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Oxidative damage and iron dyshomeostasis in thalassemic patients

Autor

Dr. Elisa Ferro

Coordinator

Prof. Salvatore Cuzzocrea

Tutor

Dr. Maria Angela La Rosa

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1. Introduction

1.1 Iron homeostasis and associated diseases

Iron represents a paradox for living systems by being essential for a wide variety of metabolic processes, but also having the potential to cause deleterious effects. Iron is required in biochemical processes such as metabolism, oxygen delivery, and DNA synthesis. Its biological functions are based on its ability to participate in one-electron reduction– oxidation reactions between the ferric (Fe3+) and the ferrous (Fe2+) states.

However, iron is a leader in free radical production, because of the ability of Fe2+ to catalyze the production of hydroxyl radicals by Fenton reaction (Gutteridge JMC, 1982). Redox imbalance determines the oxidation of membrane phospholipids, glycoxidation, and DNA damage that trigger apoptosis or carcinogenesis (Breimer LH, 1990; Huang X, 2003). Thus, organisms were compelled to solve one of the many paradoxes of life, that is, to keep “free iron” at the lowest possible level and yet in concentrations allowing its adequate supply for the synthesis of hemoproteins and other iron-containing molecules by the evolution of specialized molecules for the acquisition, transport, and storage of iron in a soluble, non toxic form to meet cellular and organismal iron requirements (Ponka P, 1999).

1.1.1 Intracellular regulation

To ensure that there is adequate iron for basal functions but no free iron that could promote formation of reactive oxygen species, iron homeostasis is regulated at both the cellular and systemic level.

Dietary non heme iron is reduced by brush border ferric reductases to Fe2+ that, is transported across the cellular membrane by divalent metal transporter 1 (DMT1), while heme iron appears to be transported intact from the gut lumen into enterocytes, through the activity of heme carrier protein 1 (HCP1). Absorbed iron is rapidly bound to transferrin (TF), an abundant, high affinity iron-binding protein. Very small amounts of iron may be loosely associated with albumin or small molecules e.g., anions such as citrate. The erythroid bone marrow is the largest consumer of iron. Erythroid precursors express cell surface transferrin receptors (TFRs) that take up Fe-TF by receptor-mediated endocytosis (Andrews NC, 2007).

A regulatory mechanism involves iron regulatory proteins (IRPs). These proteins bind iron regulatory elements (IREs) of mRNAs encoding protein involved in iron uptake, storage, utilization and export. The binding of IRPs to IREs serves either of two purposes, depending upon the location of the IREs. IRPs bind to cis-regulatory IRE at 5′UTR of target mRNAs and decrease their rate of translation (FPN1, FTL, HIF-2α) or in 3′ UTR to promote mRNA stability (TFR1).

The production of ferritin, the iron storage protein, a 24-subunit polymer composed of L-ferritin (light or liver ferritin) and H ferritin (heavy or heart ferritin), rapprests a regulatory system accepting excess iron and allowing for the mobilization of iron when needed (Andrews NC, 2007).

A further protective mechanism involves also IRPs, which, when iron is limiting, bind to RNA stem-loop IREs found in 5'UTR of ferritin mRNAs causing a down-regulation of ferritin synthesis in relation to the iron status. Similarly to ferritin, IRP binding to IREs found in the 5'UTR of mRNAs encoding ferroportin, and the heme biosynthetic enzyme aminolevulinate synthase sterically blocks the initiation of

translation by interfering with ribosome assembly at the start codon (Andrews NC, 2007).

Hereditary hyperferritinemia cataract syndrome (HHCS) is a rare autosomal dominant disease (OMIM#600886), caused by mutations of the iron-responsive element (IRE) in the 5'UTR of the ferritin light chain gene (FTL; 134790), clinically characterised by a combination of elevated serum L-ferritin unrelated to body iron stores and early onset of bilateral cataracts (Cazzola M, 1997). We described a new Italian family with seven members affected by HHCS. It is the second Italian family, the first in South Italy, with a c.-168G <T (+32G>C) mutation that is located in FTL promoter with high serum ferritin levels (>980 ng/ml), early-onset of bilateral cataracts, even in a one-year-old child with serum ferritin levels of 2080 ng/ml (Ferro E, 2018).

1.1.2 Systemic regulation of iron and hepcidin

Maintenance of stable extracellular iron concentrations requires the coordinate regulation of iron transport into plasma from dietary sources in the duodenum, from recycled senescent red cells in macrophages and from storage in hepatocytes.

The hormone hepcidin, is the master regulator of iron absorption and distribution to tissues. During studies of antimicrobial properties of various human body fluids, it was isolated from human urine and named hepcidin, based on its site of synthesis (the liver, hep-) and antibacterial properties in vitro (-cidin) (Park CH, 2001).

Hepcidin is encoded by the HAMP gene, hepcidin antimicrobial peptide (OMIM 606464) with cytogenetic location in 19q13.1 (Ganz T, 2006). HAMP gene have also

been identified in other vertebrates including mice, rats, pigs and several species of fish. Mutation in HAMP gene caused the **Juvenile Hereditary Hemocromatosis Type 2** (OMIM#613313), an autosomal recessive disease that leads to severe iron loading and organ failure before 30 years of age (Roetto A, 1999).

Hepcidin is synthesized predominantly in hepatocytes, but other cells and tissues, including macrophages, adipocytes and brain have low levels of expression.

It exists as a (84 amino acids), prohormone (60 amino acids), and (25 amino acids). In addition to the 25–amino acid form, the urine also contains 20– and 22–amino acid forms truncated at the N-terminus (Hugman A, 2006).

Most of the iron in plasma is destined for erythropoiesis in the bone marrow. The daily loss of iron from the body is small (1–2 mg/day) and diets usually contain more iron than is necessary to replace the losses. Dietary iron is absorbed predominantly in the duodenum and absorption increases in response to increased iron requirements due to systemic iron deficiency, anemia or hypoxia. Humans and other mammals lack effective mechanisms to excrete excess iron, and therefore the sole means of maintaining iron balance is by regulating intestinal iron absorption to match systemic iron requirements.

Dietary iron (Fe^{3+}) is reduced by an apical ferric reductase duodenal cytochrome b (DCYTB) to ferrous iron (Fe^{2+}) and imported into the enterocyte via the apical iron transporter, divalent metal transporter-1 (DMT1), stored as ferritin or released into the plasma by Ferroportine (FPN), a basolateral surface membrane iron exporter. An increase in serum iron results in hepatic production of hepcidin, which interacts with FPN on the enterocyte membrane inducing its internalization and degradation (Nemeth E, 2004).

FPN is also expressed on macrophages, and hepatocytes and hepcidin-induced FPN down-regulation, may explain the trapping of iron in macrophages and hepatic stores, in situations associated with increased hepcidin, such as in inflammation or infection (Nemeth E, 2004; Ganz T, 2006).

Then, hepcidin inhibits iron entry into the plasma compartment from the three main sources of iron, dietary absorption in the duodenum, the release of recycled iron from macrophages and the release of stored iron from hepatocytes. Studies support the existence of independent pathways regulating hepcidin in response to iron stores status, systemic iron availability, tissue hypoxia, erythropoietic activity and inflammatory stimuli (Ganz T, 2011).

The rapid progress in understanding hepcidin regulation as it relates to the pathophysiology of the **Hereditary Hemochromatosis (HH)** has clarified many of the cellular and molecular mechanisms of regulating body iron stores. The autosomal dominant form of HH (Type 4, OMIM#606069) is due to mutations in the FPN1 (alternative title SLC40A1) those patients with a clinical-pathological presentation similar to the autosomal recessive forms of HH have mutations that render FPN1 insensitive to down-regulation by hepcidin.

In the four autosomal recessive forms, due to mutations in the genes encoding HFE (HFE, Type 1 OMIM#235200), hemojuvelin (HJV, Type 2A OMIM#602390.), hepcidin (HAMP, Type 2B OMIM#613313) and transferrin receptor-2 (TFR2, Type 3 OMIM#604250), the level of plasma or urinary hepcidin is inappropriately low for the degree of systemic iron stores (Fleming MD, 2008) underlinig that these proteins play a role in hepcidin regulation.

On the contrary, hepcidin overproduction causes anemia of inflammation and **Iron Refractory Iron Deficiency Anemia (IRIDA**, OMIM #206200), a rare automal

hereditary disease of iron metabolism caused by mutations in TMPRSS6 gene (22q12-q13). It encode matriptase-2 (MT-2) a regulatory protein involving in down-regulation of HAMP expression (Ganz T, 2013).

The bone morphogenetic proteins (BMP) pathway and its signaling through SMAD is the key pathway for the regulation of hepcidin transcription (Darshan D, 2009). BMP receptors are members of the transforming growth factor-beta (TGF-β) superfamily of signaling molecules and are tetramers of serine/threonine kinase receptors, usually with two type I and two type II subunits. BMPs control a variety of biological processes during embryonic and postnatal development. BMP binding to a coreceptor facilitates the interaction with a type I BMP receptor with a constitutively active serine/threonine kinase BMP type II receptor. Phosphorylation of the type I receptor, such as BMP2, BMP4, and BMP6, initiates an intracellular signaling cascade involving phosphorylation of stimulatory SMAD. This results in phosphorylation of cytoplasmic Smad1/Smad5/Smad8 which is associated with the common mediator Smad4 that translocate into the nucleus and, binding the specific sequence responsive element of BMPs (BREs), induces HAMP syntesis (Fleming MD, 2008, Darshan D, 2009).

The hemojuvelin (HJV, also known as HFE2 or RGMC) is a glycophosphatidyl inositol (GPI)-linked membrane protein that is a member of the repulsive guidance molecule (RGM) family. HJV is expressed in the liver, and Juvenile Haemochromatosis Type 2A patients with HJV mutations and Hjv knockout mice exhibit significantly reduced hepatic hepcidin expression, thereby implicating HJV in the regulation of hepcidin synthesis (Papanikolaou G, 2004). The chronic injection of s-HJV in mice, in fact, causes iron overload reducing the hepcidin syntesis (Ganz T, 2006).

HJV exists in two forms, membrane HJV (mHJV) and soluble HJV (sHJV). mHJV has been identified as a co-receptor of BMP and facilitates the activation of the BMP-type I/type II receptor complex that activates the HAMP gene (Malyszko J, 2009) whereas sHJV inhibits hepcidin expression by competitive action against mHJV (Babitt JL, 2006; Lin L, 2005).

The HJV-BMP axis was demonstrated to be crucial for its role in iron-dependent transcriptional regulation mechanism of hepcidin. The generation of sHJV appears to be increased by iron treatment and hypoxia. Both stimuli lead to reduced hepcidin production and increased iron flow into the plasma. HJV, in fact, is a cleavage target of MT-2, and by the furin family of proprotein convertases, both regulated by HIF-1 α and HIF-2 α (Babitt JL, 2006; Silvestri L, 2008). The cleavage of HJV on the membrane surface, suppresses signaling through the BMP-Smad signaling pathway and thus resulting in reduced transcription of HAMP. Together, these mechanisms of hepcidin regulation ensure an adequate iron supply to meet the demands of hemoglobin and myoglobin production (Darshan D, 2009).

In addition to the liver, HJV mRNA is also highly expressed in skeletal muscle and heart. It was previously hypothesized that skeletal muscle and/or heart could serve as a source of sHJV to suppress hepcidin synthesis in response to iron deficiency or hypoxia. However, mice with a specific knockout of *HJV* in skeletal and cardiac muscle do not have altered hepcidin expression or systemic iron balance, at least under basal conditions or with dietary iron changes. Whether strenuous exercise or hypoxia may uncover a role for muscle hemojuvelin remains uncertain hepatic expression of HJV appears to have the most important physiologic role in systemic iron homeostasis regulation (Core AB, 2014).

Liver hepcidin expression is inappropriately low in mice and humans with *HFE* or *TFR2* mutations, suggesting that both HFE and TFR2 positively regulate liver hepcidin expression (Gross CN, 1998).

The HFE protein has been shown to interact with TfR1 at a site that overlaps with the binding site for transferrine (Tf). The level of circulating diferric Tf is a likely means by which information about body iron stores and demand is communicated to the hepcidin regulatory machinery. HFE competes with olotransferrin for TFR1 receptor binding. The HFE/TFR1 complex is in dynamic equilibrium with the HFE/TFR2 complex. This balance is modified by concentrations of olotransferrine, when the concentration of olotransferrine increases, this "shifts" HFE from the TFR1 receptor to which it is bound. HFE remains free to bind to TfR2 and the olotransferrin binding to TfR1 allows the iron to enter the cell (Darshan D, 2009).

All cytosolic proteins involved in this pathway have not been identified, while it appears that HFE and TFR2 interact at some level with the BMP-HJV-SMAD pathway to regulate liver hepcidin expression, but the precise molecular mechanisms of how HFE and TFR2 contribute to hepcidin regulation remain an active area of investigation (Rishi G, 2015; Rishi GC, 2013).

However, iron homeostasis is also affected by inflammatory signals that, by increasing hepcidin expression, lead to anemia of inflammation mediated by the IL-6-Stat 3 axis (Ganz T, 2013). STAT3 signaling pathway functions through STAT3RE, a response element located upstream of the HAMP gene. The proinflammatory cytokine IL-6 binds to its specific transcriptional factor STAT3 by the signaling kinase JAK2. Then, phospho-STAT3 transfers to the nucleus and binds the STAT3RE of HAMP to increase hepcidin transcription. Other cytokines, such as IL-22 and oncostatin M, can also promote hepcidin synthesis by activating the STAT3 pathway. Moreover, another

study performed in HepG2 cells suggested that GATA4 may bind to the HAMP promoter to enhance its transcriptional activity. Mutation of the GATA4 binding site impaired the IL-6 induction of HAMP transcription, but had no effect on BMP6-Smad signaling (Wang C, 2017).

Alternative mechanism of HJV down-regulation by TNF-α has been proposed (Salama MF, 2012).

1.1.3 Tissue hypoxia and erytroid regulators of hepcidin

In eukaryotic cells, oxygen is the terminal electron acceptor of the mitochondrial electron transport chain for ATP production in oxidative phosphorylation (Seagroves TN, 2001). The importance of oxygen homeostasis has led to the evolution of multiple mechanisms by which cells and organisms maintain an adequate O_2 supply. Oxygen deprivation in tissues causes a metabolic switch from oxidative phosphorylation to anaerobic lactate/ATP production with increased glycolysis. Systemic adaptive responses to hypoxia involve haematological responses, constriction of pulmonary arteries and hyperventilation (Smith TG, 2008; Weir EK, 2005). Within cells, hypoxia activates hypoxia-inducible factors (HIFs), the major signal transducers that mediate the transcription of about 400 hypoxia-sensitive genes (Schodel J, 2011). These include VEGF, GLUT-1 and other genes such as EPO, EPOR, HAMP, TF, and TFRC in different organs (kidney, liver, intestine, blood and bone marrow), and help erythropoiesis control, iron and oxygen homeostasis (Semenza G, 2009).

In the HIF family HIF-1 and HIF-2 are generally considered to be master regulators of the transcriptional response to hypoxia, while the role of HIF-3 under

hypoxic conditions is far less clear. HIF-1α plays a key role in the initial response to hypoxia whereas HIF-2α drives the hypoxic response during chronic exposure to low O_2 tension (Milosevic J, 2009; Thomas R, 2007). This HIF "switch" suggests physiological and pathological adaptation for cell survival.

The most extensively studied HIF-1 is a heterodimer composed of two subunits, an oxygen/iron-sensitive HIF-1α, which is post-translationally regulated, and a constitutively expressed HIF-1β. In normoxia, the α subunits are constantly degraded by prolyl hydroxylases (PHDs) (Bruick RK, 2001). PHDs utilize oxygen as a substrate and iron as a cofactor to hydroxylate proline residues that are recognized by the tumour suppressor protein, von Hippel–Lindau, and marked for ubiquitination and proteasomal degradation (Ivan M, 2001; Jaakkola P, 2001).

The mitochondrial electron transport chain is the first oxygen sensor in hypoxia, increasing the generation of reactive oxygen species (ROS) that, upon release in the cytosol, begin HIF-1α stabilization (Brunelle JK, 2005). Under hypoxia, impaired PHD activity contributes to subunit stabilization. The stabilized HIF-1α translocates to the nucleus and, on binding to the β-subunit, forms the heterodimer that induces expression of the gene target for cellular hypoxia response. Protein phosphorylation is also required for signal transduction and HIF-responsive-element DNA binding (Ivan M, 2001).

Approximately 65–70% of body iron is found in erythrocytes in the form of hemoglobin and this represents the largest source for iron in the body. Consequently, body iron demand is closely linked to the rate of erythropoiesis (Darshan D., 2009).

HIF was proposed as link between iron homeostasis and erythropoiesis. Hypoxia and HIF signaling are the primary regulators of *EPO*. Studies in mice demonstrated

that HIF-2α binding to HRE in the Epo promoter is the master regulator of renal EPO expression (Kapitsinou PP, 2010).

Hepcidin suppression by bone medullary erythropoiesis was historically ascribed to the action of a putative “erythroid regulator”. Convincing evidence against a direct effect of erythropoietin on hepcidin suppression were reported (Pinto JP, 2008).

HIF-1 has also been proposed as a regulator of iron homeostasis through repression of the hepcidin gene expression (Peyssonnaux C, 2007; Anderson ER, 2012). More recently, HIF-2 has emerged as a regulator of intestinal iron absorption that directly trans-activates the expression of iron transporter genes in enterocytes. Nevertheless, recent evidence indicates that hepcidin suppression by the HIF pathway is indirect, through the EPO-mediated stimulation of erythropoiesis (Mastrogiannaki M, 2012; Mastrogiannaki MM, 2013).

Growth differentiation factor 15 (GDF15) has been proposed as a mediator of decreased hepcidin expression (Tanno T, 2007). It was therefore hypothesized that GDF15 may exert its repressive effect together with another secreted factor. Subsequently, was founded Twisted Gastrulation Protein 1 (TWSG1) (Tanno T, 2009) produced during the early phases of erythropoiesis. TWSG1 was thought to be an antagonist of BMPs, inhibiting the BMP-Smad signaling pathway, thereby reducing the expression of hepcidin. However, hepcidin was strongly inhibited after bleeding, but no significant change was observed in GDF-15 or TWSG1 levels, suggesting that this repression was caused by other erythroid factors (Casanovas G, 2013, Theurl I, 2010).

The major negative regulator of hepcidin in conditions of stress or ineffective erythropoiesis, was identified more recently as erytroferrone (ERFE, also known as

FAM132B), a glycoprotein hormone secreted by erythropoietin-stimulated erythroblasts (Kautz L,2014).

Phlebotomy or EPO administration in ERFE-knockout mice failed to suppress hepcidin, demonstrating that ERFE is absolutely necessary for acute hepcidin response to increased erythroid activity. Exogenous ERFE significantly inhibited hepcidin expression in primary hepatocytes. Recently, a first-generation assay for human ERFE (hERFE) was developed, proving the pathological increases of ERFE with a parallel decrease of hepcidin in β-thalassemia patients (Ganz T, 2017).

GDF11 is another possible inhibitor of hepcidin expression. It is secreted by late-stage erythroid progenitors, and it is also significantly increased in β-thalassemia mice and in patient sera. Whether GDF11 can directly inhibit the synthesis of hepcidin in hepatocytes remains to be studied (Wang C, 2017). All these are erythroid factors that may participate in controlling hepcidin expression, but their precise roles require further study.

1.2 Redox imbalance and genotoxicity in thalassemias, secondary dyshomeostasis diseases

The β-thalassemias are autosomal recessive disorders characterized by absent or reduced synthesis of ß-globin chains. The imbalance of α- and β-globin chains causes a premature death of erythroid progenitors in the bone marrow and in the spleen for the excess of α-globin chains, which precipitate as tetramers in the red blood cells. This leads to severe anemia, tissue hypoxia, marked hemocatheresis, hepatosplenomegaly, and ineffective erythropoiesis (Galanello R, 2010).

Tissue hypoxia and erythropoiesis downregulates the expression of hepcidin and increases intestinal iron absorption (Semenza GL, 2009; Haidar R, 2010)

Chronic anemia and iron overload are the main complications of β-thalassemic patients. β-thalassemia intermedia is clinically heterogeneous. Generally, the mildest form of anemia do not require a steady transfusion treatment (Galanello R, 2010). Thalassemia major patients are regularly transfused to improve haemoglobin levels, suppress bone marrow activity and reduce gastrointestinal iron absorption. This treatment is designed to maintain Hb levels between 9.5 and 10 g/dl, in accordance with the guidelines for the clinical management of thalassemia and the Thalassemia International Federation (Galanello R, 2010). Blood transfusion therapy increases non-transferrin bound iron (NTBI) and can lead to multi-organ complications due to haemosiderosis (Delea TE, 2007). Clinical effects of the abnormal iron stores are liver disease (liver fibrosis and cirrhosis), cardiac dysfunction, arthropathy, gonadal insufficiency and other endocrinal disorders.

Hypogonadism, the most common endocrinopathy in thalassaemic patients (Chirico V, 2015), may result from iron deposits in the hypothalamic–pituitary cells or in the gonads. Other common endocrine complications in thalassemic patients are short stature, thyroid and parathyroid dysfunctions and diabetes.

Osteopoenia and osteoporosis represent the most common bone metabolic disorders and the prominent causes of morbidity in thalassemia. Iron overload-induced liver fibrosis can be exacerbated by intercurrent transfusion-dependent viral infection (Vento S, 2006). Chronic liver hepatitis can evolve to cirrhosis and HCC that is very frequent in thalassemia (Borgna-Pignatti C, 2004).

Since the 1970s, the use of lifelong iron chelation therapy with compounds such us desferrioxamine, deferiprone, and deferasirox, has increased the life expectancy of transfused dependent thalassaemic patients, limiting iron overload complications (Gabutti V, 1996).

Iron overload induces reactive oxygen species (ROS) overproduction (Esposito BP, 2003). Many pathological aspects of the hemoglobinopathies are mediated by oxidative stress due to an imbalance between radical species and enzymatic and non enzymatic antioxidant compounds. Redox-active iron catalyzes the generation of not only hydroxyl radicals, by the Fenton reaction, but also of organic reactive species, such as peroxyl (ROOS), alkoxyl (ROS), thiyl (RS), orthiylperoxyl (RSOOS) radicals (Papanikolaou G, 2005). Iron keeps its catalyst role bound to free heme as well as within the hemoproteins. The haemoglobin release during hemolysis enhances the redox imbalance by oxoferryl intermediates (Ryter SW, 2000). A further source of free radical generation is the direct reaction between Fe+ and oxygen with the in vivo production of ferryl (Fe2+–O) or perferryl (Fe2+–O2) iron intermediates contributing to a greater redox imbalance. Unlike other reactive oxygen species, $^{\cdot}$OH is the only

radical able to react with all components of DNA leading to formation of 8-Oxo-2′-deoxyguanosine (8-oxo-dG). It is one of the main oxidation products of guanosine (dG) and leads to misreading and misinsertion of nucleotides during DNA synthesis, leading to G→T and G→C conversions. The 8-oxodG levels are elevated in some human pre-neoplastic lesions and cancerous tissues (Gedik CM, 2002). Commonly considered as a valid DNA oxidation biomarker, it is indicative of a potential mutagenic and carcinogenic process. Recently, 8-oxo-dG levels in the urine have been associated with a risk colorectal cancer (Guo C, 2016).

In addition to the direct reaction between DNA and hydroxyl radicals, iron genotoxicity can be due to lipid peroxidation end-products. Lipid hydroperoxides are transient intermediate species in the living cells, since they are rapidly converted into free radicals, carbonyl compounds and epoxides. In particular, decomposition of hydroperoxides may form much more toxic breakdown products such as RO, ROO and reactive aldehydes, included malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and 13-hydroperoxy-octadecadienoic acid (13-HPODE). The carbonyl compounds have a strong promutagenic effect forming DNA adducts. In particular 4-HNE forms exocyclic etheno-DNA-base adducts (Nair J, 2007). These are highly promutagenic lesions and allow transitions as well as transversions, whereas MDA-DNA adducts can cause both base pair substitutions and transversions (Frank A, 2004).

Higher levels of 1,N6-ethenodeoxyadenosine and 3,N4-ethenodeoxycytidine were detected in the livers of thalassaemic patients (Meerang M, 2009) and the genotoxic effect of lipid peroxidation end-products was observed in the urine and lymphocytic DNA of thalassaemic patients (Meerang M, 2008). Furthermore, significant lipid

peroxidation was assessed in β-thalassemic subjects by the analysis of the plasma level of thiobarbituric acid reactants (Meral A, 2000; Cighetti G, 2002; Naithani R, 2006).

We confirmed this cellular damage in β-thalassemic subjects by the fluorescent-activated cell sorter (FACS) detection of lipid hydroperoxides and highlighted a significant increase in talassemia intermedia untransfused patients with more severe anemia and lower iron overload than major polytransfused patients (Ferro E, 2012). To date, redox imbalance in thalassaemic patients has mainly been attributed to iron but chronic tissue hypoxia is a source of oxidative damage, as shown by mouse exposure to altitude and by observations in hypoxic chronic obstructive pulmonary disease patients (Semenza G., 2009; Semenza G., 2011). Lipid peroxidation causes hemolysis, which worsens the already severe anemia and further enhances redox imbalance due to haemoglobin release.

The mitochondrial membrane potential ($\Delta\Psi m$) is a key indicator of mitochondrial function. The electrochemical gradient across the mitochondrial membrane is indicative of an active proton gradient that drives ATP (Samudio I, 2008). The hypoxia effect is highlighted also by the higher lymphocytic ROS levels and lower mitochondrial transmembrane potential in untransfused subjects, with more severe anemia and lower iron overload, in comparison to major polytransfused patients (Ferro E, 2012).

Tissue hypoxia, in addition to enteric iron absorption increases (Haidar R, 2010), is a source of ROS, because lower levels of PO_2 induce mitochondrial ROS overproduction (Semenza GL, 2009). This additional source of oxidative damage has been underestimated in thalassemia, while we observed, for the first time, that redox imbalance was inversely related to Hb levels in these patients (Ferro E, 2012).

Redox imbalance determines the oxidation of membrane phospholipids, glycoxidation, and DNA damage that trigger apoptosis or carcinogenesis (Breimer LH, 1990; Huang X, 2003).

Thalassemic patients show chromosomal aberrations and micronuclei (Côté GB, 1979; Offer T, 2005), which are cytogenetic biomarkers of cancer (Bonassi S, 2000; Iarmarcovai G, 2008). Iron overload is associated with an increased cancer risk. Malignancies, that were considered rare in β-thalassemia, have been detected at higher frequency, possibly because of the increased age of the affected population. In addition to hepatocellular carcinoma (HCC), frequently diagnosed in haemochromatosis but also in thalassemias, other types of cancer including leukaemias are observed (Zanella S, 2016; Baldini M, 2012; Sherief LM, 2015).

Cytokinesis-block micronucleus (CBMN) and comet assay are used to assess genotoxicity in the peripheral blood lymphocytes, after in vitro activation.

Circulating throughout the body and recording genotoxic insults received in various parts of the organism, the lymphocytes are the preferred cell model to assess DNA damage, as they are an 'ideal biological dosimeter'. Lymphocytes 'store' the results of these contacts and accumulated damage is not remedied in the quiescent phase of the cell cycle (G0), it can be detected when the cells undergo mitotic division in vitro. Thus, an increased lymphocytic MNi frequency can be the result of cumulative effects, due to recent or previous exposures (Vral A, 2011).

The cytokinesis-block micronucleus (CBMN) assay in human lymphocytes has become one of the most widely used methods for measuring chromosomal damage in human cells in vitro and in vivo (Bolognesi C and Fenech M, 2013; Kirsch-Volders M, 2014). In human biomonitoring studies CBMN has been used for decades as a standard biodosimetry assay to assess the genotoxic effects of exposure to chemical

compounds in occupational and environmental settings. The assay is also applied in *in vitro* genetic toxicology to test new compounds for regulatory purposes (OECD, 2009).

The Micronuclei (MNi) originate from acentric chromatid/chromosome fragments or from loss of whole chromosomes during telophase. Since it represents a measure of both chromosome breakage and chromosome loss, an increased frequency of micronucleated cells can reflect exposure to genotoxic agents with clastogenic or aneugenic modes of action (Albertini RJ, 2000). MNi frequency, other than as a index of genotoxicity, can be considered an early biomarker of cancer risk (Bonassi S, 2007). Moreover, by the nuclear division index (NDI) and proportion of necrotic and apoptotic cells, CBMN assesses mitogen response and cytotoxicity (Fenech M, 2007).

HUMN, which stands for HUman MicroNucleus, is also known as the international Collaborative Project on Micronucleus Frequency in Human Populations. HUMN was launched in 1997 because of world-wide interest in the application of the micronucleus (MN) method to assess environmental effects on chromosome damage in blood and epithelial tissues in human populations.

We reported higher DNA damage expressed by %TDNA (parameter of Comet assay) and micronuclei frequency (MNi, parameter of CBMN assay) in β-thalassemia major patients compared to untransfused and healthy controls (Ferro E, 2012).

The comet assay (single-cell gel electrophoresis) is a simple, rapid, reliable, and very sensitive method to measure genotoxicity. The high sensitivity of the comet assay alkaline version is mainly attributed to the capability to recognize the temporary presence of alkali-labile abasic sites, produced by DNA glycosylase during the repair mechanism that restores the double strand integrity (Collins AR, 2012). The comet assay is a well-established technique in genotoxicology and in 2012 ComNet project

was launched, which aims at investigating whether the comet assay is a reliable, validated biomarker assay that can be used in human biomonitoring (Collins A, 2012).

During the last 30 years, the comet assay has become widely used for the measurement of DNA damage and repair in cells and tissues. A landmark achievement was reached in 2016 when the Organization for Economic Cooperation and Development adopted a comet assay guideline for in vivo testing of DNA strand breaks in animals (OECD, 2016).

An early application of the comet assay was in investigating antioxidant status. When cells are treated with H_2O_2, breaks are induced, and the effectiveness of break production depends on the level of antioxidant defences within the cells. These defences include glutathione (a sulphydryl-rich tripeptide, present at high concentration, and of special importance in the nucleus) (Markovic J, 2010). This method was also used in nutritional intervention trials, in studies of workers occupationally exposed to genotoxic chemicals, and in investigating the effect of repair gene polymorphisms (Collins AR, 2012). The comet assay is a valuable tool for quantifying DNA damage in populations exposed to various types and doses of genotoxic agents, and can usefully contribute to the 'biological effect dosing' of occupational and environmental exposures. Increased DNA damage was found in human populations exposed to air pollution, radiation, and pesticides (Collins A, 2014).

Recently, the comet assay together with the micronucleus assays, has become the most popular technique in genotoxicity studies on nanomaterials (Doak SH, 2017). It is a highly versatile assay and the large number of studies on various types of genotoxic agents, more than 700 articles indexed in PubMed with comet assay results per year, makes it a suitable screening assayfor DNA damage (Peter M, 2018).

2. Objectives

Redox imbalance and genotoxic damage iron-induced are commonly observed in β thalassaemic patients. Were previously reported that anemia-induced hypoxia is an additional source of oxidative damage that has been underestimated in thalassemia. The hypoxia effect is highlighted by the higher lipid hydroperoxides, lymphocytic ROS and lower mitochondrial transmembrane potential in untransfused thalassemia intermedia patients with more severe anemia and lower iron overload in comparison to major polytransfused patients. Furthermore, we observed significantly high genotoxicity in regularly transfused subjects (Ferro E, 2012).

A better knowledge on the behaviour of iron homeostasis parameters and its relationship with oxidative imbalance and genotoxicity may allow a better understanding of thalassemia, a pathology in which hypoxia, erythropoiesis, iron overload and inflammation coexist. Based on these findings, I aimed herein to investigate, the pathophysiological mechanisms of iron dyshomeostasis in transfused and untransfused thalassaemic patients by assesing hepcidin and co-modulatory factors, such us HIF1α and Glut 1 (Ferro E, 2016), soluble transferrin receptor (sTFR), soluble hemojuvelin (sHJV), GDF15 ([a]Ferro E, 2017) and erytroferrone (ERFE); the role of anemia in oxidative and genotoxic damage in regularly transfused thalassaemic patients in relation to blood transfusion regimen and iron chelation therapy to improve the effectiveness of therapeutic management ([b]Ferro E, 2017); the genotoxic/cytotoxic effect of thalassemia complications and their drug treatments, in addition to universally know oxidative damage induced by iron overload, in politransfused patients ([c]Ferro E, 2017).

3. Methods

3.1 Patients

One hundred and five (58 women and 47 men) transfusion dependent and fifteen (6 women and 9men) non-transfusion dependent patients with thalassemia were recruited from Messina University Hospital, Riuniti Hospital in Reggio Calabria and Sant'Agata Militello Hospital, Italy. Patients gave informed consent to participate in the study and approval was obtained from the Ethics Committees of the hospitals. All transfusion dependent patients underwent iron chelation therapy which was not suspended before sampling. Drugs were administered as follows, 33 patients were treated with DFX (30 mg/kg once daily in the morning per os), 27 with DFO (40 mg/kg per day subcutaneously overnight from 8,00 pm until 8,00 am), 17 with DFP (75 mg/kg per os in three daily doses) and 28 with combined DFO + DFP (40 mg/kg of DFO for three days of the week and 75 mg/kg of DFP in three daily doses on the remaining four days). A control cohort was formed by enrolling twenty healthy individuals matched with patients for age and gender.

Five milliters of blood were collected from each patient and serum aliquots were stored at -80°C until use. Sampling time was immediately before packed red blood cell (RBC) transfusion, in order to better assess the effects of anemia. In the patient group, samples were collected about 2 hours after taking DFX or DFP and to about 3 hours after the end of DFO treatment.

The transfusion regimen for each subjects was based on haematological parameters; the average interval between transfusions was 15–20 days. The number of total transfused RBCs (ml/kg/year) was calculated using the equation, RBCs = Annual blood intake × 0.65 (haematocrit 65%)/kg body weight.

Iron intake was calculated by multiplying the total transfused RBCs by 1.08 (Cappellini MD, 2008) and it not was reported in Tables for its overimposable trend with the latter.

Were determined levels of Hepcidin, Hipoxia Inducible Factor 1α (HIF-1α), Glucose transporter 1 (Glut 1), Growth differentiation factor 15 (GDF15), soluble transferrin receptor factor (sTfR), erytropoitin (EPO) levels. Hemocromocytometric parameters, serum ferritin, iron, transferrin, transferrin saturation (%TSAT), pre-transfusion haemoglobin, Reticolocytes ‰ were determined as routine exams.

A subgroup of 20 (10 male, 10 female) transfused (T) patient samples were collected just before blood transfusion (T0) and 4/6 days after transfusion (T1) when erythroid suppression was expected to be maximal. In addition were recruited also 24 (12 male, 12 female) untransfused (U) and 20 (10 male, 10 female) healthy controls. In T group the 50% of patients were splenectomized while in U were 29.16%. In these sample were determined in addition to hemocromocytometric and iron parameters, erytroferrone (ERFE) and Hipoxia Inducible Factor 2α (HIF-2α), soluble transferrin receptor factor (sTfR), erytropoitin (EPO) and martial indexes, fetal hemoglobin (HbF) ratio and nucleated red blood cells (NRBC%), a marker of ineffective erythropoiesis.

Cardiac and hepatic MRI T2* were detected to evaluate tissue iron stores by standardized and validated procedures of MIOT (Myocardial Iron Overload in Thalassemia) project (Ramazzotti A, 2009). Cardiac iron overload was defined by MRI T2*global values as, mild (14-20 ms), moderate (10-14 ms) or severe (<10ms). Hepatic iron overload was defined by MRI T2* values as, mild (6.3-2.7ms), moderate (2.6-1.4 ms) or severe (< 1.4 ms).

In according to the absence-presence of detectable serum HCV-RNA, patients were characterised as HCV-RNA– or HCV-RNA+ subjects, respectively. The patients were also valuated for disease complications with high prevalence in β-thalassemia as hepatic disease, hypothyroidism, hypogonadism, diabetes mellitus, short stature and bone disease (osteopenia and osteoporosis), along with their respective drug therapies.

3.1 Hypoxia and Iron homeostasis endpoints

The serum aliquots stored at -80°C were used for the measurements of hepcidin (EIA-4256, DRG Instruments GmbH, Frauenberstrabe), GDF15 (SK001108-01; Adipo Bioscience, Santa Clara, USA), soluble hemoyuvelin (sHJV, E91995Hu; USCN Life Science, Houston, USA), soluble transferrin receptor (sTfR, EIA-5258 Instruments GmbH, Frauenberstrabe), hypoxia inducible factor 2 (HIF2α, SEU450Hu; Cloud-Clone Corp., Katy, USA) and erytroferrone (ERFE, SED466Hu; Cloud-Clone Corp., Katy, USA). The determinations were performed in duplicate by ELISA kits following the recommended protocol.

Absorbance was measured using a microplate reader (BIORAD), and standard curves were constructed by the Microplate Manager Biorad program V.5.1.

HIF-1α and Glut 1. Cytoplasmic and nuclear fractions of HIF-1α were extracted from lymphocytes as previously described (Ferro E, 2016) and, before Western blot analysis, their protein content was quantified by using the Bradford method.

After denaturation in SDS-sample buffer, cytoplasmic (30 µg) and nuclear (5 µg) proteins were separated by 8.5% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked overnight at 4°C with 5% non-fat dry milk; then membranes were probed with rabbit anti-HIF-1α polyclonal antibody (Sigma-Aldrich) (diluted 1,500 in TBS-T). Mouse anti-beta-actin (diluteed 1,10000 in TBS-T) and rabbit anti-PCNA (diluted 1,1000 in TBS-T) were used as endogenous controls for cytoplasmic and nuclear proteins respectively. Lastly, incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (diluted 1,300 in TBS-T) or horseradish peroxidase-conjugated anti-mouse secondary antibodies (diluted 1,20000

in TBS-T) (Sigma-Aldrich) was performed. The proteins were visualized by chemiluminescence with an ECL kit on Kodak film. Blots were scanned and quantified by densitometric analysis with the AlphaImager 1200 System (Alpha Innotech, San Leandro, CA).

HIF-1α determination was validated by the measurements of Glucose Transporter 1 (Glut-1), directly regulated by HIF-1α. Glut-1 receptor expressed on the cell surfaces was also detected cytofluorimetrically using a rabbit anti-Glut-1 polyclonal antibody (AnaSpec, CA, USA). The antibody was added to the cell suspensions diluted 1,200 in PBS and incubated for 1 h at 37 °C.

Subsequently the cell suspensions were centrifuged, washed with PBS, added to an anti-rabbit secondary antibody, FITC-loaded and incubated for 1 h at room temperature. In the cell population that was selected based on physical parameters, the emission values were detected in the FL-1 channel. In all FACS analyses, the weighted averages of emission values for 100 cells were calculated and expressed in AFU.

3.2 Oxidative imbalance biomarkers

Lymphocyte culture. Five ml of heparinized blood were collected from each patient, 2 ml were centrifuged and the serum aliquots were stored at -80°C until use. The remaining 3 ml were used for lymphocyte separation by Lymphoprep Separation Medium^{TM} (Axis-Shield Oslo, Norway). The buffy layer was removed, washed with PBS and collected by centrifugation. Lymphocytes (~1 x 10^4 cells ml^{-1}) were

suspended in PBS containing 10 mM D-glucose (pH 7.4) and four aliquots were prepared for cytofluorimetric assays.

Intracellular ROS were evaluated using the membrane-permeable lipophilic 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) (final concentration 1 μM). The probe undergoes deacetylation by intracellular esterases and specifically releases green fluorescent signals (530 nm) when activated by ROS, allowing the detection of redox imbalance (Ferro E, 2012).

8-Oxo-2′-deoxyguanosine (8-oxo-dG) was detected by the fluorochrome FITC-labeled avidin. The probe binds to 8-oxo-dG with a highly specificity due to the structural analogies between the keto form of the oxidized base and biotin. The method, previously adapted to the flow cytofluorimetric analysis was performed in lymphocytes permeabilized in methanol (15 min at -20 °C) and loaded with the avidin–FITC conjugate (1 h at 37 °C). The emission signals were collected in the green fluorescence channel (FL-1).

Mitochondrial membrane potential (ΔΨm) was evaluated by measuring the incorporation of the fluorescent probe rhodamin 123 (R123). The chemical properties of the cationic fluorochrome allow the mitochondrial membrane crossing and storage in the matrix only in functional mitochondria that possess a ΔΨm indicative of an active proton gradient maintained during oxidative phosphorylation. The probe was added to cells suspensions (final 0.2 μM) and incubated (10 min at 37 °C). The emission signals were collected in the red fluorescence channel (FL-2).

Labile Plasma Iron (LPI) which, unlike the Fe 3+-transferrin-bound (TBI), catalyses ROS formation, was analysed following the method devised by Esposito (Esposito BP, 2003). This fluorimetric assay measures iron-specific redox cycling capacity. Redox reactions were detected by the oxidation of the non-fluorescent probe

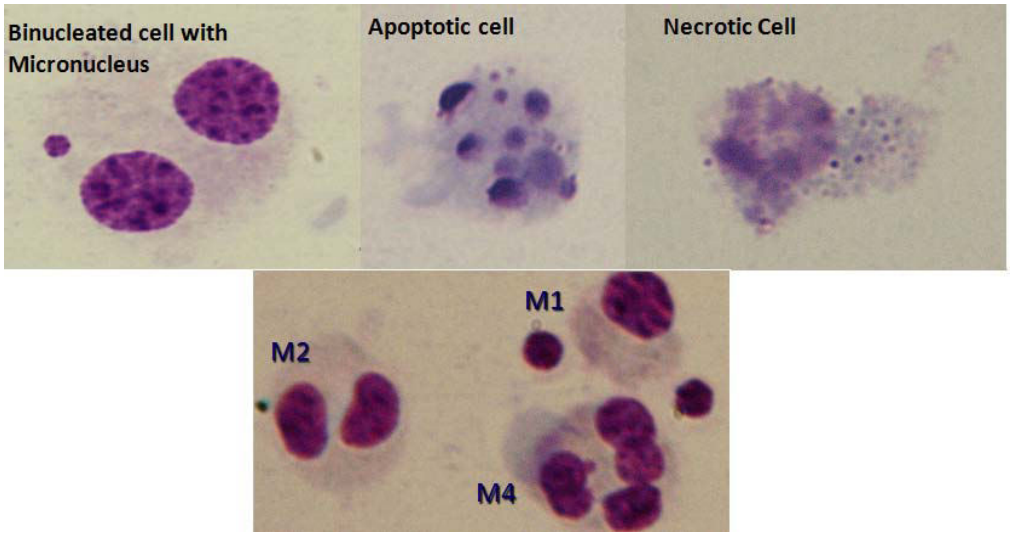
dihydrorhodamine (DHR) to its fluorescent form rhodamine. The assessment of labile forms of iron, independently of other DHR oxidation mechanisms, samples were analysed in parallel by the addition of the iron chelator DFO which quenches only iron induced fluorescence. Briefly, serum samples were assayed in quadruplicate in 96-well plates by adding the DHR (50 μM) in reagent solution (pH 7.3) containing 40 μM of ascorbate to regenerate Fe2+ after its oxidation to Fe3+ DFO (50 μM) was added to this solution in two of the wells. A Fe:NTA (1,7 mM) complex, starting from freshly prepared ferrous ammonium sulphate and nitrilotriacetic acid (NTA) (pH 7.0), was used to build a calibration curve (0.2–4 μM). Emitted fluorescence was recorded every 2 minutes starting from 15 up to 40 minutes using excitation/emission filters 485/ 538 nm (Tecan, Brescia, Italia). The differences between samples with and without DFO, due to redox-active iron, were used to calculate fluorescence units per minute (FU/minute). LPI values (μM) were extrapolated from the calibration curves.

In FACS analyses, the weighted average of emission values for 100 cells was calculated and expressed in arbitrary fluorescence units (AFU).

3.3 CBMN and Comet assay

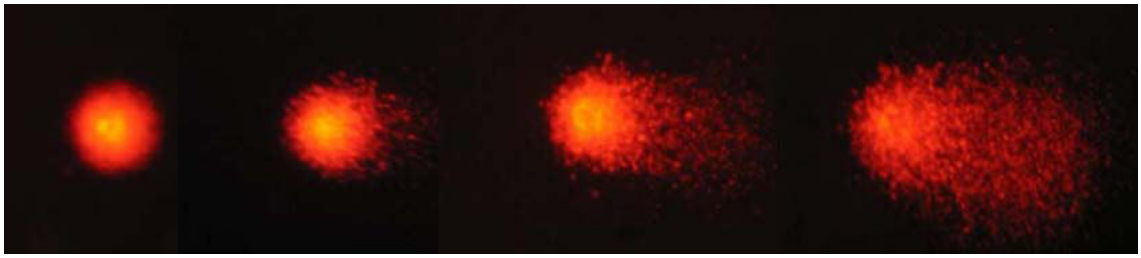
CBMN assay. For each sample, the cells were resuspended in two 10 ml falcon tubes containing RPMI 1640 with L-Glutammine, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 10% fetal bovine serum and 2 % phytohemagglutinin (PHA). The cultures were incubated at 37 °C in humidified 5% CO_2 atmosphere for 72 h. Cytocalasin B (final concentration 4,5 μg ml^{-1}) was added 44 h after PHA stimulation (Ferro E, 2012). Then, the cells were fixed in 3:1 methanol/glacial acetic acid, dropped

onto clean microscopic slides, air-dried, and Giemsa stained. Using a bright field microscope (Leica DM5500B, Mannheim, Germany), each sample was scored at 100X magnification mono-, bi- and multinucleated cells, necrotic and apoptotic cells in 500 lymphocytes. MNi, NPBs and NBUDs were scored in 1000 binucleated lymphocytes. The used Scoring Criteria of CBMN are described in detail by Fenech (Fenech M, 2007). To determine cytostatic effects, NDI was calculated by the frequency of mono-, bi, and multinucleated cells, using the formula (M1 + 2M2 + 3M3 + 4M4)/N, while cytotoxicity was assessed according to the proportion of necrotic and apoptotic cells.



Comet assay. The comet assay was performed in duplicate according to the method proposed by Tice et al. (Tice RR, 2000). DNA unwinding and lysis were performed for 20 min and 1 h, respectively, whereas electrophoresis was carried out for 30 min at 300 mA and 25 V (0.86 V/cm). Analyses were carried out using a DM IRB fluorescence microscope at 400× magnification (Leica Microsystem). Images of at least 100 randomly selected nuclei (ethidium bromide stained 2 mg/mL in water) were analysed by the CASP automated image analysis system (http,//www.casp.sourceforge.net). In addition to percentage of DNA in tail

(%TDNA), comet assay results were reported as DNA density, whose values were obtained computing the ratio between percentage head DNA and head diameter, both standardized endpoints of comet assay, supplied by CASP analysis. This unusual parameter, considered by us as marker of chromatin compactness, was used to evaluate abnormal comets without or with small tail and with a particularly large head and confirmed as genotoxicity biomarker by the inverse relationships with MNi (Ferro E, 2012). The structures that were almost exclusively present in the patient spots were formed in the unwinding phase and were presumably due to the ability of the highly damaged DNA strands to spread radially at alkaline pH even in the absence of an electric field. Unlike classical parameters that analyse exclusively the tail of comet to evaluate DNA mobility (i.e. %TDNA, tail moment and Olive tail moment), the DNA density highlights a greater sensitivity, allowing to assess the early mobility of highly damaged DNA. It is useful to underline that, paradoxically, despite the presence of this damage, the radial spread causes an underestimation of the classical parameters of comet assay as it limits the formation of long tails with high percentage of DNA when the electrophoresis is performed.



4. Statistic

Continuous variables were expressed either as mean ± standard deviation and as median and interquartile range. Examined variables were not normally distributed, such as verified by Kolmogorov Smirnov test; consequently the non-parametric approach has been used.

In order to assess any significant differences between patients group (U, T) and controls (C), between splenectomized (S) and non-plenectomized patients (NS) or between groups according to the absence/presence of others disease complications, Mann Whitney test was applied for all examined numerical parameters. Categorical variables, expressed as percentages, were compared by the X^2 test.

Spearman test was applied to assess the interdependence relationship between numerical parameters. Multivariate regression analysis was performed using *a priori* models. Statistical analyses were performed using STATISTICA program (version 10.0) and $P<0.05$ was considered to be significant.

5. Results

5.1 Section 1- hepcidin and co-modulatory factors

Table 1 reports biological data, haematological and all parameters assayed in untransfused (U) and transfused (T) thalassaemic patients and the control group (C).

In transfused patients, the total RBCs transfused were 131.48±38.16 mL/kg/year; cardiac and hepatic MRI T2* was 32.47±12.52 ms and 10.47±9.71 ms, respectively, 21.0% of the patients had myocardial siderosis and 36.8% had hepatic siderosis (<20 and <6.3 ms, respectively).

The data of ERFE and HIF2α, recently determined, in different samples of a patients subgroup, in association with others biochemical endpoints, were reported in **Table 2**. These parameters were assessed in a subgroup of transfused patient samples that were collected just before blood transfusion (T0) and 4/6 days after transfusion (T1) when erythroid suppression was expected to be maximal. Firstly were reported parameter valuesoftransfused subjects in the two times assayed, pre-trasfusion (T0) and post-transfusion (T1) respectively with their respective statistic results followed by the values of U and C subjects.

There were no significant differences (P<0.05) in age and male/female ratio between the U, T and C groups (**Table1)**.

To directly assess the level of hypoxia in thalassemic patients we measured HIF-1α in the nuclear and cytoplasmic compartment. HIF-1α results were validated via FACS analysis of Glut-1 receptor, which is directly regulated by HIF-1α.

Figure 1 illustrates a western blot analysis in both compartments showing marked inter-patient group differences and between these and controls. Low levels of HIF-1α

protein were observed in cytosolic extract of patients, especially U patients. In the more representative nuclear extracts, higher levels of HIF-1α were observed in T patients, as shown by the values of densitometric analysis, which are reported in **Table 1.** Despite the less severe anemia and ineffective erythropoiesis, as shown by Hb, reticulocyte % and sTfR levels, HIF-1α and Glut-1 were significantly higher in T compared to U thalassemic patients (**Table 1**).

The severe anemia and higher erythropoietic activity of U than T subjects, were confirmed also by higher values of U patients values for ERFE, HbF, RBC, EPO and Ret‰ reported in **Table 2**. The absence of significant results for NRBC% values could be attributed to the great internal variability within the groups, but subjects ratio with NRBC% values different from zero were 100% in T0, 62 in T1 and 62.5% in U group.

Contrary to HIF-1α , HIF-2α levels were significantly higher in U than T group and were affected by transfusion as underlined by the fluctuation between T0 respect to T1 sampling time. Similarly to HIF-2α, ERFE and others anemia and erytropoietic parameters, except Ret%, were increased in T0 compared to T1 patients. Some of the most significatives fluctuation over the intertransfusion interval were also emphatized in **Figure 2.**

As expected, significant differences were observed between the patient groups and healthy subjects for the iron overload indices. Only transferrin concentration in thalassemic patients was lower than in the control group. Although serum ferritin levels did not differ significantly between the patient groups, serum iron and transferrin saturation demonstrate the more marked iron overload in T in comparison to U patients **(Table1).** In **Table 2**, regarding iron indices, non fluctuation were observed between T0 and T1.

Hepcidin was more highly expressed in T patients, while similar values were observed in U patients and healthy subjects. Despite this, the hepcidin/ferritin ratio, calculated to evaluate the appropriateness of hormone synthesis, showed significantly decreased hepcidin expression in both patient groups in comparison with controls (**Table 1**).

sHJV was, also, significantly increased in thalassaemic patients compared to controls with higher values in U than in T patients (**Table 1**).

As expected, GDF15 was higher in thalassaemic patients than in controls and, although no significant differences were observed between patient groups (**Table 1**),the values of U patients were on average 1.2- to 2.7-fold higher than the values in T patients for all percentiles (5th - 95th).

HCV infection. Blood transfusion-dependent thalassaemic patients are at high risk of HCV infection, which leads to a significant liver insult; 32% of the examined transfused patients were HCV-RNA+. Stratification of T subjects, according to the presence of detectable serum HCV-RNA, demonstrated a significant decrease in sHJV (Z=2.51, *P*=0.01, **Figure 3A**) and hepcidin levels (Z=2.37, *P*=0.01, **Figure 3B**) in HCV-RNA+ patients. In contrast, active HCV infection caused increased levels of GDF15 (Z=2.46, *P*=0.01, **Figure 3C**), EPO (Z=2.26, *P*=0.02) and serum iron (Z=2.26, *P*=0.02, **Figure 3D**).

Hypersplenism and splenectomy. Hypersplenism, a common condition in β-thalassemia patients, increases the need for blood transfusion to counteract the worsening of anemia. After stratification of patients into splenectomized (S: 55%) and non-splenectomized (NS: 45%), emerged that the hypersplenism led to higher blood consumption (S: 118.1 ± 23.7 vs. NS: 149.6 ± 25 ml/kg/year; Z = 4.45, $P < 0.001$). The increase in iron intake in NS patients led to higher ferritin levels (S: 1026.9 ±

1311.2 vs. NS: 1494.4 ± 1381.9 ng/ml; Z = 2.32, *P* = 0.02) and, although not statistically significant, to lower hepatic MRI T2* levels (S: 12.0 vs. NS: 9.91 ms).

This results were confirmed in the subgroup of patients that were selected for the determinations reported in **Table 2**, in which the statistic results underlined all parameters affected by splenectomy in transfused (in pre-transfusion time) and untransfused patients (**Table 3**).

Relationships between the endpoints. Were performed by Spearman test in the most numerous T group correcting for the HCV infection factor and considering only splenectomized patients.

HIF-1α and Glut-1 were strongly correlated (R=0.69; *P* <0.001). Both were inversely related to Hb, hepcidin/ferritin ratio, hepatic MRI T2* and directly to serum ferritin. Only values for HIF-1α were reported in **Table 4**.

The sHJV levels were inversely related to GDF15 and serum ferritin, while were directly related to the hepcidin/ferritin ratio and cardiac MRI T2*. We did not find a relationship between sHJV and hepcidin. GDF15 was inversely related to the hepcidin/ferritin ratio and it had a positive relationship with reticulocyte %, sTfR and serum ferritin (**Table 4**).

Hepcidin, in turn, was related to iron-levels-linked parameters, as shown by the direct relationship with serum ferritin and transferrin saturation and the inverse relationship with serum iron and transferrin. Hepcidin/ferritin ratio was also inversely related to hepatic MRI T2*(**Table 4).**

Table 4 also reports several expected correlations, such as Hb levels with sTfR and reticulocyte %, in addition to relationships not yet described, like those between sTfR and cardiac MRI T2*. Besides, serum ferritin was directly related to the increase of iron deposits in the myocardium and liver (MRI T2*). The strong link between

transfusion treatment and iron overload was confirmed by the inverse relationship between the amount of transfused RBCs and hepatic MRI T2*.

The determinations in **Table 2** were correlated separately in T0 patients. ERFE and HIF-2α were strongly correlated (R=0.70, *P* <0.001), both were not affected by splenectomy and was reported all relationships that concer them,

-ERFE is related to age (R=0.58 *P* =0.006), EPO (R=0.41, *P* =0.001), sTfR (R=0.57, *P* =0.007), serum ferritin (R=-0.73, *P* <0.001), RET-He (R=0.65, *P* =0.001), RBCs transfused (R=0.48, *P* =0.022) and to blood transfusion interval (R=-0.50; *P*=0.029);

-HIF-2α is related to age (R=0.45, *P* =0.044), EPO (R=0.73, *P* <0.001), RET-He (R=0.46, *P* =0.040) and to RBCs transfused (R=0.46 P=0.037).

5.2 Section 2- the role of anemia in oxidative and genotoxic damage

Examining a large group of transfusion-dependent patients, we assessed the role of anemia in oxidative and genotoxic damage in thalassemic patients. The study aimed to improve the effectiveness of therapeutic management of thalassaemic patients.

Oxidative biomarkers included ROS, 8-Oxo-2′-deoxyguanosine (8-oxo-dG) and mithocondrial membrane potential (ΔΨm), whereas genotoxicity was evaluated by alkaline single cell gel electrophoresis (Comet assay) and the cytokinesis-block micronucleus (CBMN) test.

To better evaluate the pro-oxidant role of anemia, minimizing the effect of redox-active iron, the endpoints were assayed on samples collected when pharmacokinetics of the iron chelators ensured maximum effectiveness. However, our protocol did not

affect chronic iron-induced cito/genotoxicity that was 'stored' in lymphocytes and it could be expressed after in vitro activation increasing lymphocytic micronuclei frequency (MNi).

Figure 4 shows the results of oxidative and genotoxicity biomarkers. In comparison to controls, higher values of biomarkers were obtained in thalassaemic patients. The graphs highlight the high intra-group variability in patients, suggesting that, due to the complexity of the disease, several variables influenced the endpoints assayed.

Moreover, in the patient group, ROS and 8-oxo-dG values were directly related to MNi, MNed and TDNA% ($P < 0.01$), underlining the inter-relations between oxidative damage and genotoxicity.

Anemia and oxidative imbalance. Although the transfusion treatment was adequate for the majority of patients, 18.9% had pre-transfusion Hb values below the recommended range (9.5–10 g/dl). In this group erythropoiesis enhancement was not effectively blocked, as confirmed by the higher reticulocytes % values in comparison to those with Hb values >10 g/dl (5.91 ± 7.28 vs. 1.31 ± 2.26, $Z = 7.91$, $P < 0.0001$).

To evaluate the roles of anemia in the assayed endpoints, patients were grouped on the basis of Hb levels (<10 vs. ≥10 g/dl). As shown in **Table 5**, highly significant differences were observed in the two subgroups since a small decrease (10.9%) in Hb (9.29 ± 0.44 g/dl vs. 10.43 ± 0.3 g/dl) remarkably increased oxidative and genotoxic damage. NDI values (a marker of proliferative activity) and cytotoxicity (measured by the number of necrotic and apoptotic cells) were also affected by the severity of anemia.

Labile Plasma Iron (LIP). The short interval between chelating treatment and blood withdrawal did not allow us to assess the prooxidanteffect of free and/or labile-

bound iron. Only 50.5% of patients had detectable LPI values while these were below the detection limit (0.2 μM) in a high percentage of patients. In the subgroup with detectable LPI median value and interquartile range were 0.43 (0.29–1.21).The LPI was >2 μM in 5 patients, probably due to poor compliance with chelation therapy. Moreover,30 sera samples from both patients and controls gave negative values of LPI. This did not allow statisticalanalysis to be performed for the entire patient groupby correlating the LPI values to the other endpoints investigated. In the patients with detectable LPI, wereno differences on the basis of iron chelation treatments (data not shown). Control group sera with detectable LPI (45%) were consistently below 0.3 μM.

In the subgroup with detectable LPI values, no significant correlations were observed between LPI and oxidative and genotoxicity endpoints. Instead, LPI was related to apoptotic and necrotic lymphocytes (R = 0.28; P = 0.03), revealing the cytotoxicity of labile iron. Moreover, as demonstrated by the significant LPI association with reticulocytes (%) (R = 0.3; P = 0.04), the degree of anemia was more severe in patients with higher LPI levels. Similar results were obtained for transferrin level, %TSAT and serum iron; the latter seemingly was more predictive of iron genotoxicity, showing direct relations with %TDNA and TM ($P < 0.05$ for all percentiles). There were no differences between iron chelation treatments and oxidative/genotoxicity biomarkers.

Hypersplenism and splenectomy. As reported in Section 1, hypersplenism rappresents a common condition in β-thalassemia that increases, 1) the markers of ineffective erythropoiesis as nucleated red blood cells (NRBC) and reticolocytes% (**Table3**); 2) the need of RBC transfused causing the consequent increase of iron intake and ferritin levels.

Hypersplenism was also responsible for the variability in the assayed endpoints, causing premature and rapid removal of damaged blood cells and the underestimation of cyto- and genotoxicity biomarkers. Bivariate analysis showed a significant difference between S and NS patients regarding 8-oxo-dG (S:13.6 ± 6.0 vs.NS: 7.2 ± 3.9 AFU; Z =2.2; $P < 0.05$). Trends were found for intracellular ROS, MNi frequency, apoptotic and necrotic cells.

Further factors that influenced the assayed parameters in the patient cohort were biological variables such as gender and age. A greater number of necrotic cells were observed in males than in females (median value 8.1 vs. 4.4; $P < 0.05$) whereas, as expected, MN rose with increasing age (>30 vs≤30 median value , 20.9 vs. 15.5; P=0.02).

Multivariate analysis. It was performed using the a priori models of multiple regression to evaluate among the most commonly used anemia and iron parameters the independent variables that most affect oxidative–genotoxic endpoints. The model included six continuous covariates (**Table 6**). Our results indicate a pivotal protective role of Hb level/blood transfusion. Due to the confounding effect of hypersplenism, the amount of RBCs transfused was not considered in the regression model.

%TDNA was inversely related to Hb values and the effect was observed throughout the lymphocyte population (all percentiles) of each patient. Moreover, higher Hb values improved lymphocyte proliferation, as assessed by NDI. None of the covariates included in the model to evaluate iron overload contributed significantly to genotoxicity endpoints. However, serum iron and hepatic and cardiac siderosis were significantly related to cytotoxicity, increasing the number of apoptotic and necrotic lymphocytes. Moreover, hepatic siderosis significantly depressed lymphocyte proliferation.

Spearman test was performed to evaluate the relationships between the all assayed parameters and oxidative and genotoxicity endpoints in splenectomized transfused subjects (**Table 7**). The results confirmed the protective effect of Hb and the amount of RBCs transfused as underlined in multivariate analysis and highlight by hypoxia and erytropoietic parameters as EPO, Reticulocyte %, GDF15 and sTfR. Besides, HIF-1α was positively related to mithocondrial membrane potential.

Among the iron indicators, serum ferritin was negatively related to ROS, MNi and directly to NDI. This correlation likely was to be attributed to better anemia control, as shown by Hb values that were, on average, 3% lower in the patients with more severe hyperferritinemia (values >500 ng/ml). Although a more intensive blood transfusion therapy increased iron load, it also reduced anemia-induced oxidative and genotoxic damage. Similarly to ferritin, sHJV levels was inversely related to %TDNA.

On the contrary, serum iron, transferrin and TSAT% highlight the oxidative damage iron-induced. The hepcidin was inversely related to mithocondrial potential as marker of organelle functionality.

5.3 Section 3-the genotoxic/cytotoxic effect of complications and therapy

To investigate the genotoxic/cytotoxic effect of iron overload, thalassemia complications and the respective therapies was created a homogenous group of 64 regularly transfused thalassemic patients. As above reported, hypersplenism, by a marked haemocatheresis, causes the removal of more damaged lymphocytes and the underestimation of cellular andgenotoxicity biomarkers, therefore wereselected only splenectomised patients. Furthermore, to reduce hypoxia-induced oxidative damage,

those with Hb levels <9.5 g/dL were also excluded. As expected from the therapeutic protocol of poly-transfused patients, all patients were undergoing iron chelation therapy that was not suspended before sampling. Twenty-two patients were treated with deferasirox, 14 with deferoxamine (DFO), 10 withdeferiprone (DFP) and 18 with combined DFO + DFP.

The total RBCs transfused were 131.48±38.16 mL/kg/year; cardiac and hepatic MRI T2* was 28.1±12.4 ms and 11.46±9.8 ms, respectively, 20 (31.2%) patients had hepatic siderosis and 7 (10.9%) had myocardial siderosis (<6.3 ms and <20, respectively).

There was a high prevalence of endocrinopathies. In particular, 26 patients had hypothyroidism (40.6%) and were undergoing levothyroxine therapy, 36 (56.2%) presented hypogonadism and 26 (40.6%) were undergoing hormone replacement therapy (HRT). Seven (10.9%) had diabetes mellitus (DM), and 10 (15.6%) had short stature. Overall, 20 (31.2%) of the patients had at least one endocrine disease and 23 (35.9%) had two or more.

Thirty-one patients (48.4%) had osteopenia, defined by the WHO as a T-score between −1 and −2.5, and 30 (46.9%) had overt osteoporosis, recording a T-score above 2.5. The thalassemic group also included 23 (35.9%) patients with hepatic disease and 20 (31.2%) with active hepatitis C virus (HCV) infection.

Similarly to result in all patients cohort (**Figure 4**) the values for the main genotoxic endpoints obtained in this subgroup by CBMN and comet assays were significantly increased in patients (MNi: 17.0(12.0–21.0), MNed: 11.55(7.5–16.5) and %TDNA: 9.68(7.5–18.5) compared with the controls (MNi: 10.77(7.6–16.2), MNed: 10.04 (12.0–25.0)and %TDNA: 8.56(5.7-10.5). Conversely, DNA density, marker of chromatin compactness was lower in T (DNA density: 42 (0.33–0.53) than in C

subjects (DNA density: 0.49 (0.46–0.52). All statistic results by Mann–Whitney test are with $P < 0.01$.

Within the T group patient cohort, there were no differences according to chelation treatments for iron parameters, iron intake, cardiac or hepatic MRI T2* or for the endpoints assessed (data not shown).

Genotoxicity endpoints also were interrelated and, conversely to % TDNA, DNA density, marker of chromatin compactness, was significantly related with MNi (**Figure 5**), enhancing the value of this parameter.

The genotoxic effects of disease complications with high prevalence in β-thalassemia were assessed. These included hepatic disease, active HCV infection, hypothyroidism, hypogonadism, diabetes mellitus, short stature and bone disease (osteopenia and osteoporosis), along with their respective drug therapies.

The conditions significantly affecting genotoxicity biomarkers are reported in **Table 7**, where patients are grouped according to the absence/presence of each complication or therapeutic treatment. As highlighted by the interquartile range, the variability was kept high in each subgroup.

In particular, HCV-induced genotoxicity was emphasised by the frequency of MNi-MNed and by %TDNA values. As shown in **Figure 6A**, the DNA damage increased up to 2-fold ($P < 0.01$ for all centiles) in HCV-RNA-positive patients, in comparison with the negative ones. While osteopenia/osteoporosis not directly affected genotoxicity endpoints, their therapeutic treatment by bisphosphonates caused significant genotoxic damage (**Table 8**). The effect in thalassemic patients with bone disease under bisphosphonates therapy compared with those without is highlighted in the box plot (**Figure 6B**). No differences in the assayed endpoints were observed in relation to hypogonadism, while a genotoxic effect was observed in the patients

undergoing HRT (40.6%). In comparison with untreated patients, their MNi frequency was significantly higher (**Figure 6C**) and the genotoxicity of the treatment was confirmed by DNA density measurements (**Table 8**).

Considering the complexity of the disease, the absence of a secondary complication or therapeutic treatment did not necessarily exclude the others. Similarly, the simultaneous presence of more complications was not excluded. The conditions significantly affecting genotoxicity biomarkers were simultaneously present in some patients and, while only one was present in 39%, the coexistence of two and three conditions was present in 15% and 13%, respectively.

As expected, the group of 45 subjects with complications and/or therapeutic treatments showed higher genotoxicity than the 20 patients without (DNA density: $Z = 3.87$, $P < 0.001$; MNi: $Z = 3.11$, $P < 0.01$ and MNed: $Z = 2.91$, $P < 0.01$). Owing to the decreased sample size for each group, patient cohort stratification according to the presence of one, two or three genotoxic conditions reduced the statistical power.

Despite this, a gradient in assayed endpoints was observed. In comparison with the patients without genotoxic conditions (median values 0.50, 13.8 and 13.2 for DNA density, MNi and MNed, respectively), the differences were significant for DNA density, whose median values were 0.37 ($Z = 3.52$; $P < 0.001$) and 0.33 ($Z = 3.75$; $P < 0.001$) in the presence of one and two conditions, respectively.

Similar significant differences were observed in MNi-MNed frequency. When two conditions coexisted, the median values were 19.8 ($Z = 2.8$; $P < 0.01$) and 18.4 ($Z = 2.7$; $P < 0.01$), respectively. MNi-MNed frequency increased further when three determinants coexisted and median values were 46.0 ($Z = 2.8$; $P <0.01$) and 41.0 ($Z = 2.9$; $P < 0.01$).

In particular, the coexistence of HCV infection and HRT for hypogonadism seemed to considerably enhance the genotoxicity. In comparison with the patients in HRT treatment only, %TDNA median value was 26.39 vs. 7.96 ($Z = 2.8$; $P < 0.01$) and DNA density was 0.36 vs. 0.45 ($Z = 2.16$; $P = 0.03$). Similar differences were observed in MNed frequency comparing these patients to those with only HCV infection (median value 20.5 vs. 14.5, $Z = 3.5$, $P < 0.01$).

4. Discussion

Iron is required in a variety of important biological processes but the redox-active iron can generate reactive oxygen species (ROS), leading to oxidative damage and initiation of signaling pathways crucial for cell survival. In order to maintain adequate and safe amounts of iron, cells require the coordination of a wide variety of genes, which tightly control both intracellular and systemic iron metabolism.

An examples of iron dyshomeostasis diseases is Iron Refractory Iron Deficiency Anemia (IRIDA, OMIM#206200), a rare autosomal hereditary disease caused by mutations in TMPRSS6 gene of a protein involving in down-regulation of HAMP expression (Ganz T, 2013). We reported a case of a 7-year-old girl with clinical presentation of IRIDA as severe hypochromic microcytic anemia, who was unresponsive to classical iron supplements The TMPRSS6 sequence analysis showed a complex genotype with a rare heterozygous missense variant (Capra AP, 2017).

Extensive research by many groups has revealed key mechanisms in iron homeostasis, as well as the association between iron dyhomeostasis and human disease. The discovery of hepcidin has provided significant insight in the regulation of iron homeostasis (Camaschella C, 2013). We gained understanding of hepcidin regulation from studies on the pathophysiology of the Hereditary Hemochromatosis, a genetic disorder whose murine models allowed to uncover main regulators of iron homeostasis.

Thalassemia pathophysiology is characterized by secondary hemocromatosis caused by down-regulation of hepcidin and increased intestinal iron absorption tissue hypoxia-induced. β-thalassemia major patients need to undergo constant blood-

transfusion therapy in order to suppress bone marrow activity but this treatment cause iron overload (Cohen AR, 2008). Untransfused thalassemic patients, are also characterized by secondary hemocromatosis but it is not as severe as in transfused subjects (Haidar R, 2010).

The regulation of hepcidin is influenced by several independent pathways in response to body iron requirements, hepatic iron stores and inflammation. Enhanced erythropoietic activity is responsible for the down-regulation of hepcidin synthesis. This mechanism is particularly prominent in untransfused patients in whom ineffective erythropoietic activity is not silenced by transfusion therapy and the main source of iron overload is the consequent hyper-absorption of dietary iron (Esposito BP, 2003).

As previously reported (Kearney SL, 2007), the suppression of hepcidin is greater in patients with thalassemia intermedia than thalassemia major suggesting that by suppressing erythropoiesis, transfusion permits hepcidin levels to increase. This effect occurs dynamically across the transfusion cycle in regularly transfused patients (Pasricha SR, 2013).

Soluble hemojuvelin (sHJV) in transfused and untransfused thalassaemic subjects. The HJV is the major regulator of HAMP expression in the liver and its gene mutations lead to severe hepcidin deficiency,which characterises the genetic disease known as Juvenile Hemochromatosis Type 2A (OMIM#602390) (Xia Y 2008). As an antagonist of mHJV, sHJV down-regulates HAMP when body iron requirements increase (Lin L, 2005).

Serum sHJV levels are increased in anemia of chronic disease (Brasse-Lagnel C, 2010), in Congenital Dyserythropoietic Anemia I (CDA I) (Shalev H, 2012), during pregnancy (Finkenstedt A, 2012) and in hemodialysis patients (HD) (Malyszko J, 2012). We determined for the first time sHJV levels in regularly transfused β-

thalassemic major (T) and untransfused β-thalassaemic intermedia (U) patients evaluating its relationships with iron and anemia parameters ([a]Ferro E, 2017).

The array of determined parameters showed severe anemia and erythropoietic activity in U, while we estimated iron overload in T patients. Among the several anemia indices, hepcidin/ferritin ratio demonstrated a significant inadequate hepcidin expression in thalassemic patients (Kearney SL, 2007), particularly in U subjects.

We assessed higher levels of sHJV in thalassemic patients compared with healthy subjects and the same trend was found in U patients rather than in T patients. This suggests that chronic anemia-induced erythropoiesis activates the "iron needed" signal despite concurrent haemosiderosis, leading to pathologically high sHJV levels and suppression of hepcidin.

On the contrary to CDA I (Shalev H, 2012), sHJV was inversely related to serum ferritin and positively related to the hepcidin/ferritin ratio in β-thalassemia major. Instead, iron overload represents an oppsosite signal to anemia and the major affector of sHJV level in polytransfused thalassemic patients.

According to this effect of iron overload, we also reported a negative relationships of sTfR and sHJV levels with cardiac siderosis (expressed by cardiac MRI T2*). The erythropoietic stimulus increases TfR levels, while an appropriate transfusion regimen silences bone medullary activity and reduces sTfR (Khumalo H, 1998), but causes tissue siderosis. In addition increased iron stores, due to probable cardiac fibrosis, reduce the extra-hepatic source of sHJV (Papanikolaou G, 2004; Niederkofler V, 2004). Further studies are needed to corroborate the use of sHJV as a predictor of cardiac siderosis.

GDF15 has been proposed as "erythroid regulator" that suppresses hepcidin expression in β-thalassemia. High levels of GDF15 have been found in thalassemia

(Tanno T, 2007), CDA I and CDA II (Tamary H, 2008; Nemeth E, 2006) and during pregnancy (Finkenstedt A, 2012). Similarly, we observed increased GDF15 levels in thalassemic patients without significant differences between the two groups. In transfused subjects, despite erythroid suppression as a consequence of transfusion, there is a transient hypoxia that fluctuates in the inter-transfusion interval achieving its maximum peak immediately before blood transfusion (Ferro E, 2016). The sera samples collected at this stage could explain the comparable GDF15 values observed both in T and U patients.

High erythropoietic activity, often related to marked haemocatheresis, leads to iron overload as suggested by the direct relationship between serum ferritin and GDF15.

At variance with the positive relationship between sHJV and GDF15 in CDAI patients as well as in pregnant women (Shalev H, 2012; Finkenstedt A, 2012), we found an inverse relationship in transfused subjects leading to suggest the main role of iron compared to erythropoietic/hypoxic signals in sHJV synthesis ([a]Ferro E, 2017).

Overall, GDF15 seems to be the main sensor of erytropoitic signal compared to sHJV. It positively correlated with reticulocyte %, sTfR and inversely to hepcidin/ferritin ratio.

In addition to the known effects of HCV-infection on hepcidin, GDF15 and serum iron (Liu X, 2015; Si Y, 2011; Kijima H, 2008; Fujita N, 2007), we observed also its negative effets on sHJV levels. In the same way to hepcidin (Miura K, 2008), this result could be attributed to the HCV-induced oxidative damage in hepatic cells that are the main producers of HJV.

Erytroferrone (ERFE) in transfused and untransfused thalassaemic subjects. It is the new discovered erytroid regulator of hepcidin in ineffective erythropoiesis.

ERFE, released by erythroid precursors in the marrow and the spleen in response to erythropoietin (EPO) stimulation, induces hepcidin suppression increasing iron availability for new erythrocytes synthesis (Kautz L, 2014; Kautz L, 2015).

As recently reported by Ganz (Ganz T, 2017), our patients had higher ERFE levels than controls with lower values in T compared to U subjects. The serum derminations of ERFE in two sampling times (pre- and post-transfusion) enabled us to better understand its behaviour in thalassemia.

ERFE levels abruptly decreased within 4/6 days from the transfusion. This effect of blood transfusion suggests a significant relation between ERFE levels and total amount of RBCs transfused required to maintain Hb levels between 9.5 and 10 g/dl (Galanello R, 2010). Furthermore, high ERFE levels were associated to low inter-transfusional interval. As as experimentally demonstrated, we confirmed that ERFE was strongly correlated to EPO, its first inductor and in addition we described new interactions either with sTfR and HIF-2α. The latter has been proved to be the master regulator of EPO expression explaining the strong relationships with ERFE (Kapitsinou PP, 2010).

Regulators factors of tissue hipoxia in transfused and untransfused thalassemic patients. Althought β-thalassemia major patients silence bone marrow activity, thanks to regularly blood-transfusion therapy, O_2 tension varies constantly and decreases during the inter-transfusion interval, with a minimum value immediately before transfusion, which abruptly interrupts the physiological hypoxic adaptation. Differently U patients are characterized by a constant low O_2 tension causing a chronic hypoxia that increases intestinal iron absorption through down-regulation of hepcidin (Ganz T, 2011).

In the HIF family, HIF-1 and HIF-2 are generally considered to be the master regulators of the transcriptional response to hypoxia. They are heterodimer composed of two subunits, an oxygen/iron-sensitive subunit-1α, which is post-translationally regulated by PHDs in function of "oxygen/iron-need" signal, and a constitutively expressed subunit-1β (Wang GL, 1995). HIF-1α plays a key role in the initial response to hypoxia whereas HIF-2 α drives the hypoxic response during chronic exposure to low O_2 tension (Milosevic J, 2009). This HIF "switch" suggests physiological and pathological adaptation for cell survival.

We reported the first determination of HIF-1α by western blot (Ferro E, 2016) and we recently assessed HIF-2α seric levels both in T and U thalassemic patients. Similarly to the evaluation of ERFE, HIF-2α was determined in two sampling times (pre- and post-transfusion).

HIF-2α and others biomarker of anemia and erythropoiesis such as sTfR, EPO, NRBC, GDF15 levels (Speeckaert MM, 2010) reflected a chronic hypoxia and a higher marrow erythropoietic activity in U than in T thalassemic patients. Conversely, HIF-1α-Glut-1 were increased in T subjects reflecting only an acute hypoxia. However, both were inversely related to pretransfusion Hb levels and to hepcidin/ferritin ratio in T subjects.

The persistent severe anemia that characterizes U patients causes a chronic status of tissue hypoxia and leads to metabolic switch from oxidative phosphorylation to anaerobic glycolysis. The impaired mitochondrial oxygen utilization creates the paradox of increased cellular O_2 availability for PHDs which promotes proteasomal degradation of HIF-1α as shown by the low levels observed in cytosolic extracts. We reported lower mithocondrial membrane potential (ΔΨm), marker of mithocondrial

functionality, in thalassemia intermedia than thalassemia major patients, in wich HIF-1α was positively related to ΔΨm.

It could be possible that it is the underlying mechanism used to switch from HIF-1α during acute hypoxia, to HIF-2α in chronic hypoxia (Milosevic J, 2009; Wu Y, 2010). This was confirmed by higher HIF-2α levels in U than T patients and by its fluctuation between pre and post-transfusion time in T group. In fact, hypoxia is transient in T patients and, by increasing in the inter-transfusional interval, reaches its maximum peak immediately before blood transfusion.

Moreover, the higher levels of HIF-1α in the nuclear compartment found in T patients compared to U patients could be also ascribed to: 1) the intensive iron chelation therapy which reducing intracellular iron availability prevents the enzymatic activity of PHDs, allowing the stabilization of HIF-1α subunits (Ginzburg Y, 2011) and by removing excessive Fe2+ reduces mithocondrial oxidative damage (Milosevic J, 2009; Wu Y, 2010); 2) the RBC transfused, positively related to HIF-1α-Glut1, that had a protective effect from oxidative stress hypoxia-induced.

Likewise to HIF-1α, high intracellular iron and oxidative mithocondrial impairment of U patients should lead to degradation of HIF-2α. However it should be noticed that models of intestinal iron overload coupled with systemic anemia indicate that systemic anemia/hypoxia is the predominant regulator of HIF-2 levels (Matak P, 2013).

Similarly to ERFE, HIF-2α increased in relation to amount of RBCs transfused requirements to maintain pre-transfusion Hb levels in according to guidelines of Thalassemia International Federation (Galanello R, 2010).

The role of anemia in oxidative and genotoxic damage. In thalassemia the oxidative damage was commonly attributed to iron overload. Its pro-oxidant effect, due to Fenton and Haber–Weiss reactions, and its role in determining organ failure is already known.

On the basis of previous observations that showed a significant inverse relationship between redox imbalance and Hb levels and higher oxidative endpoints in U than in T patients (Ferro E, 2012). herein was highlighted the role of anemia in oxidative/genotoxic damage of polytransfused thalassaemic patients subjected to iron chelation by an array of redox imbalance and genotoxicity indicators.

According to the values reported in well–chelated thalassemic patients (Daar S, 2009), in our polytransfused patients LPI values were generally low (0 – 0.4 μM) and some sera gave negative results.

Daily LPI recrudescence may occur during the washout period of the drugs (Zanninelli G, 2009), conseguently chelating treatment could not ensure a complete removal of the redox–active iron. Indeed, we observed a significant increase in apoptosis and necrosis cell ratio in relation to LPI values ([b]Ferro E, 2017).

Our data lead to suggest how even small Hb increments (i.e. ~11%) are sufficient to significantly reduce oxidative and genotoxic damage. They indicate the importance of a regular transfusion regimen coupled to iron chelation therapy that complies strictly with the guidelines for thalassemia management (Cappellini MD, 2014). A transfusion regimen designed to maintain pre-transfusion Hb levels as close as possible to 10 g/dL is essential for the correct management of transfused β-thalassemia patients. The increase in iron load, due to an appropriate transfusion treatment is contained by life–long chelation therapy and partially offset by reduced intestinal iron absorption hypoxia–activated (Ferro E, 2016; Ganz T, 2011). This was confirmed by

unexpected relationships between serum ferritin, iron intake by RBC transfused, cardiac and hepatic MRI T2* with cyto/genotoxicity endpoints.

Consistent with others studies (Casale M, 2013), we demonstrated the protective effect of splenectomy that allows anemia and siderosis containment in thalassemia. The eradication of splenic hemocatheresis ensures higher Hb levels, reducing the need for RBC transfusion and, consequently, decreasing ferritin and iron load. This effect was further supported by the higher values of hepatic MRI T2* found in splenectomised versus non-splenectomised patients ([b]Ferro E, 2017).

The genotoxic/cytotoxic effect of complications and therapy. In order to reduce DNA damage induced by hypoxia, the genotoxic/cytotoxic effects of disease complications were tested in T patients, splenectomised and with Hb values≥ 9.5 gr/dL.

Active HCV infection was significantly related to higher MNi frequency and of %TDNA ([c]Ferro E, 2017) confirming the increased chromosomal instability previously reported (Machida K, 2010). Considering the high risk of liver malignancies in iron overload conditions and the strong link between chronic HCV and HepatoCellular Carcinoma (HCC) (Sukowati CH, 2016), our results are considerably important. Liver fibrosis and its progression to cirrhosis and cancer are mainly due to siderosis, in comparison with HCV infection (Angelucci E, 2002). Despite this, the co-presence of both factors synergistically increases the HCC risk (Borgna-Pignatti, 2004) and antedates its onset (Ryder SD, 2003).

Accelerated hemopoiesis with marrow expansion, endocrinopathies, direct iron toxicity on osteoblasts and chelation-induced osteoblast impairment are responsible for a reduction in bone mineral density (Toumba M, 2010). Osteopoenia and osteoporosis were widely found in our patients, some of whom were treated with

bisphosphonates. We have shown a significant genotoxic effect ([c]Ferro E, 2017) of the drug which is consistent with that described for postmenopausal women (Bayram M, 2006).

Although previous studies have considered the potential genotoxicity of Hormone Replacement Therapy (HRT) for hypogonadism, results available are inconsistent (Casella M, 2005; Ozcagli E, 2005). Our data highlighted this drug-induced effect on DNA damage of thalssemic subjects ([c]Ferro E, 2017).

Finally, also β- and α-globin genotype, pro-oxidant and anti-oxidant genetic factors, gene variants of the iron pathways proteins and modifying HbF production, could affect disease severity worsening or improving iron dyshomeostasis and the consequent redox-imbalance and genotoxicity.

Recently polymorphism (rs236918) of the PCSK7 (OMIM#604872) gene, that encodes for a convertases furin-like operating the sTfR shadding, has been associated to iron homeostasis by genome-wide association study (Konrad O, 2011). It was associated with the development of severe liver fibrosis in patients with hereditary hemochromatosis (Stickel F, 2014; Pelucchi S 2016). Our preliminary results for PCSK7 polymorphism analisys showed that carrier had higher serum iron, TSAT% values and oxidative imbalance, as expressed by mithocondrial membrane potential and 8-oxo-dG values, than non carrier subjects with superimposable Hb levels and amount of RBC transfused.

6. Conclusion

Taken together, our data support the conclusion that redox imbalance and genotoxicity in β-talassamia patients is multifactorial.

The increased life expectancy from improved disease management might increase the chance of cancer. The significantly higher risk of haematological malignancies, recently observed in thethalassemic cohorts, corroborate our results that showed a strong link between severe anemia and genotoxicity. Despite the complexity of β thalassemia and the many confounding factors, thanks to the several relationships between oxidative stress biomarkers and biochemical and hematological parameters, our study indicates that appropriate blood transfusion therapy is essential to restrain oxidative and genotoxic damage.

The prevention of oxidative/genotoxic damage should not overshadow the role of iron overload that has to be efficiently contained by life–long chelation therapy.

In addition to iron and tissue hypoxia, disease complications and their drug treatments play a key role in genotoxicity. In particular, when disease complications and/or drug treatments coexist, the resulting genotoxic effects seem to be at least additive.

Splenectomy improved the anemia and siderosis containment in polytransfused patients with β-thalassemia major.

Furthermore, the array of iron homeostasis parameters evaluated had clarify and improve our knowledge of complex interplay of cellular factors regulating iron homoeostasis in thalassemia. In particular, among the routine laboratory parameters,

soluble transferrin receptor (sTfR) was most rappresentative of anemia-erythropoiesis status, oxidative damage and even of heart siderosis in T patients.

Further studies are required to elucidate the high redox-imbalance highlight in chronic hypoxic conditions of U patients. Non-transfusion-dependent thalassemias (NTDT) rappresents a heterogeneous group for genetic base of anemias and management strategies. On the contrary to erythroid regulators as ERFE and HIF2α, sTfR levels were higher in splenectomized compared to unsplenectomized NTDT patients. Similarly to others iron indices, our data lead to suggest a splenectomy effect on sTfR levels. In the light of these considerations, our observations confirmed in greater NTDT patients cohort (Porter JB, 2017) emphasizes the need for a iron chelation therapy program also in NTDT patients to counteract the high redox-imbalance.

8. Tables

Table 1. Biochemical and haematological data with differences between untransfused and transfused subjects (U vs T), untransfused and controls (U vs C), transfused and control group (T vs C). Were reported only significant (P<0.05) statistical results.

Variables Average ± SD	Untransfused (U)	Transfused (T)	Controls (C)	Statistic
Age	36.5 ± 10.31	38.14 ± 7.68	36.1 ± 10.5	
Male/Famale %	6/9	58/47	10/10	
HIF-1α§	0.74	1.69	0.33	(U vs.T) Z=4.06; P<0.001 (U vs C) Z=2.50; P=0.01 (T vs C) Z=5.23; P<0.001
Glut-1§§	0.42±0.07	1.08±0.07	0.14±0.13	(U vs.T) Z=3.92; P<0.001 (T vs C) Z=4.92; P<0.001
Hb, g/dL	8.72±0.86	9.58±0.62	13.92±0.98	(U vs T) Z=2.64; P =0.006 (U vs C) Z=3.83; P <0.001 (T vs C) Z=5.20; P <0.001
Reticulocyte %	2.63±1.73	2.35±4.33	0.5±0.44	(U vs T) Z=2.67; P =0.006 (U vs C) Z=4.13; P <0.001 (T vs C) Z=2.36; P =0.01
Serum Ferritin, ng/mL	714.55±360.99	1599.34±1389.87	54.31±32.78	(U vs C) Z=3.72; P <0.001 (T vs C) Z=4.87; P <0.001
EPO, mIU/mL	48.29±24,11	67.977±74.06	12.952±2.70	U vs T) Z=2.13; P =0.039 (U vs C) Z=2.33; P =0.01 (T vs C) Z=3.05; P <0.001
Transferrin, mg/dL	187.50±23.25	198.01±36.66	260.00±35.43	(U vs C) Z=3.09; P <0.001 (T vs C) Z=4.00; P <0.001
Transferrin saturation (%)	63.20±16.18	85.60±19.78.	24.12±9.34	(U vs T) Z=2.59; P =0.003 (U vs C) Z=2.76; P <0.001 (T vs C) Z=3.78; P =0.002
Serum Iron, µg/dL	171.50±73.85	242.55±54.73	77.24±25.12	(U vs T) Z=2.27; P =0.01 (U vs C) Z=2.78; P =0.003 (T vs C) Z=4.68; P <0.001
sHJV, mg/ L	2.25±1.28	1.52±1.12	0.34±0.15	(U vs T) Z=2.05; P =0.03 (U vs C) Z=3.9; P <0.001 (T vs C) Z=3.93; P <0.001
GDF15, pg/ml	14.45±17.18	10.05±8.31	0.22±0.1	(U vs C) Z=3.28; P =0.001 (T vs C) Z=4.7; P <0.001
sTfR, µg/mL	7.73±2.39	5.16±2.68	0.96±0.29	(U vs T) Z=2.23; P =0.02 (U vs C) Z=3.28; P <0.001 (T vs C) Z=4.06; P <0.001
Hepcidin, ng/mL	16.47±11.38	31.14±14.60	14.06±8.25	(U vs T) Z=3.48; P <0.001 (T vs C) Z=4.26; P <0.001
Hepcidin/Ferritin ratio	0.02±0.02	0.04±0.06	0.69±0.95	(U vs C) Z=3.72; P <0.001 (T vs C) Z=5.12; P <0.001

*** *P* values were calculated by Mann-Whitney U Test for continuous variables and by Pearson Chi-square for categorical variables**

§ The values are obtained by densitometric analysis in 5 µg of nuclear proteins.

Table 2. Biochemical and haematological data with differences between transfused just before blood transfusion (T0) and after transfusion (T1), untransfused and transfused subjects in two sampling time (U vs T0 and U vs T1), controls and transfused group (C vs T0 and Cvs T1) and control and untransfused group (C vs U). Were reported only significant (P<0.05) statistical results.

Variables Median (IQR)	Pre-Transfusion (T0)	Post-Transfusion(T1)	Statistic	Untransfused (U)	Statistic	Control (C)	Statistic
ERFE, ng/mL	106.14 (26.01)	15.73 (3.47)	P <0.001	130.45 (23.73)	UvsT0 and T1: P <0.001	0.31 (0.55)	CvsT0 : T1 and U: P <0.001
HIF2α, ng/mL	2.28(0.66)	0.62(0.53)	P <0.001	7.46 (3.11)	UvsT0 and T1: P <0.001	0.35(0.11)	CvsT0 and U: P<0.001 Cvs T1: P=0.010
Hb, g/dL	10.20 (0.93)	12.10 (1.13)	P <0.001	9.20 (2.02)	UvsT0: P =0.029 UvsT1: P <0.001	13.70 (1.25)	CvsT0 : T1 and U: P<0.001
Hb F %	4.55 (3.03)	3.35 (1.18)	P <0.001	12.40 (62.75)	UvsT0 and T1: P <0.001	0.40 (1.10)	CvsT0 : T1 and U: P<0.001
RBC, $10*10^6$/µL	3.89 (0.24)	4.50 (0.43)	P <0.001	4.79 (0.70)	UvsT0: P <0.001	4.90 (0.35)	CvsT0 and T1:P<0.001
NRBC%, /100WBC	1.60 (4.83)	0.40 (2.73)	P =0.039	1.40 (8.6)		0	CvsT0 : T1 and U: P<0.001
Ret‰	7.45 (13.95)	6.40 (10.92)		32.10 (51.75)	UvsT0 and T1: P <0.001	1.25 (0.50)	CvsT0 : T1 and U: P<0.001
RET-He	21.75 (4.82)	22.80 (5.23)		19.70 (1.75)	UvsT0: P =0.005 UvsT1: P <0.001	31.50 (3.50)	CvsT0 : T1 and U: P<0.001
EPO, mIU/mL	44.30 (26.05)	19.10 (15.60)	P =0.001	49.10 (45.00)	UvsT0: P =0.009 UvsT1: P <0.001	9.25 (8.53)	CvsT0 : T1 and U: P<0.001
Serum Ferritin, ng/mL	742.00 (947.00)	734.50 (829.75)		194.00 (139.50)	UvsT0 and T1: P <0.001	43.00 (44.53)	CvsT0 : T1 and U: P<0.001
Serum Iron, µg/dL	226.50 (61.25)	245.50 (81.00)		104.00 (73.25)	UvsT0 and T1: P <0.001	76.00 (41.75)	CvsT0 and T1: P<0.001 Cvs U: P=0.031
Transferrin, g/L	1.58 (0.34)	1.64 (0.35)		2.02 (0.715)	UvsT0: P =0.011 UvsT1: P =0.045	2.67 (0.43)	CvsT0 : T1 and U: P<0.001
TSAT%	93.15 (28.33)	103.35 (34.29)		38.86 (32.9)	UvsT0 and T1: P <0.001	19.00 (7.5)	CvsT0 : T1 and U: P<0.001
sTfR, mg/L	3.66 (2.68)	3.59 (1.87)		4.38 (2.90)	UvsT0: P =0.006 UvsT1: P =0.008	1.10 (0.25)	CvsT0 : T1 and U: P<0.001

***P* values were calculated by Mann-Whitney U Test**

Table 3. Biochemical and hematological data with only significant (P<0.05) statistical results comparing splenectomized and unsplenectomized subjects in T0 and U groups.

	Transfused T0			Untransfused U		
	Unsplenectomized	**Splenectomized**	**Statistic**	**Unsplenectomized**	**Splenectomized**	**Statistic**
RBCs transfused, mL/Kg/years	158.00 (39.00)	113.00 (8.00)	P<0.001	/	/	
Blood Transfusion Interval, days	14.00 (2.00)	18.50 (3.00)	P<0.001	/	/	
ERFE, ng/mL	104.01 (17.21)	109.92 (38.83)		131.12 (21.27)	125.00 (33.65)	
HIF2α, ng/mL	2.07 (0.49)	2.48 (0.77)		7.45 (2.37)	7.46 (3.97)	
Hb, g/dL	10.20 (0.80)	10.25 (1.10)		9.20 (2.40)	9.20 (1.70)	
Hb F %	4.90 (2.80)	3.80 (1.30)		12.20 (37.45)	73.90 (79.90)	
RBC, 10*106/µL	3.89 (0.20)	3.84 (0.34)		4.88 (0.66)	4.53 (1.51)	
NRB %, /100WBC	0.40 (0.50)	5.90 (19.70)	P<0.001	0.35 (1.40)	25.20 (126.10)	P<0.001
Ret‰	3.90 (1.50)	16.65 (42.70)	P=0.002	26.50 (10.70)	136.40 (117.60)	P=0.001
RET-He	22.80 (4.30)	20.45 (5.00)		18.70 (2.80)	20.10 (2.40)	P=0.010
EPO, mIU/mL	46.15 (6.50)	30.00 (36.70)		53.00 (36.50)	38.00 (65.00)	
Serum Ferritin, ng/mL	771.00 (938.00)	594.50 (1137.00)		169.00 (153.50)	267.00 (112.00)	
Serum Iron, µg/dL	213.00 (97.00)	242.50 (38.00)		90.00 (37.00)	157.00 (82.00)	P=0.010
Transferrin, g/L	1.42 (0.39)	1.66 (0.52)		2.05 (0.61)	1.57 (0.65)	
TSAT%	93.16 (52.53)	94.70 (24.03)		29.93 (16.01)	69.17 (22.44)	P=0.003
sTfR, mg/L	3.66 (1.15)	3.11 (4.13)		4.34 (2.27)	8.14 (2.97)	P=0.004

Table 4. Results of Spearman test performed in transfused group, only significant (P<0.05) statistical results were reported.

R ***P***	**sHJV**	**GDF15**	**Hb**	**HIF1α**	**EPO**	**Ret.%**	**RBCs Transfused**	**sTfR**	**Hepcidin**	**Serum Ferritin**	**Hepc./ Ferritin ratio**	**Serum Iron**	**Transferrin**	**TSAT %**	**Cardiac MRIT2***	**Hepatic MRIT2***
sHJV	—															
GDF15	-0.59 *P*<0.001	—														
Hb			—													
HIF1α	-0.66, *P*=0.001			—												
EPO			0.45 *P*<0.001		—											
Ret %		0.53 *P*=0.03	-0.36 *P*=0.02			—										
RBCs Transfused				0.35 *P*=0.032		-0.45 *P*=0.046	—									
sTfR		0.35 *P*=0.025	-0.37 *P*=0.01		0.63 P<0.001	0.51 *P*<0.001		—								
Hepcidin									—							
Serum Ferritin	-0.47 *P*=0.025	0.44 *P*=0.040		0.36 *P*=0.030	- 0.30 *P*= 0.010			-0.35 *P*=0.012	0.52 *P*<0.001	—						
Hepcidin/Ferritin ratio	0.57 *P*=0.006	-0.70 *P*<0.001		-0.43 *P*=0.034					0.33 *P*=0.023	-0.57 *P*<0.001	—					
Serum Iron							0.43 *P*=0.002		-0.42 *P*=0.003			—				
Transferrin							0.38 *P*=0.036		-0.46 *P*=0.002	-0.49 *P*<0.001		0.66 *P*<0.001	—			
TSAT %									0.48 *P*=0.008	0.44 *P*=0.01			-0.44 *P*=0.01	—		
Cardiac MRI T2*	0.44 *P*=0.010							0.41 *P*=0.024		-0.51 *P*=0.003					—	
Hepatic MRI T2*				-0.43 *P*=0.030			-0.36 *P*=0.044			-0.59 *P*<0.001	0.39 *P*=0.035					—

Table 5. Oxidative and genotoxic damage in patients grouped according the levels of Hb (g/dL) .

	Hb<10 **Retic. % 2.8 (1.7-6.9)** **N°51**	**Hb ≥10** **Retic. % 0.42 (0.2-1.3)** **N°54**	***P***
ROS[§]	38.7 (19.6-63.3)	24.0 (16.8-54.6)	0.018
8-oxo-dG[§]	34.3 (19.6-45.9)	26.6 (19.4-38.1)	0.042
ΔΨm[§]	108.63 (60.56-180.34)	182.81(122.32-214.58)	0.018
MNi	21.0 (13.0-26.0)	13 (8-16)	0.0005
MNed	20 (13-24)	13 (9-17)	0.0008
NDI	2.0 (1.7-2.2)	2.28 (1.9-2.5)	0.026
Apoptotic + Necrotic cells	42 (31-56)	34 (21-43)	0.038
TDNA%	18.0 (10.3-29.7)	8.3 (5.8-10.0)	0.001

The values are reported as median and in the brackets is reported the interquartile interval.

§ The values are expressed as FAU, fluorescence arbitrary units

Table 6. Multiple regression analysis for oxidative and genotoxic effects. For each covariate are reported β value and, in the bracket, *P* value.

Covariates	ROS	8-oxi-dG	MNi	NDI	Necrotic and apoptotic cells	TDNA%25th	TDNA%50th	TDNA%75th
*	0.348 (0.047)	0.402(0.046)	0.381(0.005)	0.419(0.001)	0.494(0.045)	0.688 (0.0003)	0.368(0.007)	0.317(0.024)
Age					0.516(0.003)			
Hb	-0.468(0.045)	-0.457(0.048)	-0.533(0.001)	0.328(0.03)		-0.371(0.01)	-0.337(0.036)	-0.449(0.045)
Ferritin								
Serum iron					0.358(0.041)			
Cardiac MRI T2*					-0.350(0.047)			
Hepatic MRI T2*				-0.296(0.041)	-0.383(0.043)			

***R^2 adjusted obtained by a priori model**

Table 7. Results of Spearman test, performed to evaluate the relationships between the all assayed parameters and oxidative and genotoxicity endpoints in splenectomized transfused subjects.

R *P*	ΔΨm	ROS	8-oxo-dG	MNi	NDI	Apoptotic + Necrotic cells	TDNA%	DNA Density
sHJV							-0.34 0.035	
GDF15			0.43 0.021					
Hb				-0.41 <0.001	0.36 <0.001	-0.23 0.035	-0.34 0.003	0.55 <0.001
HIF1α	0.43 0.008							
Reticulocyte %				0.45 0.001	-0.40 0.003	0.32 0.001		
EPO				0.32 0.016	-0.30 0.020			
sTfR				0.28 0.034				-0.30 0.032
Hepcidin	-0.42 0.012							
Serum Ferritin	0.40 0.012	-0.39 0.01		-0.32 0.008	0.45 0.001		-0.26 0.046	0.31 0.010
Hepcidin/Ferritin ratio				0.46 <0.001	-0.46 <0.001			
Serum Iron							0.25 0.032	-0.24 0.042
Transferrin				0.28 0.014	-0.27 0.016			
TSAT %							0.36 0.001	
Total RBCs Transfused					0.36 <0.001		-0.46 <0.001	

Table 8. Effects of thalassemia complications and drug treatment on genotoxicity endpoints in the patient cohort. The values in absence (-) or presence (+) are expressed as median and, in the bracket, interquartile interval. Results of Mann Whitney test are reported as *P* values.

Absence (-) Presence(+)	Active HCV infection (-=44, +=20)	*P*	Biphosphonates (-=33, +=31)	*P*	HRT for Hypogonadism (-=38, +=26)	*P*
MNi, number of micronuclei/1000 binucleated cells	- 16.0 (10.8-23.5) + 21.0 (20.0-26)	<0.01	- 16.0 (11.5-21.0) + 25.0 (13.0-26.8)	<0.01	- 15.0 (10.3-20.8) + 23.0 (18.5-25.5)	<0.01
MNed cells, number of micronucleated cells/1000 binucleated cells	- 15.5 (10.8-20.0) + 20.0 (19.0-21.0)	<0.01	- 16.0 (11.5-19.5) + 20.5 (13.0-24.5)	0.02	- 14.5, (10.3-19.0) + 20.0 (17.0-22.0)	<0.01
%TDNA, % tail DNA/100 nuclei	- (9.2,6.5-13.2) + (20.9,8.9-28.9)	0.01				
DNA density, ratio between % head DNA and head diameter/100 nuclei			-(0.41 (0.34-0.52) + 0.34 (0.33-0.39)	0.02	- 0.41 (0.35-±0.53) + 0.33 (0.32-0.40)	<0.01

The patient cohort were grouped according to the absence (−)/presence (+) of each complication or therapeutic treatment (in the bracket the patients number) of each subgroup. For each endpoints were reported the values expressed as median, interquartile interval (in the bracket) and *P* values of the Mann–Whitney test.

9. Figures

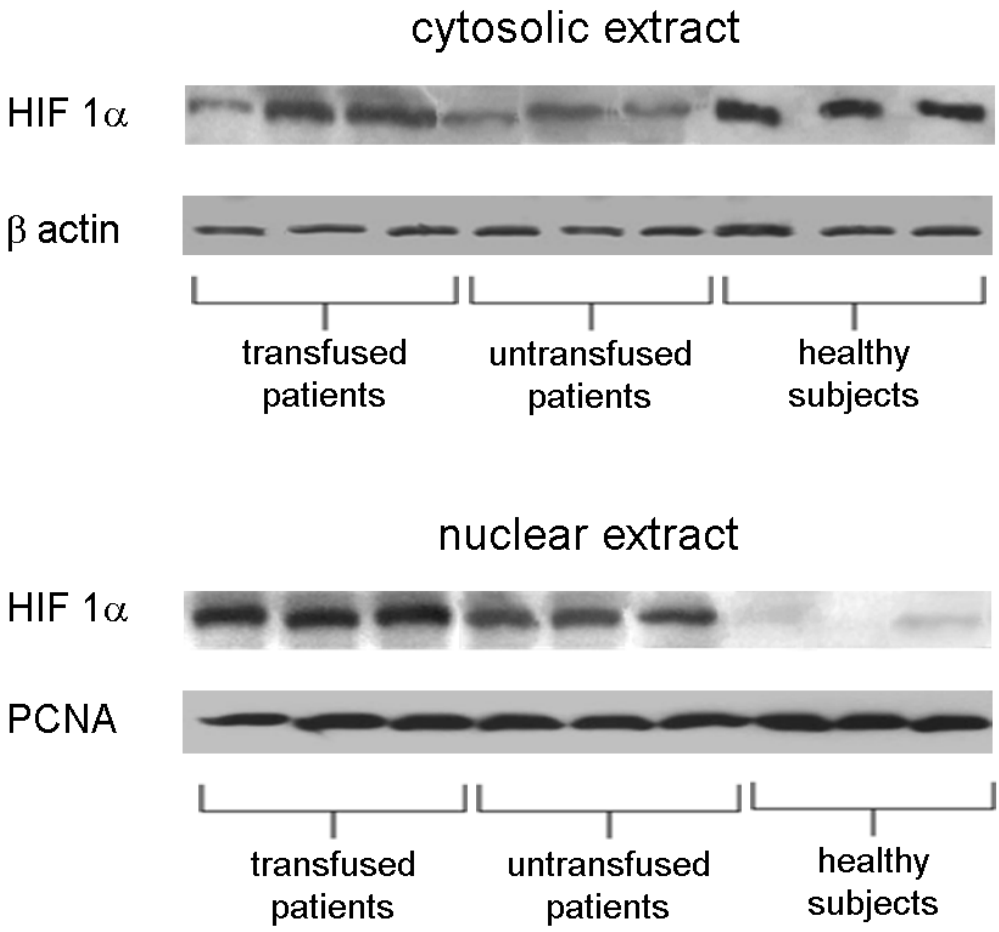


Figure 1. Representative results of western blot analysis in nuclear and cytoplasmic extracts.

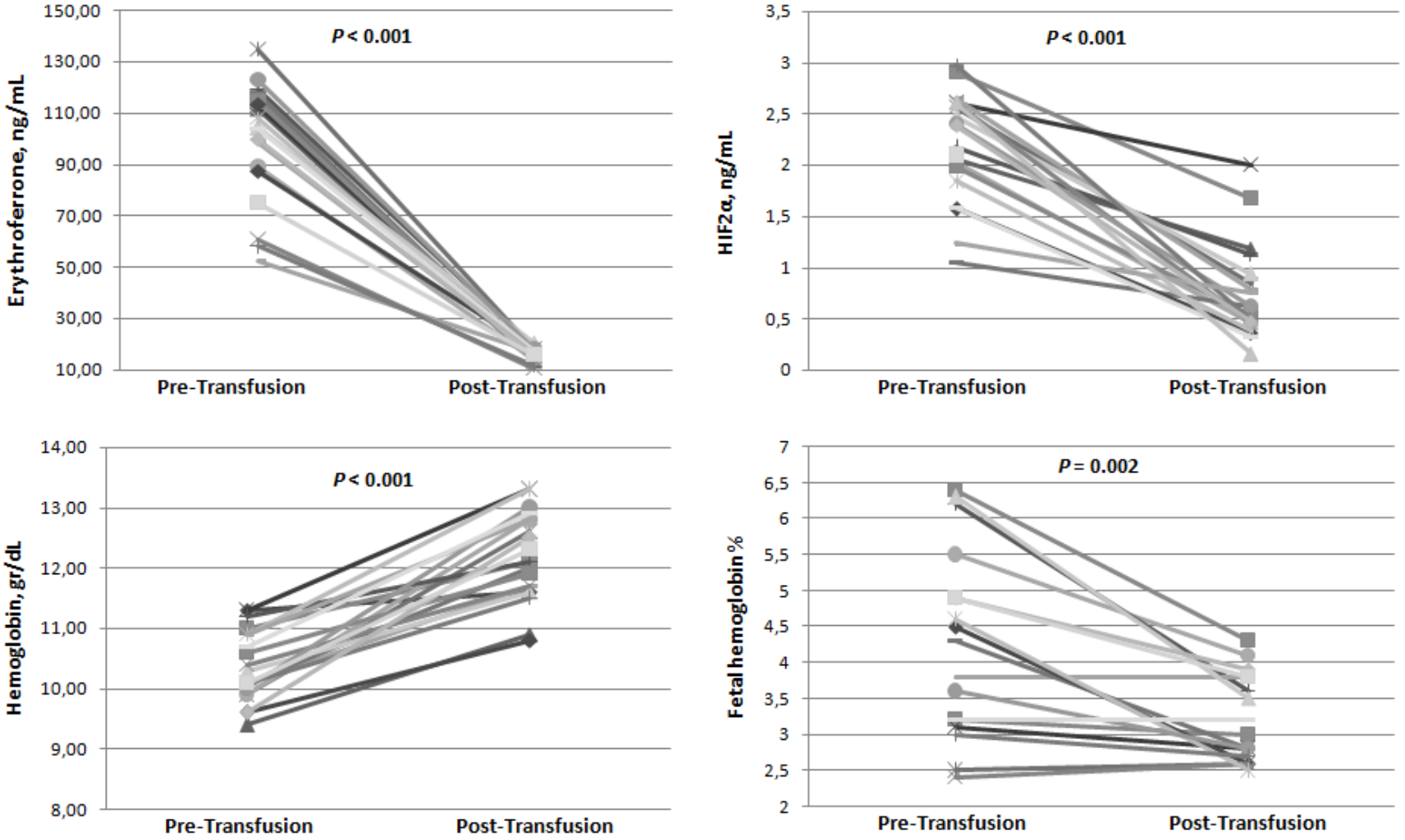


Figure 2. The most significatives fluctuation over the intertransfusion interval between Pre-Transfusion and Post-Transfusion sampling time.

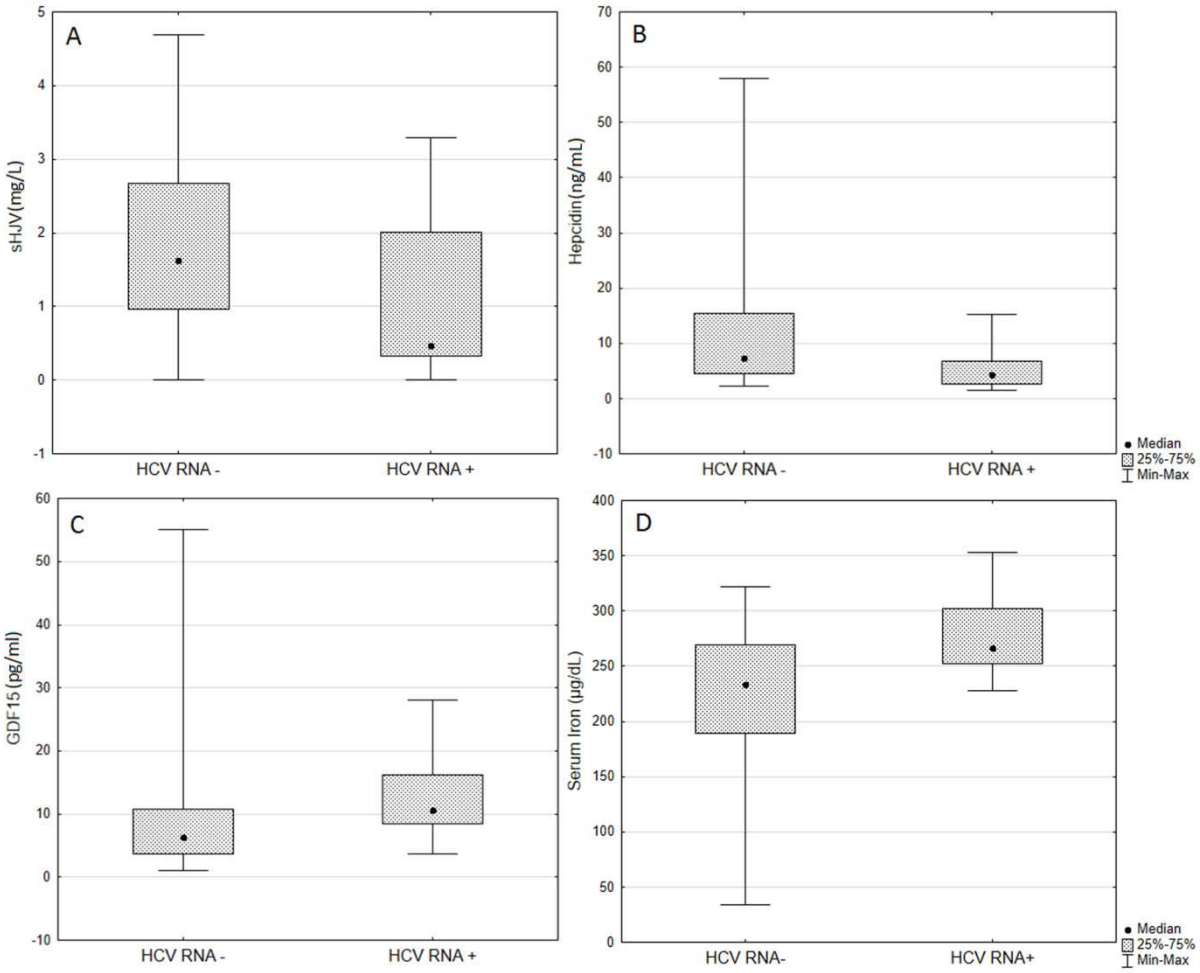


Figure 3. Results of Mann-Whitney test performed between patients with detectable serum HCV-RNA (HCV-RNA+) and without detectable serum HCV-RNA (HCV-RNA–) that underline the impactof active HCV infectionon the assayed endpoints, (A) soluble hemojuvelin (sHJV, Z=2.51, P=0.01), (B) hepcidin (Z=2.37, P=0.01), (C) GDF15 (Z=2.46, P=0.01) and (D) serum ironlevels (Z=2.26, P=0.02).

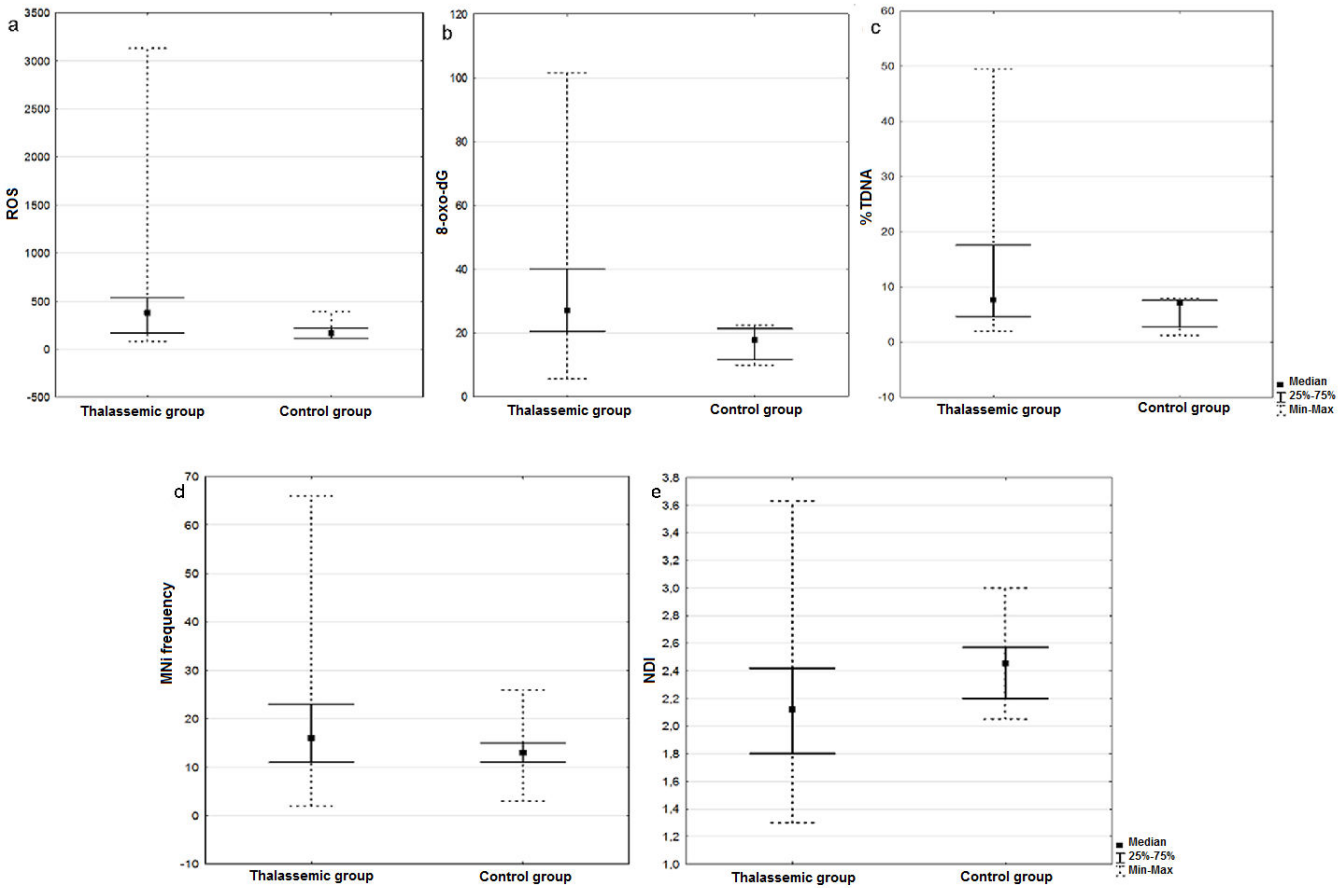


Figure 4. Results of bivariate analysis to compare lymphocytic oxidative damage and genotoxic effect in transfused patient cohort and controls. (A) ROS as assessed in function of DCF emission values ($P = 0.0001$). (B) 8-oxo-dG as assessed in function of emission values of Avidin-FITC emission values ($P<0.0001$). (C) Oxidative DNA damage to Comet-assay evaluated by %TDNA ($P = 0.01$). (D) MNi frequency ($P = 0.027$). (E) Nuclear division index NDI used as markers of proliferative activity ($P = 0.001$).

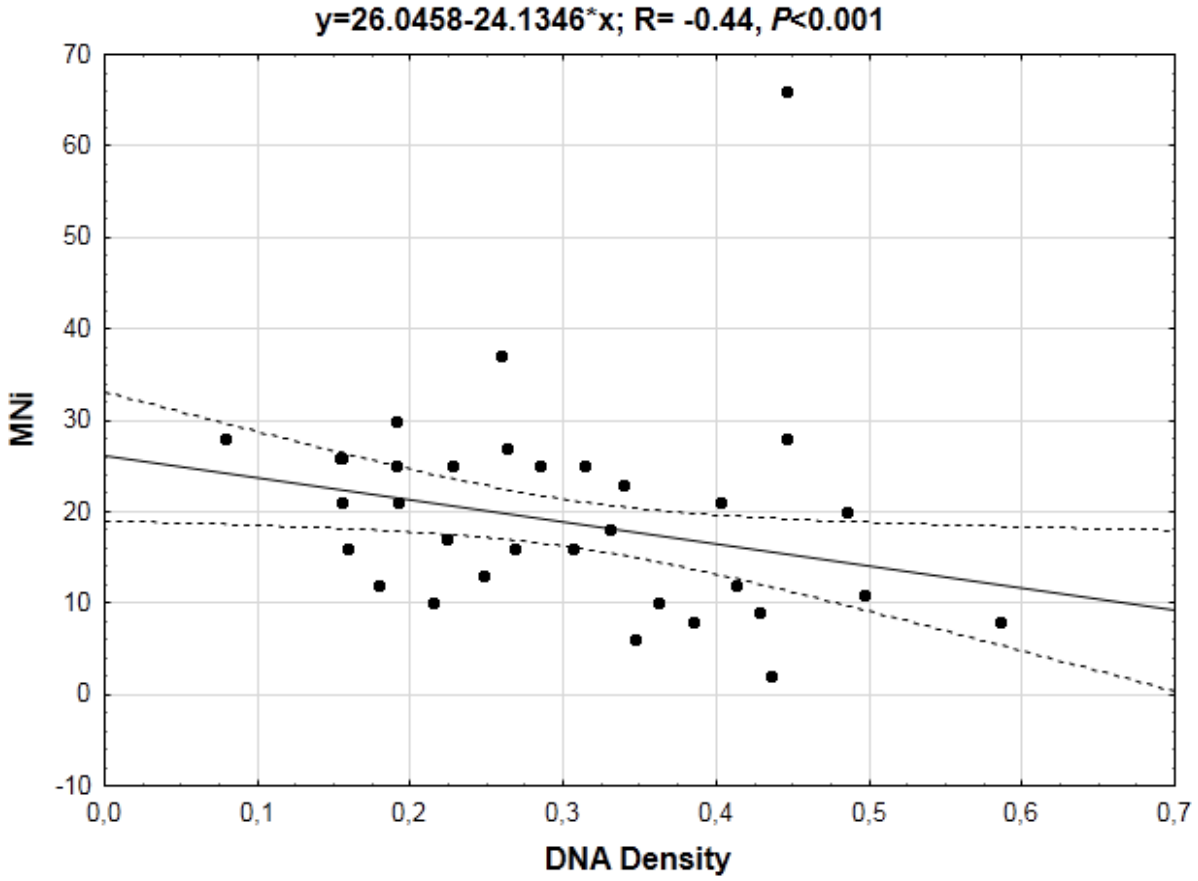


Figure 5. The scatterplots show regression equation, confidence interval and results of Spearman test between DNA density, assessed by comet assay, and MNi, respectively.

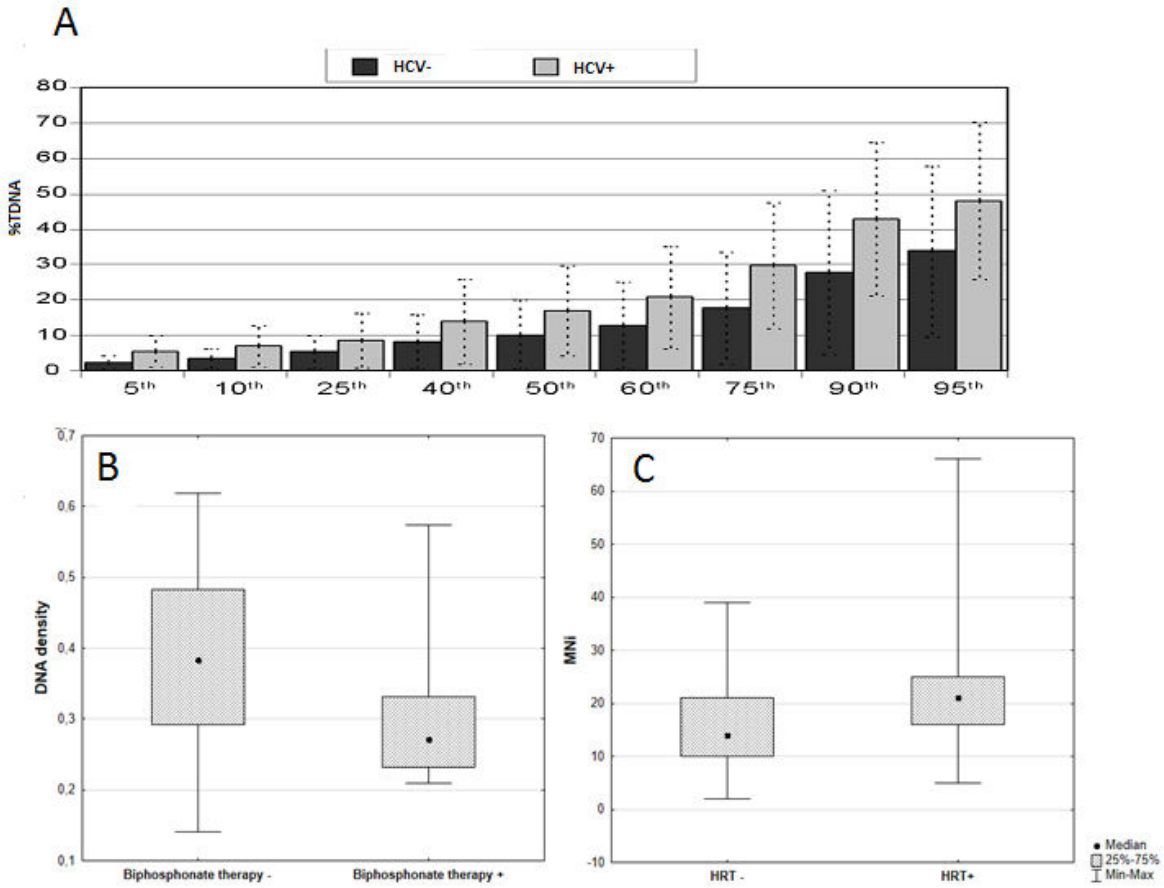


Figure 6. A) Percentile values of %TDNA, assessed by the comet assay, in thalassaemic patients in the presence or absence of active HCV infection. The results are reported as means ± SE ($P < 0.05$ for all centiles to Mann–Whitney test).

Box plots highlight the genotoxic effects of A)bisphosphonate therapy assessed via DNA density that, measuring the chromatin compactness, is inversely related to double- and single-strand breaks as well as to alkali labile sites and C) HRT in thalassaemic patients assessed via MNi frequency.

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