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Dual Role of Heme Iron in Cancer; Promotor of Carcinogenesis and an Inducer of Tumour Suppression.

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List of abbreviations:

ROS: reactive oxygen species; ALA: 5-aminolevulinic acid; ALAS1: 5- aminolevulinate Synthase 1, ALAD: ALA dehydratase; PBGD: Porphobilinogen deaminase; UROS: uroporphyrinogen III synthase; UROD: uroporphyrinogen decarboxylase; CPOX: coproporphyrinogen oxidase; PPOX: protoporphyrinogen oxidase; FECH: ferrochelatase; PpIX: protoporphyrin IX; FLVCR: feline leukemia virus receptor; HCP1: Heme carrier protein 1; DMT-1: divalent metal transporter-1; Dcytb: Duodenal cytochrome b; FPN: feroportin; HIF: hypoxia inducible factor; HO: heme oxygenase; CO: carbon monoxide; TfR:

transferrin receptor; ABCG2: ATP-binding cassette transporter G2; PHD: Prolyl hydroxylase; HEPH: hephaestin; NSCLC: non- small cell lung cancer; HRG1: Heme responsive gene 1; HCP 1: heme carrier protein 1; PUFA: polyunsaturated fatty acid; MDA: Malonaldehyde; 4-HNE: 4- Hydroxynonenal; TP53: tumour protein 53; HFE: homeostatic iron regulator; ICAM-1 (intracellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule), VWF (von Willebrand factor), PKC: protein kinase C; TLR4: toll-like receptor-4; KC: keratinocyte chemokine; TNF: tumour necrosis factor; IBD: Inflammatory bowel disease; CRC: colorectal cancer; DFO: deferoxamine; RSL3: RAS selectively lethal.

Key words: Heme, carcinogenesis, reactive oxygen species, ferroptosis, red meat, dysbiosis, inflammation, tumour suppression, inflammatory bowel disease, P53 down regulation.

Abstract:

Heme is a crucial compound for cell survival but with the potential to be toxic and carcinogenic to cells. It is considered to have negative effect in human owing to the vast number of researches supporting its carcinogenic properties. However, with the recent advancement of knowledge regarding ferroptosis, the iron mediated cell death, heme can be postulated to induce tumour suppression through ferroptosis. This review summarizes the literature on the carcinogenic and anti-carcinogenic properties of heme with specific emphasis on the alterations observed on heme synthesis, metabolism and transport in tumour cells. Heme supports carcinogenesis via modulation of immune cell function, promoting inflammation and gut dysbiosis, reducing the tumour suppressive potential of *P53* gene, and promoting cellular cytotoxicity and reactive oxygen species generation. The carcinogenic and anti-carcinogenic properties of heme are proven to be both dose and oxygen concentration dependant. At low doses, heme is apparently harmless and even helpful in maintaining the much-needed redox balance within the cell. However, when heme exceeds physiological levels, it could initiate and propagate carcinogenesis, due to its ability to produce reactive oxygen species (ROS). To conclude, the same phenomenon of heme mediated ROS generation could be manipulated to initiate tumour suppression via ferroptosis, but the therapeutic doses are yet to be determined.

Introduction

Heme, an iron protoporphyrin complex, is of utmost importance for cell survival because of its involvement in numerous essential biological processes, including oxygen transport, energy production and drug metabolism. Apart from being a crucial compound for cell survival, heme has the potential to be toxic and carcinogenic to cells (Fiorito et al. 2020). There is a considerable body of evidence suggesting that heme may induce carcinogenesis via inducing cytotoxicity, production of reactive oxygen species (ROS), modulating immune cell function, promoting dysbiosis and inducing inflammation (Glei et al. 2006; Ishikawa et al. 2010; Min and Ahn 2005; Fiorito et al. 2020). Even though carcinogenic properties of heme are evident to be prominent than the anti-carcinogenic properties, heme could tumour suppression through ferroptosis (Chiang, Chen, and Chang 2018). This review summarizes the literature on the carcinogenic and anti-carcinogenic properties of heme with specific emphasis on the alterations observed on heme synthesis, metabolism and transport in tumour cells. In addition, topics such as heme induced ROS, inflammation, dysbiosis, and its role as a ferroptotic mediator have been reviewed in detail.

Heme iron synthesis, absorption, metabolism and their alterations in cancer

In vivo Heme Synthesis and its alterations in cancer

All cells in the human body have heme synthesizing ability. The reactions involve condensation of succinynl-CoA and glycine to produce 5-aminolevulinic acid (ALA) and then several reactions including the enzymes 5- aminolevulinate Synthase 1 (ALAS1), ALA dehydratase (ALAD), porphobilinogen deaminase (PBGD), uroporphyrinogen III synthase (UROS), uroporphyrinogen decarboxylase (UROD), coproporphyrinogen oxidase (CPOX), protoporphyrinogen oxidase (PPOX) and ferrochelatase (FECH) resulting in the production of heme (Chiabrando et al. 2014; Chiabrando, Mercurio, and Tolosano 2014). Of these

enzymes, ALAS1, UROD and PBGD were found to be overexpressed in cancer tissue. Further, tumours accumulated protoporphyrin IX (PpIX), final intermediate in heme biosynthetic pathway, upon ALA administration (Yang et al. 2015). These findings suggest that tumours characteristically show higher PpIX synthesis ability, which may indicate increase in heme synthesis. The above hypothesis was further reinforced by the discovery of higher activity of heme-containing proteins and increased heme content in tumour cells when compared to normal cells (Hooda et al. 2013) (Fiorito et al. 2020). A possible explanation for the enhanced heme synthesis in tumour tissue is that tumour cells exploit heme for the maintenance of mitochondrial electron transport chain for energy production (Fiorito et al. 2020). However, this hypothesis contradicts with the well-established “Warburg effect”, which explains that tumour cells rely mainly on glycolysis for energy production as opposed to normal cells, which are dependent on mitochondrial electron transport chain (ETC). “Warburg effect” takes place predominantly in hypoxic conditions. and it supports abnormal proliferation and metastasis of tumour cells by suppressing the production of reactive oxygen species (ROS) and thereby reducing the oxidative stress on the cells. On the other hand, in the presence of oxygen and excess heme or heme precursors, tumour cells are forced to shift from glycolysis to ETC, thereby increasing the production of ROS. This feature has been manipulated to induce apoptosis in cancer cells by adding heme precursors, promoting heme synthesis and shifting the energy metabolism towards aerobic ETC (Michelakis, Webster, and Mackey 2008; Bonnet et al. 2007; Sugiyama et al. 2019). Thus, heme is a potent apoptosis inducer of tumour cells due to its ability to cause oxidative stress on the cells by promoting oxidative phosphorylation.

Heme Absorption and Metabolism in Normal Duodenal Cells

Approximately, 40% of the iron acquired by an individual consuming meat, is in heme iron form and the rest is in the form of non-heme iron. Majority of both heme and non heme iron is absorbed by the duodenum by a tightly regulated process (Figure 1). Heme iron is thought to be absorbed as intact metalloprotein, making heme iron easier to absorb and more bioavailable than non-heme iron (Figure 1). Heme uptake into the epithelial cell is thought to occur mainly by two mechanisms. The first mechanism is via the heme receptor-mediated endocytosis and the second is through the heme transporters HCP1 (Heme carrier protein 1) (West and Oates 2008). Feline leukemia virus subgroup C receptor-related protein 1 (FLVCR) and ATP-binding cassette transporter G2 (ABCG2) are responsible for heme export from cells (Figure 1)

Intracellular heme is degraded into Fe2+, carbon monoxide and bilirubin by heme-oxygenase 1 (HO-1) and heme-oxygenase 2 (HO-2), which is located in the endoplasmic reticulum of the intestinal epithelial cell (Xue and Shah 2013). HO-1 and HO-2 are products of different genes. HO-2 catalyses heme degradation in physiological conditions, and HO-1 becomes active when there is an excess amount of intracellular heme, to accelerate the heme breakdown. The labile iron produced by this reaction is scavenged by ferritin, a protein which stores iron. In addition, other intracellular and extracellular mechanisms come into operation when excess iron is detected (Maines 1997). The extra extracellular iron triggers liver to produce Hepcidin, the principal regulator of iron absorption and distribution to tissues, binds with FPN (ferroportin) and causes FPN internalization and degradation, thereby reducing the iron exportation from the cell into the bloodstream (Xue and Shah 2013). Increased intracellular iron triggers Hypoxia Inducible Factor (HIF-2α), which in turn causes an increase of Prolyl Hydroxylase activity (PHD), leading to decreased transcription of DMT-1 and Dcytb thereby causing decreased dietary iron absorption (Xue and Shah 2013). (Figure 1).

Differences in Heme Absorption and Metabolism in Small and Large Intestines

Even though majority of heme iron is absorbed from the duodenum, heme appears to exert more carcinogenic effects on the colonic epithelium than on the duodenal epithelium. Therefore, it is important to consider the differences of colonic mucosa from duodenal mucosa with respect to heme iron absorption and metabolism. According to a study conducted in pig models to compare the capacities of luminal iron absorption between colon and duodenum, the iron absorptive capacity of the colonic mucosa was noted to be approximately 14% of absorptive capacity of duodenal mucosa (Blachier et al. 2007). The normal pig colonic epithelial cells express less DMT-1, TfR and ferritin when compared with the duodenal epithelial cells when exposed to luminal iron. However, ferroportin (FPN) was highly expressed in colonocytes as well in duodenal cells, in similar amounts (Blachier et al. 2007). Therefore, it can be assumed that colonic epithelial cells have a less iron internalizing capacity from the lumen and from circulation and less facility for storage within the cell, when compared to the duodenal mucosa. Furthermore, since FPN is expressed in amounts close to the duodenal epithelium, it can be assumed that iron exportation mechanism out of the colonic epithelial cell is as efficient as in the duodenal cells. According to this study, porcine colonic epithelial cells seem to be less susceptible to the detrimental effects mediated by excessive intracellular iron, which is quite the opposite of what is evidenced in literature. However, this study had been focused on inorganic iron (iron sulphate) and the mechanisms of heme iron and inorganic iron absorption are different from each other (Figure 1). Therefore, further studies aimed at exploring the expression of DMT-1, PCFT/FTC1, FLVCR, TfR, FPN, Ferritin, HO-1 and HO-2 in normal human colonic epithelial cells in response to heme iron exposure, may help to understand why colonic mucosa is more susceptible to the mal-effects of heme iron compared to the small intestinal mucosa.

Alterations of heme transportation and metabolism in cancer

Dysregulation of heme iron absorption, transport and metabolism are observed to be hallmark features of several types of cancers (Xue and Shah 2013; Fiorito et al. 2020). Colorectal carcinoma (CRC)n cells are shown to express increased amounts of DMT-1 and TfR1, involved in luminal and circulatory iron absorption machinery. TfR1 is expressed in the normal colonic epithelium, but this amount is significantly increased in early stages colorectal carcinoma (Prutki et al. 2006). Overexpression of TfR provides a growth advantage for tumour cells and enhances c-myc (oncogenic transcription factor) mediated tumorigenesis. On the other hand, inhibition of TfR causes decreased cellular proliferation and arrest of cells at the G1 phase of the cell cycle (O'Donnell et al. 2006). Ferritin, which acts as an iron detoxifier by storing excess intracellular iron, is increased in colorectal adenomatous polyps (Nelson et al. 1994). Hepcidin, which binds and internalizes FPN (intracellular iron exporter), causing its degradation (Figure 1), is expressed in increased amounts in CRC tissue (Ward et al. 2008). The FPN and HEPH (Hephaestin) proteins which support cellular iron export are decreased in advanced CRC (Xue and Shah 2013). Thus, compared to normal colonic cells, the cancer cells in the colon tend to absorb and accumulate more iron within the cell.

Non-small cell lung cancer (NSCLC) exhibits upregulation of a collection of proteins promoting heme synthesis, transport, and function. Some of these proteins include Heme biosynthesis enzyme ALAS, heme transporter uptake proteins HRG1 (Heme responsive gene 1) and HCP1. Reduced heme biosynthesis and uptake were associated with limitation of lung cancer cell migration, proliferation and colony formation. On the other hand, an increased supply of heme to cancer cells resulted in intense oxygen consumption and intracellular energy production, which may in turn promote cancer cell proliferation (Hooda et al. 2013).

Heme oxygenase -1, the enzyme responsible for intracellular heme degradation, shows higher expression in vast array of tumour tissues such as lymphoma (Schacter and Kurz 1982) , brain tumours (Hara et al. 1996), melanoma (Torisu-Itakura et al. 2000), prostate adenocarcinoma (Maines and Abrahamsson 1996), pancreatic adenocarcinoma (Berberat et al. 2005). This phenomenon is evidenced to be a promoter of ROS (reactive oxygen species) mediated tumour cell proliferation, which can be exacerbated secondary to radiotherapy and chemotherapy (Was, Dulak, and Jozkowicz 2010).

Most cancer cells often possess an increased ability to uptake iron and decreased capacity to export it (Zhang and Chen 2019). It was observed that there was decreased expression of FPN (Ferroportin), resulting in an increase of labile iron pool in breast cancer, leading to increased carcinogenic potential (Jung et al. 2019). In a study using more than 800 female patients, it was concluded that downregulation of expression of *FPN* gene was associated with a significant decrease in patient survival and metastasis-free disease (Pinnix et al. 2010b). In addition, patients with high FPN and low hepcidin levels were identified as a cohort with a ten-year survival rate exceeding 90% (Pinnix et al. 2010a; Lamy, Durigova, and Jacot 2014). Similarly, expression of *FPN* gene was downregulated in ovarian and prostate cancer (Basuli et al. 2017; Tesfay et al. 2015) whereas expression of hepcidin, FPN's negative regulator, was upregulated in several cancers including hepatocellular, ovarian and prostate cancers (Tanno et al. 2011; Kali, Charles, and Seetharam 2015; Kijima et al. 2008). Therefore, as opposed to normal cells, cancer cells are highly capable of acquiring iron and heme iron into the intracellular compartment and prefers to retain most of the iron within the cell in order to facilitate its carcinogenic potential. Thus, heme iron concentration and heme related molecule expression is evidently an important factor in establishing tumorigenesis.

1. Heme iron induced cytotoxicity, reactive oxygen production and carcinogenesis

Heme evidently exerts cytotoxic and genotoxic effects on cells (Glei et al. 2006; Ishikawa et al. 2010). However, these effects are argued to be mediated by the reactive oxygen species produced by heme, rather than by heme itself (Balla et al. 1991). Heme proteins are essential for a variety of vital physiological functions in the body, such as oxygen transport and storage (hemoglobin and myoglobin), drug metabolism, biosynthesis of steroids (cytochrome P450), signal transduction (CooA, FixL, Guanylate cyclase), catalase/ peroxidase antioxidant defence enzymes and mitochondrial respiration (cytochrome) (Nagababu and Rifkind 2004). All these processes involve oxidation and reduction reactions (redox reactions) of heme iron, generating reactive oxygen species (ROS). The redox reactions are more efficient and rapid when free heme is involved compared to bound heme proteins, making free heme more toxic to cellular membranes and macromolecules (Aft and Mueller 1983; Gutteridge and Smith 1988; Tappel 1955). Cellular damage caused by ROS is further enhanced in the presence of free iron (Halliwell and Gutteridge 1984), as evidenced by drastically enhanced killing of iron-rich Staphylococcus aureus by hydrogen peroxide, as opposed to iron- free organisms (Repine, Fox, and Berger 1981). Further, oxidative damage of both prokaryotic and eukaryotic cells is minimized when the cells are in an iron depleted state (Gannon et al. 1987), implying that iron plays a leading role in oxidative cytotoxicity.

Heme catalyses the degradation of the cell membrane proteins and intra cellular proteins to small fragments of peptides (Aft and Mueller 1984). Heme driven cell membrane protein degradation occurs independent to lipid peroxidation and is characterized by amino acid oxidation and extensive cross-linking (Vincent 1989). Furthermore, low density lipoprotein is converted to cytotoxic by-products by the action of heme [23]. Heme and hemin induced DNA damage have been explained in several studies. Oxidative stress generated by hemin has been shown to cause deletion of a specific area of mitochondrial

DNA, resulting in changes in expression of mitochondrial cell death proteins and thereby apoptosis [24]. In the presence of oxygen and 2-mercaptoethanol (antioxidant), hemin causes strand scission in DNA. Circular supercoiled plasmid DNA was shown to transform into open circle or linear forms within 30 minutes, in the presence of hemin. These transformations are initiated at hemin concentrations as low as10µM and the rate of open circle and linear form formation increases rapidly as hemin concentration increases. With increasing incubation time, DNA was degraded into smaller fragments [15]. Majority of heme mediated carcinogenic effects are caused by ROS via the following mechanisms:

Lipid peroxidation

Lipid peroxidation is a result of a free radical chain reaction, oxygen being the most important factor. The human colon is considered to be a relatively hypoxic compartment (PO_2 less than 10mmHg) (Morrissey et al. 1998; Karhausen, Ibla, and Colgan 2003; Kelly and Colgan 2016). Therefore, oxidation of polyunsaturated fatty acids (PUFA) and formation of toxic free radicals in meat presumably occur while being exposed to atmospheric oxygen than while in the colonic lumen. Oxygen in its form in atmospheric air cannot react with PUFAs. Iron in heme acts as a major catalyst to the reaction, which converts atmospheric oxygen to ROS (Min and Ahn 2005). Some of the ROS thus produced are superoxide anion ($O_2^{\cdot}$-), hydroxyl radical (OH), hydrogen peroxide ($H_2O_2^{\cdot}$) and hydroperoxyl radical ($HO_2^{\cdot}$) and iron-oxygen complexes (ferryl and perferryl radical). Some of these radicals are highly reactive and initiates lipid peroxidation in red and processed meat (Min and Ahn 2005). The number of bis-allylic carbons in lipid molecules determines their susceptibility to peroxidation. Weakest C-H bond which is most susceptible for peroxidation lies at the bis-allylic position of the lipid molecule, and thus it is the most reactive site for hydrogen abstraction (Wagner, Buettner, and Burns 1994). ROS formed by the catalytic action of iron,

especially ferryl and perferryl radicals, have a high potential for abstracting the hydrogen iron from these bis-allylic positions (Bielski 1991). Once ROS abstracts the hydrogen atom from the lipid chain, an unpaired electron is left on the carbon. This carbon radical tries to become stable by forming a conjugated diene. The fate of this conjugated diene is dependent upon the oxygen concentration in the system. In aerobic conditions, it reacts with oxygen to form Lipid peroxyl radicals (LOO·). On the other hand, in relatively anaerobic conditions, such as in the human colonic compartment, the conjugated diene may react with cell membrane components such as lipids and proteins and DNA (Min and Ahn 2005). This causes changes in the fluidity and increases the permeability of cell membrane resulting in intracellular enzymes to leak into the extracellular compartment leading to chemotaxis of inflammatory cells into the area paving the way for inflammation (Perse 2013). Thus, the cells exposed to heme iron-mediated lipid peroxidation are more susceptible to inflammation, which is known preceding event of carcinogenesis (Terzić et al. 2010; Min and Ahn 2005).

Heme iron catalyses the production of Malondialdehyde (MDA) and HNE (4-Hydroxy-2-nonenal) by lipid peroxidation. They are markers of formation of free radicals, lipid peroxidation and oxidative stress on cells (Gawel et al. 2004). Both MDA and HNE can act as signal transducers and modulating cell proliferation and gene expression (Marnett 2002; Uchida 2003). MDA is known to interact with DNA both in vitro and in vivo and can form adducts with deoxyadenosine, deoxyguanosine and deoxycytidine (Marnett 2002). These adducts induce oncogenic mutations in vitro (Marnett 2000; Cadet et al. 2003). Therefore, heme mediated formation of ROS and lipid peroxidation may contribute cellular toxicity and genetic mutations.

Oxidation of Proteins

Heme iron related ROS can oxidize proteins to alter both their structure and function (Grune et al. 2003). This can result in the hindrance of enzymatic and binding functions of proteins as well as influence cellular uptake of proteins and inactivation of DNA repair enzymes. Though the moderately oxidized proteins are efficiently degraded by proteasomes, severely oxidized proteins accumulate with in the cells (Grune et al. 2003). Accumulation of damaged proteins in the cell over a long period causes inhibition of the proteasome, leading to accumulation of damaged, misfolded and defective proteins in the cells and hinders the cellular lysosomal activity. This process affects the protein metabolism, leading to structural and functional alterations of the cellular organelles (Brunk and Terman 2002).

Oxidation of DNA

In addition to protein oxidation, ROS produced due to action of heme iron could oxidize DNA bases forming oxidized thymines, cytosines, adenines and guanines which can trigger mutagenesis (Abalea et al. 1998; Bjelland and Seeberg 2003; Glei et al. 2002). One of the major by-products of DNA oxidation is 8-oxodG, which can cause G to T transversions during replication if the damage is not repaired by base excision repair (Bjelland and Seeberg 2003). These DNA oxidative by-products can be metabolized into 8-oxodGTP, which in turn will be incorporated into DNA during replication and repair leading to A to C transversions (Henderson, Evans, and Cooke 2010) which may potentiate carcinogenesis. DNA oxidation subsequently results in hypomethylation of DNA (Perse 2013). DNA hypomethylation is a usual phenomenon observed in a number of carcinomas including colorectal adenocarcinomas, cervical, hepatocellular carcinoma , prostate adenocarcinoma and ovarian carcinoma (Bjelland and Seeberg 2003; Ehrlich 2009). Therefore, heme can promote ROS

induced damage to cellular proteins, lipids and genetic material inducing carcinogenesis (Figure 2).

2. *Excess heme undermines the Tumour suppressive action of TP53 and promotes carcinogenesis*

The heme in excess amounts are shown to diminish the action of Tumour Protein 53 (TP53 or P53), a major tumour suppressor, by directly binding to the protein (Shen et al. 2014). Heme binds to TP53 protein, thus interfering with P53-DNA interactions. This results in an increase in nuclear export of P53 as well as cytosolic degradation (Shen et al. 2014). On the other hand, chelation of heme iron results in stabilization of P53 protein, increases P53 protein expression and effective tumour suppression via activating P21 (Shen et al. 2016; Zhang et al. 2008; Zhang and Chen 2019). Parallel to the above findings, $HFE^{-/-}$ mice, mimicking human hereditary haemochromatosis and iron overload, had downregulation of P53 expression (Bahram et al. 1999). However, the dynamics of heme iron-P53 interactions apparently differ in cancer cells and non-neoplastic cells. In colon cancer cells, P53 expression was decreased following exposure to high concentrations of heme, whereas in normal colonic epithelial cells, P53 expression was upregulated with high heme exposure (SMK Gamage 2020). There are also reports claiming that there is P53 upregulation with high iron exposure in hepatocytes (Dongiovanni et al. 2010). Therefore, heme dependant P53 expression seems to be different in non-neoplastic cells and cancer cells. In non-neoplastic cells, heme induce tumour suppression by P53 activation and in cancer cells, heme drives carcinogenesis by supressing P53 activity (Figure 2).

3. Heme induces modulation of immune cell function, inflammation, gut dysbiosis and cancer

Heme activates innate immune cells and non-hematopoietic cells, increase vascular permeability, trigger leukocyte migration from the intravascular compartment to tissues and surge acute phase reactants in rats and mice models (Lyoumi et al. 1999; Wagener et al. 2001). Heme also causes neutrophil activation and migration, activation of endothelial cells, induction of adhesion molecules such as ICAM-1 (intracellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule), VWF (von Willebrand factor), E- selectin and P-selectin (Wagener et al. 1997; Graça-Souza et al. 2002; Dutra and Bozza 2014). Heme induced neutrophil activation is secondary to protein kinase C (PKC) activation and ROS generation leading to the modification of cytoskeleton activity and expression of adhesion molecules, which are essential for neutrophil migration (Graça-Souza et al. 2002). Neutrophil migration is further facilitated by the fact that heme acts as a chemotactic molecule (Porto et al. 2007). Heme is capable of inhibiting apoptosis of neutrophils and thereby enhancing their life span and the possible mal-effects of their actions (Arruda et al. 2004). Production of Leukotriene B4 by macrophages is also increased due to heme, which induces adhesion and activation of leukocytes to the tissue, thus enabling them to exit into the tissue (Monteiro et al. 2011). Heme causes Toll-like Receptor 4 (TLR4) activation, keratinocyte chemokine (KC) induction and increased Tumour Necrosis Factor (TNF) secretion secondary to macrophage activation (Figueiredo et al. 2007). Taken together, heme acts as a signalling molecule which alters immune response via receptor mediated mechanisms. Prolonged action of these inflammatory mediators, secondary to heme exposure over a period , can cause detrimental effects on tissue homeostasis and may also lead to chronic inflammation.

There is a strong association between prolonged gut inflammation and colon carcinogenesis. Inflammation of the colon could occur secondary to dysbiosis, in which there

is a derangement in the colonic bacterial flora. Availability of nutrients in the gut and the capacity to internalize them is the main survival factors for microbes within the colon (Kamada et al. 2013). Bacterial heme uptake system is made up of an array of proteins to sense the upregulation of heme related alterations in the environment, high affinity binding to heme and efficient release of heme into the cytoplasm (Tong and Guo 2009). Majority of microbes are equipped with efficient iron uptake, storage and metabolization machinery, which is crucial for thriving (Becker and Skaar 2014; Andrews, Robinson, and Rodríguez-Quiñones 2003). Heme being an efficient delivery mode of iron into the cell, microbes with heme uptake ability use heme as a growth factor to flourish in the intestinal cavity, thereby altering the normal colonic flora (Constante et al. 2017). In a heme rich environment, bacteria with the highest heme utilizing ability thrive suppressing bacteria, which do not have such ability, resulting in an imbalance in the gut microbiome. This is relevant in inflammatory bowel disease (IBD) where availability of trace elements such as iron, is limited due to sequestration by the host to limit inflammation (nutritional immunity). Limited availability of the iron provides a survival advantage to the bacteria with efficient iron uptake ability, leading to an imbalance of the microbial flora of the gut, especially during inflammation.

Association between intestinal dysbiosis and IBD has a predisposition to premalignant lesions in the intestine (Harpaz et al. 2013; Louis, Hold, and Flint 2014; Nakatsu et al. 2015; Constante et al. 2017). It has been also noted that dietary heme causes dysbiosis and exacerbation of colitis and initiates adenoma formation in mice (Constante et al. 2017). On the other hand, gut dysbiosis is noted consistently in patients with IBD (Manichanh et al. 2006; Peterson et al. 2008; Winter and Bäumler 2014). Interestingly, if on a heme-rich red meat diet, patients with ulcerative colitis develop more relapses (Jowett et al. 2004). It is known that patient with IBD are at a higher risk of acquiring CRC (Kim and Chang 2014)

and with the available evidence, it is apparent that CRC can occur secondary to heme mediated intestinal dysbiosis leading to IBD (Figure 2).

4. Heme iron induced ferroptosis and tumour suppression

Regulated cell death is of importance for the normal development, homeostasis of cells and for the prevention of hyperproliferative states such as carcinomas. Ferroptosis is an iron dependant form of cell death which is reliant upon lipid peroxidation secondary to Fenton reaction and production of ROS by iron (Bebber et al. 2020). Being iron dependant and developing characteristic lipid peroxides in membranes are hallmarks of ferroptosis, as iron chelation by deferoxamine (DFO) and inhibition of ROS production halts ferroptosis induction (Dixon et al. 2012). As discussed earlier, heme plays a key role in the production of ROS, Fenton reaction and lipid peroxidation as it is a carrier of an active iron moiety (Fortes et al. 2012) and thus could have significant ferroptotic potential. Interestingly, the same lipid peroxidation by-products observed in heme mediated lipid peroxidation, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), are found in the process of ferroptotic cell death and they are currently being investigated for their definitive role in ferroptosis (Dalleau et al. 2013; Ayala, Muñoz, and Argüelles 2014; Chaudhary et al. 2010; Gamage et al. 2018; Feng and Stockwell 2018).

Approximately 30% of all cancers, including colorectal cancers, harbour *RAS* mutations (KRAS, NRAS and HRAS) (Arrington et al. 2012). The known ferroptotic inducers are Erastin, RSL3 (Ras selective lethal), Acetaminophen and Sulphasalizine. Interestingly, ferroptosis inducers are selectively lethal to oncogenic *RAS* mutant cell lines (Dixon et al. 2012). According to a recent study, the addition of iron chelators to *RAS* mutant cells 6 hours following treatment with erastin, still provided considerable protection of cells against ferroptosis (Dixon et al. 2012). This suggests that a continuous iron dependant ROS

formation over a considerable period is required for ferroptosis. Thus, constant exposure of RAS mutant cells to heme iron could theoretically induce ferroptosis of the cells. This phenomenon could be manipulated in order to selectively destroy RAS mutant tumour cells and trigger tumour suppression (Figure 2) (Dixon et al. 2012).

In conclusion, heme supports carcinogenesis via modulation of immune cell function; promoting inflammation and gut dysbiosis; reducing the tumour suppressive potential of *P53* gene and promoting cellular cytotoxicity and reactive oxygen species generation. The carcinogenic and anti-carcinogenic properties of heme are proven to be both dose and oxygen concentration dependant. At low doses, heme is apparently harmless and even helpful in maintaining the much-needed redox balance within the cell. On the other hand, when heme exceeds the physiological levels, it could initiate and propagate carcinogenesis, due to its ability to produce ROS. However, the same phenomenon of heme mediated ROS generation could be manipulated to initiate tumour suppression via ferroptosis, but the therapeutic doses are yet to be determined.

Figure legends

Figure 1- Mechanism of heme and non-heme iron absorption and metabolism in duodenal epithelial cells. Heme iron is absorbed by receptor-mediated endocytosis and heme iron transporters HCP1 and metabolized by heme oxygenase 1 and heme oxygenase resulting in ferrous ion which is either stored in ferritin or exported out of the cell via FPN. *FLVCR: feline leukemia virus receptor; HCP1: Heme carrier protein 1); DMT-1: divalent metal transporter-1; Dcytb: Duodenal cytochrome b; FPN: ferroportin; HIF: hypoxia-inducible factor; HO: heme oxygenase; CO: carbon monoxide; TfR: transferrin receptor.*

Figure 2- Summary of pathways via which heme induces carcinogenesis and tumour suppression. The dual action of heme is apparently dependent upon the concentration of heme available and the availability of oxygen. Thus, there is a possibility of manipulating the heme concentration and oxygen status to induce tumour suppression. *P53: tumour protein 53; DNA: Deoxyribonucleic acid; ETC: electron transport chain; ATP: adenosine triphosphate; ROS: reactive oxygen species; 4HNE: 4-Hydroxynonenal; MDA: Malonaldehyde*.

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