nutritional pyrrole source, which is essential for the haemoglobin synthesis. Human haemoglobin contains 6 tryptophan residues, which are located in the α- and β-subunits of the haemoprotein (Fontaine et al. 1980; Alpert et al. 1980; Albani et al. 1985).

Recently, a cross-sectional study, which included 105 South African HIV-infected patients and 60 HIV-negative controls, reported a positive correlation ($p < 0.001$, $r = 0.378$) between the nutritional indicators haemoglobin and tryptophan (Bipath et al. 2018).

Malnutrition may be one possible reason for tryptophan depletion as it is for anaemia in general. This condition is underdiagnosed and especially low- and middle-income countries are affected.

The required minimum daily intake of tryptophan is estimated with 175 mg for adult women and 250 mg for adult men (Peters 1991; Murray 2003; Bipath et al. 2016).

5.2 Tryptophan serum levels and ferritin

Present data show that tryptophan serum levels correlated with the iron storage protein ferritin ($p = 0.038$, $r = 0.100$). The correlation coefficient was relatively small indicating only a weak correlation. A 10% increase of tryptophan was shown to be associated with a 6.45% increase of ferritin. Individuals with iron deficiency (ferritin < 15 µg/L and/or a TSAT < 20%) were found with lowered tryptophan concentrations.

In a previous study, apoferritin, the hollow shell protein component of ferritin, was shown to contain tryptophan residues (Addison 1983). This could be one possible reason for the positive correlation between tryptophan and ferritin found here.

Another possible explanation for the observed relationship between tryptophan and iron metabolism could be that both metabolic pathways may be influenced by acute or chronic inflammatory conditions.

Ferritin is known as an acute phase response reactant, which can result in hypoferremia due to iron sequestration in the reticuloendothelial macrophages with subsequent iron-restricted erythropoiesis (Ganz & Nemeth 2009); diagnosis however is difficult The gold standard of a Perl’s stained bone marrow aspirate is not commonly performed as it is of little use in diagnosing an anaemia of chronic disease (ACD) and may even be inaccurate in 30% all interventions (Barron et al. 2001; Koulaouzidis et al. 2009).

Both ID and ACD often appear together but often transferrin is elevated in ID while it is normal or lower in ACD (Koulaouzidis et al. 2009; Cullis 2011). In these cases, the serum transferrin receptor (sTFR) to the log of the serum ferritin could enable differentiation between ACD and ID – is it < 1, anaemia of chronic disease is probable, while a ratio > 2 indicates low iron stores regardless of ACD (Malope 2001; Skikne 2008).

5.3 Tryptophan, immune activation and markers of inflammation

In their 2004 study, Nemeth et al. used human liver cells, mice as well as volunteers, proposing that IL-6 is a relevant cytokine in stimulating hepcidin under conditions of inflammation (a type II acute-phase response) and causal for the hypoferremia in these circumstances. The human volunteers were administered IL-6 intravenously; heavily stimulating hepcidin excretion, causing a significant lowering of serum iron and transferrin saturation.

As similar states also occur with age-related cytokine dysregulation and were observed in some types of cancer, influencing this pathway may be helpful for stimulating erythropoiesis, hence ameliorating ACD (Ershler & Keller 2000; Barille et al. 2000).

In a previous published study, lung cancer patients with inflammation and immune activation were shown to have lower tryptophan serum levels and an increased kynurenine/tryptophan ratio, indicating that the kynurenine pathway (KP) activation is accelerated under inflammatory conditions.

Most of these processes of tryptophan degradation (three out of four, amounting to >90%) take place in the liver under non-inflammatory activity (Bender 1983; Kurz et al. 2012). This is suggested to influence the kynurenine pathway massively due to the fact that tryptophan is the precursor molecule of serotonin (5HT, 5-hydroxy-tryptamine), which has manifold functions in the CNS but also mediates immune and inflammatory activation and influences peripheral diseases such as fibromyalgia, IBS and chronic fatigue syndrome (Hudson & Pope 1996; Frazer & Hensler 1999; Berger et al. 2009).

Furthermore, tryptophan is also a precursor for melatonin, which is known to modulate the circadian rhythm (Oxenkrug & Ratner 2012). Metabolites of inflammation processes may interfere with the kynurenine pathway and may lead to dysfunction, which is caused by accumulation or lack of metabolic substrates and products.

This pathophysiological mechanism is suggested to be linked with cancer, major depression, bipolar disease, bodyweight issues and porphyria, immune diseases, neurological and neurodegenerative disorders, yielding a possible target for therapeutic intervention (Badawy 2013; Stone & Darlington 2013; Jayawickrama et al. 2015; Sheen & Soliman 2015).

Moreover, also 3-HK, 3-hydroxykynurenine promotes programmed death of neuronal cells by producing ROS (Polyzos & Ketelhuth 2015), and is linked with diseases such as Morbus (Mb) Alzheimer-, Mb Parkinson and Mb Huntington. (Valente-Silva & Ruas 2018). These diseases should be considered as chronic inflammatory conditions (Lanser et al. 2020).

5.4 Tryptophan, kynurenine, inflammation response and psychiatric and neurological diseases

It is important to note, that the inflammatory induced tryptophan metabolism is closely linked to psychiatric and neurological diseases. This metabolic pathway can be triggered by a limited tryptophan level, which causes serotonin deficiency and at least influences the diseases mentioned above. Quinolic acid (QA) is associated with free radicals and is also an agonist of the glutamate sensitive N-methyl-D-aspartate (NMDA) receptors. This can result in brain cell death and problems of glutamatergic transmission (Lugo-Huitron et al. 2013).

Low levels of kynurenic acid, which are proposed to be neuroprotective, seem to have a negative influence. For laboratory measurements, a ratio of QA to KYNA is suggested for the assessment of the prognostic outcome. Of course, also other aspects in genesis of these conditions and the multifactorial model have to be kept in mind (Lanser et al. 2020).

An accelerated tryptophan catabolism appears to be involved in the pathogenesis of ACD. Cytokine-induced (i.e., interferon-γ, tumour necrosis factor-α) tryptophan degradation via the enzyme indoleamine 2,3-dioxygenase (IDO) suppresses the erythropoiesis (Schroecksnadel 2006). IDO is highly active under inflammatory conditions and very responsive to cytokines, lipid molecules, pathogen parts (such as prostaglandin and lipopolysaccharides) and interferons especially interferon-γ (INF-γ).

As a consequence, the enhanced tryptophan breakdown to kynurenine results in a deficiency of this essential amino acid (Weiss et al. 2044). Given that, tryptophan is known to bind non-covalently to the negative acute-phase protein albumin (Salter 1989). An inflammation-induced reduction of albumin concentrations could also have a potential influence on the serum tryptophan concentrations (Yeung et al. 2015).

The levels of kynurenine and tryptophan are age-dependent and allow both, aggravating and limiting the so-called “inflammaging”-related diseases (Sorgdrager et al. 2019). It seems to be very important to keep the balance between protective and aggressive factors, pro- and anti-inflammatory mediators in order to be vital for healthy aging and health in general (Franceschi et al. 2017).

Therefore, an advanced understanding of these pathophysiological processes is considered to be helpful in reaching this goal.

5.5 Tryptophan, kynurenine and the gut-brain axis

As mentioned earlier, the tryptophan and kynurenic pathways are linked to the gut microbiome and the brain metabolism. Therefore, associations with various psychiatric and neurological diseases can be drawn.

In a recent study, Laurans et al. (2018) showed how a genetic alteration of the indoleamine 2,3-dioxygenase activity may lead to obesity and metabolic alterations subsequently limiting the activity of the gut-IDO and leading to an increasement of kynurenine and a decrease of microbiome-metabolites.

Obesity and type 2 diabetes were also found together with higher levels of plasma kynurenine and also higher levels of kynurenine in faeces, which correlated with the plasma triglycerides concentrations and the triglycerides/HDL cholesterol ratio. Similar outcomes were described by Lanser et al. (2020).

Although most studies concerning the gut and brain-gut axis were rather small, the obtained data indicate the importance of this connection. The gut bacteria are associated with tryptophan, but also γ-aminobutyric acid (GABA) and noradrenalin as well as other metabolites. A study by Strasser et al. (2016) comprised 33 highly-trained persons distributed in a probiotic or placebo group; they were given a dose of 1×10^{10} CFU of a multi-species probiotic mixture once a day. After 12 weeks, the probiotic group displayed post-exercise tryptophan levels, that were quite similar to the ones before, while the placebo group had tryptophan levels decreased by 11%. They concluded that these probiotics reduced the exercise-induced tryptophan breakdown.

The current problem is that many of these observations were made in cross-sectional studies, which cannot serve as a basis for a cause-effect conclusion (Strasser et al. 2017). Therefore, all these considerations should be extended in further longitudinal prospective studies.

In addition, the potential link between tryptophan, haemoglobin and iron parameters found in the present work could be subject of a basic research study, which covers the tryptophan metabolism of erythrocytes. This approach might have potential to gain better insight into the interaction of the metabolic tryptophan pathway and the erythropoiesis.

5.6 Tryptophan breakdown index and non-anaemic persons

In our study, patients with anaemia were found with lower serum tryptophan levels and a distinct increased tryptophan breakdown index compared to non-anaemic individuals. This finding is in agreement with an earlier study by Weiss et al. (2004), which reported lower tryptophan concentrations in patients with ACD compared to healthy controls.

However, differences between the study designs must be mentioned. Weiss et al. studied 22 hospitalised patients with a variety of chronic diseases and ACD (median age 78 years) and 22 non-anaemic controls (median age 75 years).

In comparison, we recruited 430 ambulatory individuals (median age 39 years) without chronic diseases in their case histories for haemoglobin and iron status assessment.

In our cohort, we differentiated between ACD and IDA and observed the lowest tryptophan concentrations and the highest kynurenine/tryptophan ratio in patients with ACD compared to individuals with IDA and non-anaemic subjects.

5.6 Thomas-Plot and diagnosis of anaemia

We used the Thomas-Plot for categorising iron deficiency, allowing discrimination between ACD and regular iron deficiency (ID), also in a functional state. It includes the reticulocyte haemoglobin content (CHr) and the soluble transferrin receptor-ferritin index and considered inflammation via the C-reactive protein (CRP).

Unlike ferritin, the sTFR does not differ between sexes and is not influenced by inflammatory processes but the assays have not yet been standardised.

Previous studies demonstrated a good analytical performance of this diagnostics approach (Thomas & Thomas 2002; Skikne et al. 2011; Enko et al. 2013), more recent ones are even more favouring the Thomas-Plot (Enko et al. 2015; Al-Rubaie et al. 2018; Krawiec & Kozuchowska 2019).

In the present study population, only relatively few ACD cases were found. One possible explanation of this phenomenon could be the fact that the cohort consisted mostly of young adults without relevant comorbidities.

5.7 Falsification/validation of the hypothesis

The hypothesis of this thesis was

Hypothesis: A possible link between tryptophan of the human metabolism and iron homeostasis exists.

- Ho: The tryptophan serum level and tryptophan break-down index differ in patients with different forms of anaemia (ACD, IDA, healthy).
- H1: The tryptophan serum level and tryptophan break-down index in patients suffering from different forms of anaemia (ACD, IDA, healthy) is the same.

In conclusion, little to moderate associations between haemoglobin, biomarkers of iron metabolism and tryptophan indicators were observed.

Patients with iron deficiency or anaemia showed lower serum tryptophan levels compared to individuals without iron deficiency or anaemia. Therefore, the main hypothesis was confirmed.

6. Limitations and conclusions

The major limitation of this cross-sectional study is that pro-inflammatory cytokines (such as interferon-γ, tumour necrosis factor-α) and the acute-phase reactant α1-acid-glycoprotein were not measured. These indicators could have the potential to give insight into the still not fully understood relationship between inflammation markers and the development of anaemias.

Also, the number of study participants could be increased to gain even further reliability.

The potential link between tryptophan, haemoglobin and iron parameters found in the present work could be subject of a basic research study, which covers the tryptophan metabolism of erythrocytes. This approach might have potential to gain better insight into the interaction of the metabolic tryptophan pathway and the erythropoiesis.

The cohort consisted mostly of young adults without relevant comorbidities; differentiating between patients with and without accompanying diseases could be an interesting point of further scientific approach. Performing a longitudinal study, especially with older patients and patients with comorbidities would help understanding the mechanisms of these conditions. Their number is quickly growing worldwide, so this would increase the quality of many lives.

It is obvious that supporting the World Health Organisation, taking the same line in their endeavours of improving the nutrition (especially in terms of iron supplementation) of people living in lower- and middle-income countries would also help reduce the burden of disease of anaemia and concurrent conditions associated with ACD.

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