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Epigenetic effects of biofortification with folate and microelements in food plants

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Coordinator: Prof. Lucia De Franceschi

Tutor: Prof. Simonetta Friso

PhD student: Domenica De Santis

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1. Abstract

The increasing interest in nutrients and microelements linked with epigenetic modifications, brought to focus on folate (vitamin B9), the transporter of methyl groups for DNA methylation the most studied epigenetic feature of DNA. The principal approaches to overcome folate and microelements deficiencies have been either mandatory fortification or voluntary supplementation by pills of synthetic B9 vitamin. These strategies, however, have potential drawbacks due to the fact that folic acid is an unnatural compound with potential adverse effects. Some research, therefore, lately focused on developing genetically engineered plants that contains more folates than natural plants, but this approach also raises a considerable concern for the potential noxious effects of metabolic products from genetically modified plants. Since folate metabolism needs microelements as enzyme cofactors, as selenium (Se) and zinc (Zn), whose content is often inadequate in natural soil, there is a growing interest in finding novel strategies to increase both natural folate and microelement contents in food plants. This agronomic biofortification approach required the development of adequately enriched fertilizer formulas. The study is aimed at developing an agronomic biofortification model of lettuce through the development of specific foliar fertilizers containing Zn, Se and para-aminobenzoic acid, a natural precursor of folate, in a setting of growth chamber, hydroponic and open field culture. Microelements analyses were performed after setting up a novel ICP-MS method by using NIST 1570A, *Trace Elements in Spinach Leaves*, as certified reference material (CRM). The improvement of the levels of microelements in plant leaves was demonstrated and a clear enrichment of folate content in lettuce plants was confirmed by UPL/MC qTOF analysis. Cell culture model experiments were used to test the effects of lettuce extracts on HepG2 transcriptome through RNA-Seq sequencing that highlighted either a u- and down-regulation of an ample

number of genes in several pathways including those linked to Se and Zn transport and folate metabolism. The epigenetic regulation at promoter region by DNA methylation was tested on selected genes involved in folate and microelements metabolism such as MT2A and FOLR1 were investigated showing a possible role of lettuce extract exposure on gene expression modulation through the epigenetic feature *i.e.* DNA methylation.

For a deeper focus on novel mechanism of cell-to-cell communication, attention was given also to exosomes, microvesicles released by all fluids and cells. Exosomes mRNA cargo was analysed after the treatment of HepG2 and also on Ea.hy926, endothelial cell model, with extracts of lettuce control and biofortified and gene expression compared between the two model showing that cells exposed to biofortified lettuce have higher number of exosome production by the cells potentially affecting significantly the cell-to-cell communication of mRNA of genes differentially modified by lettuce extracts exposure.

Biofortification using a novel Se, Zn and p-ABA-enriched formula is effective to ameliorate the content of such microelements and 5methylTHF in lettuce and lead to gene expression and gene specific DNA methylation modulation in HepG2 and Ea.hy926 cell culture models. Future perspectives include specific investigation of biofortified lettuce nutritional and microelements bioavailability and specific epigenetic modulation in humans for possible correction of widespread microelements and vitamin deficiencies with the purpose of reducing the risk of several major chronic diseases related to nutritional deficiencies.

2. Abbreviation

AZA   5-Aza-2' deoxycytidine

CRM   Certified Reference Material

CCT   Cell Collision Technology

DNMT   DNA methyltransferases

FOLR1   Folate Receptor α

HYDRO   Hydroponic Modality

ICP/MS   Inductively Coupled Plasma-Mass Spectrometry

KED   Kinetic Energy Discrimination

MT2A   Metallothionein 2A

MTHFR   Methylenetetrahydrofolate reductase

p-ABA   Para-Aminobenzoic Acid

ppm   Part per million

RFC1   Reduced Folate Carrier 1

Se   Selenium

SLC19A1   Solute Carrier Family 19 type A1

SLC39A13 Solute Carrier Family 39 type A13

ZIP13   Zinc Transporter 13

Zn   Zinc

THF   Tetrahydrofolate

UPLC/MS   Ultra Performance Liquid Chromatography/Mass Spectrometry

3. Introduction

3.1 Epigenetics

Epigenetics refers to those heritable changes in phenotype due to gene expression regulation without alterations of the primary DNA sequence (Allis and Jenuwein 2016). Conrad H. Waddington, a developmental biologist, coined this term in 1942 and recently this term includes gene expression regulatory mechanisms such as histone modifications, e.g. acetylation, methylation, phosphorylation, biotinylation, ubiquitination, also called *"DNA decoration"* as well as DNA methylation and RNA interference (RNAi). These mechanisms play essential roles in cell proliferation, differentiation, survival and are fundamental during human development (Gilbert 2012). DNA methylation is generally considered one of the most important epigenetic modification, and it occurs when a methyl group ($-CH_3$), derived from the universal methyl donor S-adenosyl-L-methionine (SAdoMet), is added to the 5'-carbon position (C5) of a cytosine in a CpG dinucleotide sequence (Daniel and Tollefsbol 2015). CpG dinucleotides are typically clustered in CpG rich areas called 'CpG islands'. These islands compose less than 1% of the genome and are typically unmethylated (Crider, Yang et al. 2012) and often take place within regulatory regions of gene promoters playing thus, a pivotal role in gene expression regulation. DNA methylation is largely controlled by enzymes known as DNA methyltransferases (DNMTs), a family of highly conserved proteins that catalyse the transfer of methyl groups. In healthy cells, DNA methylation controls the integrity of the genome, genomic imprinting, X-chromosome inactivation, inhibition of repeated elements and transposons transcription. In cancer cells an aberrant DNA methylation pattern, such as genomic hypomethylation and a concurrent gene specific hypermethylation has instead been described (Ehrlich 2006). Considering the important role of DNA methylation, an aberrant pattern of this

epigenetic feature of DNA is involved also in the pathogenesis of several other complex chronic diseases including those affecting the cardiovascular system (Udali, Guarini et al. 2013, Friso, Carvajal et al. 2015). Since epigenetic markings are also heritable they can represent the mechanisms whereby an adult organ's genome would retain the memory of early-life environmental exposures by long-term alterations in gene expression programming (Ong, Moreno et al. 2011). Epigenetics is now considered one of the most important mechanisms possibly regulating the unknown aetiology of many diseases in which the environmental exposure to either nutritional or other factors may play an important role in etiological aspects of the ample complexity of environment-associated diseases (Powell 2000).

3.1.1   The role of promoter DNA methylation

For many years, methylation was believed to play a crucial role in repressing gene expression (Saif, Kasmi et al. 2018). Cytosine methylation can interfere with transcription factor binding, yet repression seems to occur largely indirectly, *via* the recruitment of methyl-CpG binding domain (MBD) proteins that induce chromatin changes (Weber, Hellmann et al. 2007). Consequently, the strength of repression can depend on the local concentration of CpGs within the promoter. Indeed, it is established that methylation of CpG-rich promoters is incompatible with gene activity. Equally uncertain is the contribution of promoter DNA methylation to tissue-specific gene expression, which predicts a dynamic reprogramming during development. Most CpG island remain unmethylated at promoter sites even in cell types that do not express the gene. However, changes in DNA methylation linked to tissue-specific gene expression have been sporadically seen on CpG-rich promoters although other studies failed to show such a connection based on the analysis

of a small set of genes (Weber, Hellmann et al. 2007). Nowadays, the role of methylation in gene expression is better defined as mediator after observing that methylation near gene promoters varies considerably depending on cell type, with more methylation of promoters correlating with low or no transcription (Suzuki and Bird 2008). Also, while overall methylation levels and completeness of methylation of particular promoters are similar in individual humans, there are significant differences in overall and specific methylation levels between different tissue types and between normal cells and cancer cells from the same tissue (Bocker, Hellwig et al. 2011).

3.2   One-carbon metabolism

One–carbon metabolism is a network of interrelated biochemical reactions that provide methyl groups for multiple physiological processes, from biosynthesis of nucleotide, amino acid homeostasis (glycine, serine and methionine), methylation of proteins, phospholipids and nucleic acids to redox defence. These reactions are performed by a great number of enzymes dependent from several vitamins and nutrients that act as co-factors or methyl donor and acceptors, among which folate (vitamin B9), cobalamin (vitamin B12), pyridoxine (vitamin B6), riboflavin (vitamin B2), niacin (vitamin B3) have a major role. Since most of them derive from dietary intake, even a little deficiency of these nutrients have a potential to alter could affect DNA methylation and at least modulate gene expression by a complex interplay among enzyme function and vitamin availability (Friso and Choi 2002). Briefly, a serine hydroxy-methyltransferase, vitamin B6 associated, catalyses the reversible transfer of a one-carbon unit from serine to tetrahydrofolate (THF) to generate glycine and 5,10 methyleneTHF. Then methylenetetrahydrofolate reductase (MTHFR), irreversibly catalyses 5,10 methyleneTHF to 5-methylTHF, the primary methyl donor for the remethylation of

homocysteine to methionine by methionine synthase via the remethylation pathway. The amino acid methionine is either derived from the diet or from the remethylation of homocysteine which then turns into S-adenosylmethionine (SAdoMet). After donating the labile methyl groups from 5-methylTHF, SAdoMet turns into S-adenosylhomocysteine (SAdoHcy), which is further converted to homocysteine. So, two metabolites of one-carbon metabolism can affect methylation of DNA and histones: S-adenosylmethionine (AdoMet), which is the universal methyl donor for methylation reactions, and S-adenosylhomocysteine (AdoHcy), which is a product inhibitor of methyltransferases (Figure 1).
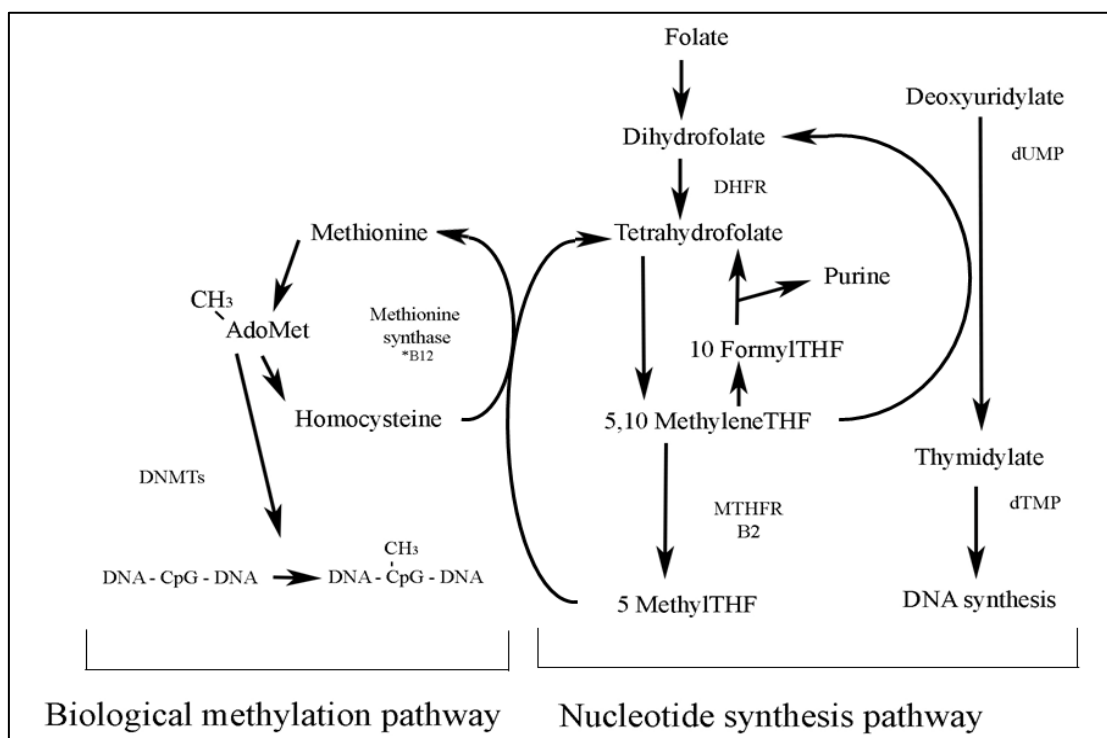
Figure 1. **Nutrients, enzymes and molecules involved in one-carbon metabolism.** One-carbon metabolism provides the methyl groups for biological methylation reactions and for nucleotide synthesis.
Nutrients that mainly influence the pathway are folate, involved in the delivery of methyl groups; vitamin B6, coenzyme of serine hydroxy-methyltransferase (SHMT); vitamin B2, coenzyme of methylenetetrahydrofolate reductase (MTHFR); vitamin B12, coenzyme of methionine synthase (MS); while among amminoacids, methionine is critical for the synthesis of S-adenosylmethionine (SAdoMet).

3.3 The role of nutrition in epigenetics

Since epigenetic phenomena are reversible and influenced by environment including nutrition, the latter is thought to be one of the most influential factors with effects on the transcriptional activity of several genes (Choi and Friso 2010). Several nutrients among which vitamins and microelements, considering their role in the provision of methyl groups, they can affect the function of DNMTs potentially altering global and gene specific promoter DNA methylation. Since epigenetic deregulation occurs early in several disease pathogenesis and is potentially reversible, intervention strategies targeting the epigenome have been proposed for cancer prevention. Although the epigenome represents a promising target for disease prevention by nutritional intervention, only few studies have addressed the influence of dietary components on these mechanisms especially if considering the effects of food or food extracts rather than single vitamins interventional approaches. Moreover, nutrition and dietary habits have been studied in relation to aging and cancer incidence and prognosis ((Li and Tollefsbol 2011); (Mercken, Crosby et al. 2013); (Meeran, Ahmed et al. 2010)). Recent research focused on the involvement of micronutrients and their role in fundamental processes such as DNA synthesis, DNA methylation, DNA repair and apoptosis. Epidemiological studies suggest that low intake of vitamins and minerals could also be a major risk factor for several types of cancers and other degenerative diseases. It has been shown that dietary deficiencies in certain micronutrients such as folate or other vitamins or microelements can result in DNA-strand breaks and DNA base lesions (Fenech and Ferguson 2001, Cousins, Blanchard et al. 2003).

3.4 Folate

Among nutrients, folate has a prominent role in the regulation of biosynthesis, repair and methylation of DNA (Hardy and Tollefsbol 2011). Folate, even called tetrahydrofolate (THF), was discovered by Lucy Wills in 1931 as a component of yeast capable of curing ''pernicious anaemia of pregnancy''. The name was coined then, in 1941 by Mitchell and co-workers after the 4 tons of spinach leaves from which it was purified, so choosing folate from the Latin word *folium* – leaf. Folate is a soluble tripartite vitamin of the B group, vitamin B9, composed by pterin, p-aminobenzoate (p-ABA) and one or more glutamate moieties (Figure 2). The pterin ring of folate exists naturally in di-hydro or tetra-hydro form, and only the latter has a cofactor activity. In his synthetic form, folic acid, the ring is fully oxidized, although it can be reduced *via* dihydrofolate (DHF) to THF. One carbon unit at various levels of oxidation, formyl (C=O), methylene (CH_2), methyl (CH_3), can be enzymatically attached to the N-5 or N-10 positions of the THF, the resulting one carbon substituted folates are enzymatically interconvertible and serve as one carbon donors for various reactions (one carbon metabolism). A short γ-linked chain of additional glutamate residues, up to approximately six, is typically attached to the first glutamate. This polyglutamyl tail is important to folate function because *in vivo* folate-dependent enzymes generally prefer polyglutamates, whereas folate transporters prefer monoglutamyl forms (Hanson and Gregory 2011) (Figure 2), thus glutamylation favours folate retention within cells. Folate is synthesized by plants and bacteria, in the first one the three parts of the THF molecule are produced separately in subcellular compartments, plastids, mitochondria and cytosol, and then joined together. Human and animals are unable to make folate *de novo* and hence depend on dietary sources, especially leaf and green plants (Figure 3).

The main function of folates is the transport of methyl groups in one carbon metabolism, essential for nucleotide biosynthesis and DNA methylation reactions (Quinlivan, Hanson et al. 2006). In humans, under normal dietary conditions, they are present in most body fluids and tissues, in fact, the folate absorbed at duodenum level is metabolized to 5-methyltetrahydrofolate (5-methylTHF, monoglutamyl form) in the intestine and/or in the liver. The 5-methylTHF is the primary form of folate taken up by non-hepatic tissue, which then must be polyglutamated for cellular retention and one-carbon cycle coenzyme function (Blancquaert, Storozhenko et al. 2013). When folic acid is consumed in fortified foods or in vitamin supplements, it is metabolized primarily to 5-methylTHF during intestinal adsorption and first passes through the liver and after this passage it behaves identically to natural dietary folate, entering in the folate pool (Crider, Yang et al. 2012).

While folate refers to all forms of vitamin including the naturally occurring forms of the vitamins, *i.e.* polyglutamates, folic acid refers to the oxidized synthetic form, monoglutamate (Arcot and Shrestha 2005). All the natural folate forms are unstable, in particular upon oxidative cleavage promoted by light that can yield large losses in post-harvesting fruits and vegetables or can be lost during processing and cooking. However, *in vivo* they may be stabilized by antioxidant compounds such as ascorbate and glutathione and by the binding of specific proteins (Witthöft, Forssén et al. 2016).

Folate deficiency can be determinate by elevate consumption of alcohol, intestinal malabsorption, inadequate intake or genetic mutations. A folate deficiency can cause anaemia, neural tube defects, cardiovascular diseases and it has been associated to many different types of cancer, such as that affecting breast, ovary, brain and colorectal (Lucock 2000). It is then important to maintain an adequate folate status and intake through the diet.

The *Recommendation Daily Intake* (RDA) for folate is 400 µg/day for adults that should be increased to 600 µg/day for female during pregnancy (Saini, Nile et al. 2016).
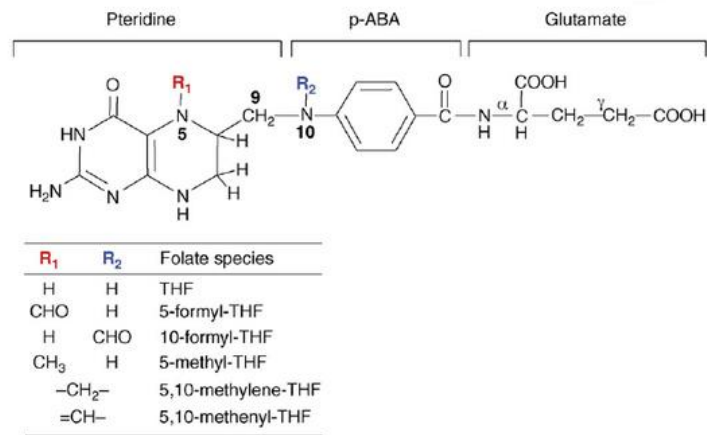
Figure 2. **Folate structure.** Folate structure and description of substituent groups to 5N or 10N in different folate forms (from *Trends in Plant Science)*
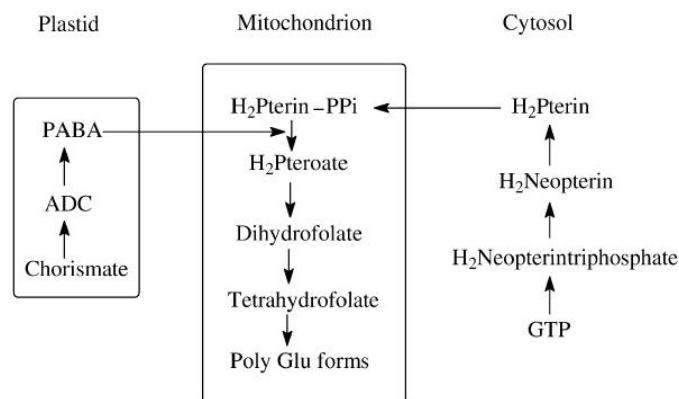
Figure 3. **Folate biosynthesis in plant.** First, the generation of pterin from GTP, second, a p-aminobenzoic acid (p-ABA) derived from chorismate, is condensed to dihydropterin pyrophosphate leading to dihydropteroate synthase; third, a first glutamate is attached to the carboxy part of p-ABA, folate is reduced and polyglutamate tail is formed.

3.5 Microelements

3.5.1 Selenium

Selenium (Se), atomic number 34 and atomic weight 78,96 u, is an essential micronutrient for humans and animals, since it plays a role as coenzyme in many cellular functions; it has

been known to have antioxidant, cardioprotective, proapoptotic, DNA repairing and anticancer properties (Rayman, Infante et al. 2008). Selenium is found in more than 20 selenoproteins and selenoenzymes, such as the redox enzyme glutathione peroxidase (GPX), which contributes to preventing oxidative cellular degradation and thioredoxin reductase that reduce oxidized molecules in animals and some plants (Funes-Collado, Morell-Garcia et al. 2013). In these ones selenium levels requirements differ among species and can be beneficial at low concentrations, favouring response to oxidative stress, produced by photosynthesis or short-wavelength light (Hartikainen 2005), promoting growth and retarding senescence (Tomasi, Pinton et al. 2015). In plant we can find Se inorganic forms as selenate and selenite, or organic forms as SeMet, selenomethionine and SeCys, selenocysteine but selenate is more easily transported from the roots and much more is accumulated in the leaves than either selenite or organic selenium and then it is predominantly metabolized to SeMet, the main Se compound in plants (Gupta and Gupta 2016). Although Se is nonessential for plant nutrition, vegetable crops are the preferred source for Se supplementation because they contain Se-organic forms, which are available for humans (Rayman, Infante et al. 2008). It has been estimated that 500-1000 million people worldwide may be deficient in Se, the *RDA* for Se is 55 µg/day. A Se deficiency has been demonstrated to cause global hypomethylation and promoter methylation of the p16 and p53 tumour suppressor genes (Hartikainen 2005).

3.5.2 Zinc

Zinc (Zn), atomic number 30 and atomic weight 65,409 u, is a ubiquitous microelement, essential component of thousands of proteins in plants and the second most abundant transition metal in organisms after iron (Fe). Its importance even for humans is due to its

presence in over three hundred different metalloenzymes (10% of human proteins) without counting the hundreds more ones that transport and traffic Zn and for its relevant role in activation of transcription factors involved in gene expression, signal transduction, transcription, replication and apoptosis (Berg and Shi 1996). Zn plays a critical role in the regulation of DNA repair mechanisms, cell proliferation, differentiation and apoptosis. Zn may also affect activities of enzymes such as BHMT, Betaine-homocysteine methyltransferase and MTR (5-methyltetrahydrofolate-homocysteine methyltransferase) involved in methionine synthase reaction, the folate-methionine cycle, by enabling the active site conformational changes necessary for homocysteine activation and methyl transfer (Sharif, Thomas et al. 2012). Zn deficiency has been shown to affect DNA repair response via regulation of one of the most important tumour suppressor proteins, p53 (Hainaut and Mann 2001). The Zn RDA for women and men are 8 and 11 mg/day. According to a WHO report, Zn deficiency ranks fifth among the most important health risk factors in developing countries (Cakmak 2007). A Zn deficiency may induce an impairment in physical development, immune system, learning ability, increased risk of infections, DNA damage and cancer development. Its low solubility in soils, in particularly in high-pH soil, rather than low total amount of Zn is the major reason for the widespread deficiency in crop plants and populations even in the richest country (Cakmak 2007).

3.6 Microelements, Se, Zn and folate transport related genes

Folates, soluble vitamins of the B family, are hydrophilic molecules that require an active membrane transport system for cellular uptake. SLC19A1, solute carrier family 19 (folate transporter, member 1), also known as reduced folate carrier 1, (RFC1), encoded by RFC

gene, Chr. 21q22.3, is ubiquitously expressed and is the major folate transporter in mammalian cells and tissues. Very high levels of RFC transcripts are detected in liver and placenta, with appreciable levels in other tissues, including kidney, lung, bone marrow, intestine, brain, and portions of the central nervous system. (Hou and Matherly 2014). By immunohistochemistry in mouse tissues, RFC was detected at the basolateral membrane of the renal tubule epithelium, the apical brush border membrane of the small intestine and colon, hepatocyte membranes. The active transport across cell membrane occurs in a bidirectional via among 5-methylTHF or 5-formylTHF and thiamine phosphate. In case of folate deficiency, the transport can be down regulated. RFC uses a bidirectional anion exchange mechanism to transport folates into cytoplasm.

Folate receptor α (FOLR1) located on Chr. 11q13.4, binds folic acid, reduced folate forms and 5-methylTHF and internalize them into the cell. FOLR1 can be found anchored to membranes via a glycosyl-phosphatidylinositol linkage or exists in a soluble form. In normal tissue FOLR1 is expressed on the apical surface of polarized epithelial cells and it isn't exposed to the blood stream. FOLR1 have been found overexpressed in several solid tumors, such as epithelial, ovary, breast, renal and lung malignant tissue.

Principal FOLR isoforms are capable of transporting folate into cells, but generally the ubiquitously expressed RFC is exclusively used for this purpose by adult tissue. In fact, most normal tissue virtually lack FOLR, and its physiological importance appears to be confined to situations where the availability of folate is limited. Under pathological condition FOLR1 may be overexpressed to increase folate uptake in order to cope with the augmented turnover of nucleic acid synthesis and reparation during accelerated cellular growth (Notaro, Reimer et al. 2016).

Se absorption, it can be affected by several dietary factors and by the chemical form of the element, can be summarized as follows: the ingested Se is transported in the blood from the intestine to the liver, then it is reduced to selenide before being transported in the blood, bound to α- and γ-globulins in target tissues and incorporated into specific selenoproteins, as selenocysteine, and, non-specifically, as selenomethionine. The mechanisms of transport of dietary selenium across the intestinal epithelial membrane is not well known: absorption of selenate appears to be by a sodium-mediated carrier transport mechanism shared with sulphur, while selenite uses passive diffusion (Fairweather-Tait, Collings et al. 1997).

Since the relevant Zn role, its transport across the cellular membranes is tightly regulated by various Zn transporters: Zn transporters (ZnTs), Zrt-, Irt-related proteins (ZIPs) and metallothioneins (MTs).

ZIP proteins or solute carrier 39A (SLC39A) family, in human are composed by 14 classes of plasma membrane proteins, allowing the Zn influx from the extracellular or luminal side into the cytoplasm and from vesicles. ZIPs are not specific and can also likely transport elements. By contrast, ZnTs or solute carrier 30 (SLC30) family, control the efflux of zinc from the cytoplasm out of the cell and from the cytoplasm into vesicles; as a counterpart of ZIPs in zinc homeostasis (Bin, Seo et al. 2018).

MTs are a superfamily of low-molecular-weight cysteine rich intracellular proteins (30% of aminoacidic residues) that bind metals and play a pivotal role as Zn specific chaperone, similar to a redox mechanism that distributes Zn to enzymes in the metabolic network, interacting with other proteins that can be crucial in the formation of metalloenzymes and transcription factors (Zalewska, Trefon et al. 2014). Humans MTs have 11 isoforms divided in 4 classes. The ubiquitous MT isoforms MT1 and MT2 have been extensively investigated

about zinc metabolism and buffering. Their synthesis is induced by many factors such as metal ions, glucocorticoids, cytokines and oxidative stress. MT seem to be very important for the regulation of p53 (Meplan, Richard et al. 2000, Ostrakhovitch, Olsson et al. 2006). MT cytosolic ligand is regulated in response to the change in cellular Zn concentration. In an ideal situation, from sinusoid, to hepatocyte via ZIP protein (Figure 4), MT chelates Zn from the environment increasing the intracellular concentration of Zn. The free form of Zn, Zn^{2+}, is able to bind the metal transcription factor (MTF-1) which translocate to the nucleus and induces the synthesis of new MTs, by binding to the metal response element (MRE) in the promoter region.

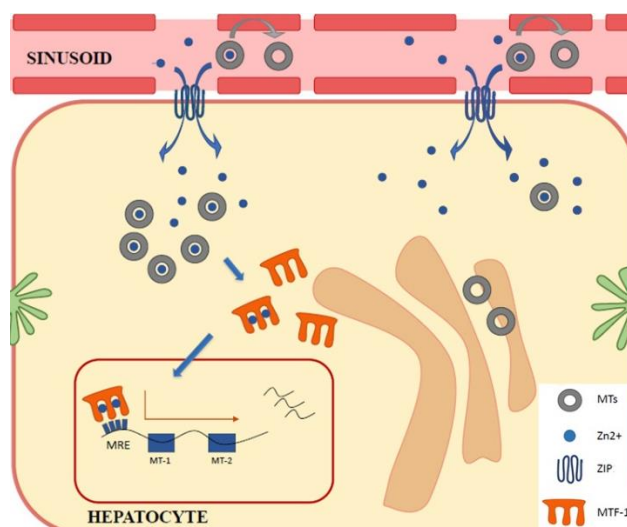Figure 4. **ZIP and MT representation.** Graphical representation of ZIP and MT proteins function in hepatic cell.

3.7 Biofortification

Food plants have been an important source of microelement and antioxidant compounds for thousands of years. Scientific data indicate that diets rich in vegetables are associated with a lower risk of several degenerative diseases, such as cancers and cardiovascular

diseases (Crosby 1963, Rimm, Ascherio et al. 1996, Steinmetz and Potter 1996). The WHO has estimated that around 65% - 80% of the world population, especially in developing countries, depends essentially on plants for their primary healthcare. Folate deficiency as well as micronutrients, are a significant worldwide public health problem in poorer countries and in some richer populations. because most plant foods have very low folates levels that are largely lost during processing and cooking (Bouis and Welch 2010). The inherent lability of nutrients derived by processing and cooking, and the consumption of them by human body, underlie the need of humans and animals for a continual supply, to avoid a subsequent deficiency (Hanson and Gregory 2011).

For these reasons was proposed a natural biofortification as agricultural tool to overtake micronutrient malnutrition worldwide substituting pills supplementation and engineered food fortification (Bekaert, Storozhenko et al. 2008). One of the most important aspects of biofortification is the sustainability compared with industrial fortification, addition of synthetic form of nutrients, microelements and pharmaceutical supplementation.

Example of folate biofortification of staple crops through enhancement of pterin and P-ABA, even called vitamin B10, levels was reported to be successful in tomato and rice, but is not sufficient to enhance folate content in potato tubers and Arabidopsis thaliana plants (Blancquaert, Storozhenko et al. 2013). In addition, natural nutrients are widely preferred over synthetic due to possible unwanted health effects of them: positive health impact, cost-effectiveness and consumer preferences studies, supported the potential impact of biofortified crops ((Selhub and Rosenberg 2016); (Kelly, McPartlin et al. 1997)).

We choose, among food plants, lettuce for its worldwide diffusion as the leafy vegetable crop most produced and consumed in the world, its easy cultivation, and for the great quantity of THF in its leaves. In addition, lettuce is eaten almost completely as a fresh salad

without risk of bioactive compounds loss or cooking methods. Reports on wheat, radish and lettuce fortified with Se, showed raises in element concentration in plants. ((Smrkolj, Pograjc et al. 2005); (Ríos, Rosales et al. 2008)).

Biofortification could be apply through fertilizer formulas added to soil or on leaves as spray. The soil approach, often, is difficult because the different pH conditions and the soil composition can affect the availability of supplied element via carrier competition.

Foliar fertilization, instead, is one of the most effective and safest approaches to enrich essential micronutrients in crop grain. Leaf-applied substances can enter the leaf either by penetration of the cuticle or via the stomatal pathway. In particular several studies have shown a more significant relationship between the measured Se increase, and the amount of fertilizer, containing Se as selenate instead of selenite form, applied directly to leaf surfaces. (Blancquaert, Storozhenko et al. 2013).

3.7.1 Cultivation modality: growth chamber, hydroponic and open field

Nowadays the increase of knowledge about plant metabolism and bioavailability of nutrients and microelements, led to the development of different cultivation modalities. The growth chamber is a useful close controlled system to test new fertilizer compositions. At the same time, the recent increased interest in hydroponic technology to produce leafy vegetables for ready-to-eat salads justified optimisation of growing system that can lead to higher yield and improved nutritional value of tested fertilizers and samples. Soilless cultivation represents an alternative to traditional agriculture, showing several possible advantages, lower production costs, higher nutrient-use efficiency and reduced foliar disease, coupled with zero leaching of nutrients to the groundwater. ((Tomasi, Pinton et al. 2015); (Manzocco, Foschia et al. 2011)). In hydroponic modality is used a well-known

nutrient solution, called Hoagland Solution, that provides every nutrient necessary for plant growth. The Hoagland solution was developed by Hoagland and Arnon in 1938 and offers a perfect condition to have a controlled management of the crop and henceforth the supply of microelements. The cultivation system can be suitable for improving nutrient content of edible leaves, although caution must be taken to avoid dangerous accumulation of element and compounds.

The open field represent the closer condition to current and widespread cultivation, although a lot of variables, from the weather to the soil composition, can affect the results of intervention of supplementation.

3.8 In vitro study

Vegetables could be relevant as dietary sources of natural antioxidants, such as, polyphenols, flavonoids, vitamins and fibre that may protect key biological constituent such as lipoproteins, membranes, and DNA (Hall 1997, Szeto, Kwok et al. 2004). Diets rich in vegetables are associated with a lower risk of cancer and cardiovascular disease (Hooper, James et al. 2008)

Potential mechanisms for cancer prevention of phytochemicals include prevention of DNA adduct formation, enhanced carcinogen elimination, inhibition of inflammatory processes, interference with tumour angiogenesis, as well as through a direct cytotoxic effect on tumour cells. This pleiotropic mechanism of action of phytochemicals imply that the chemo preventive properties that are associated with vegetables consumption are complex and likely arise from synergistic combination from several distinct molecules, not only within a given food but also from the overall composition of the diet. Clearly the identification of

specific foods or food groups that have beneficial effects on certain types of cancer represent an important issue in order to bonify current chemopreventive strategies based on increased consumption of vegetables. (Lee, Lee et al. 2004).

Lettuce is an important dietary leafy vegetable that is primarily consumed fresh or in salad mixes due its healthier perception. The health benefits of lettuce have also been attributed to the presence of vitamin C, phenolic compounds and fibre content.

There are a number of bioactivities conducted to evaluate the therapeutic significance of Lactuca sativa including; anticonvulsant, sedative-hypnotic, antioxidant, analgesic and anti-inflammatory activities.

In this study, we tested whole extracts of biofortified and not lettuce, on highly differentiated cell line of hepatocarcinoma, (HepG2) which not only resembles morphologically normal hepatocytes (Bouma et al., 1989) but has also carcinogenic phenotype, and on a human endothelial cell line, EA.hy926, that originally derived from human umbilical vein and showed vascular endothelial cell characteristics, where microelements were been already investigated (Steinbrenner, Bilgic et al. 2006).

3.8.1 Exosomes and RNA messengers transport

Cells, even tumor cells, release different kind of extracellular vesicles (EVs), among which, exosomes (40-130 nm) derived from a specialized compartment of the endosomal-lysosomal pathway, deliver a complex cargo of materials including polypeptides, micro-RNA and mRNA (Figure 5). Noted that exosomes are released also by cultured cells into media and are biologically active entities they can be isolated in vitro to study a variety of pathways. Isolation and profiling of exosomes from cell culture systems and body fluids provide important information about the biological system investigated, they can also

activate signaling pathways, enhance cancer progression, suppressing immune response and deliver nucleic acids to distant cells. So, targeting and studying molecular components of exosomes could be advantageous in order to increase the efficacy of therapeutic approaches as potential novel biomarkers for the diagnosis and prognosis of disease progression, too (Barros and Carvajal 2017).
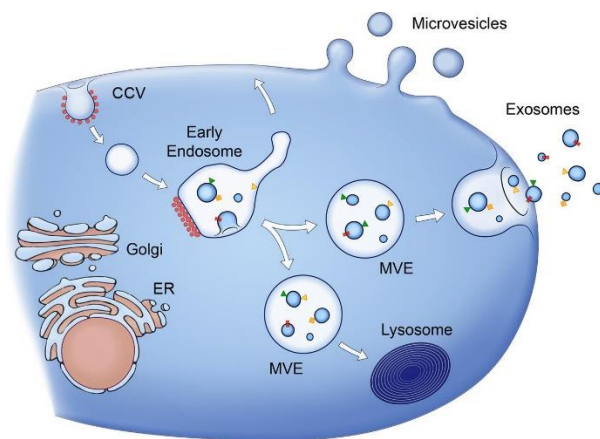

Figure 5. **Exosome biosynthesis.** Biosynthesis of exosome derived from early endosome complex inside the cells. Their release is determined from the fusion of multi vesicular endosome with cellular membrane or with lysosome.

3.9 Aim of the study

The main scope of the project was to evaluate after developing a novel system of agronomic biofortification, the effects of food plants extracts grown on epigenetic mechanisms and transcriptomics by using cell culture models. First specific aim was to develop a new foliar spray fertilizer formula enriched with zinc, selenium and p-ABA with the aim of increasing folates and microelements content. Second specific aim was to test in cell culture models the effects of biofortified plants on gene expression modulation through epigenetic features of DNA. Third specific aim was to analyse the specific effects of plant extracts on specific metabolic pathways by evaluating the cell exosomes content after exposure to food plants extracts.

4. Materials and methods

4.1 Plant material, fertilizer formulas and growing conditions

Plants cultivation, fertilizer formulas preparation and biofortification were executed in collaboration with Fabbrica Cooperativa Perfosfati Cerea (FCP, San Pietro di Morubio (VR), Italy).

Powder sodium selenite (Na_2SeO_4) and p-ABA (H1 vitamin) were purchased by ACEF, whereas zinc sulphate ($ZnSO_4$) by FCP (Cerea, Italy). Spray fertilizer foliar formulas were prepared in water at different concentrations of selected compounds as shown in Table 1 and were supplied after 33 or 31 days of cultivation. For formulas containing $ZnSO_4$ and p-ABA, a triplicate administration was also performed for some concentrations (Table 1). Control samples were cultivated in "Base" type NPK 6.12.18 soil (nitrogen/phosphorus/potassium) and were supplemented with a water foliar spray. All the treatments from A to H tests were performed in triplicate

	A	B	C	D	E	F	G	H	Mix
p-ABA (ppm)	50	250	500	1000	1500(500X3)*	3000(1000X3)*	-	-	2000
Na_2SeO_4(ppm)	2	4	6	15	30	60	-	-	60
$ZnSO_4$ (ppm)	10	100	1000	2000	1500 (500X3)*	3000(1000X3)*	6000(2000X3)*	12000(4000X3)*	4000

Table 1. **Foliar spray fertilizers.** Concentration of spray foliar fertilizer formulas, for $ZnSO_4$ and p-ABA in E, F, G, H treatments were carried out a triplicate administration (*).

4.1.1 Growth chamber

Preliminary trials, in soil, were conducted on *Lambole* and *Gentilina* lettuce varieties in growth chamber under the following conditions: 12 hours' day length, temperature of 22/17° C (day/night), relative humidity of 55-65%. The soil used for the cultivation was

"Base" type NPK 6.12.18 with low chlorine title, total nitrogen 6.2 %, ammonia nitrogen N-NH_4 4.1%, urea nitrogen N-NH_2 1.0% and nitric oxide N-NO_2 1.2%; phosphorus pentoxide P_2O_5 12.4 %, soluble phosphorus pentoxide 11.0%, potassium oxide K_2O 18.2 %, sulphur dioxide SO_2 31.4% (FCP, Cerea, Italy).

Subsequent experiment performed in growth chamber were carry out on *Gentilina* variety with Mix treatment, p-ABA 2000 ppm, Na_2SeO_4 60 ppm and $ZnSO_4$, in pool triplicate with each pool was constituted by 10 plants to normalize potential phenotype differences.

4.1.2 Hydroponic crop

After preliminary tests, lettuce plants, "*Lambole*" variety, were grown in hydroponic modality in growth chamber under conditions previously described in "Hoagland' solution" composed as listed: nitrogen (N) 210 ppm, potassium (K) 235 ppm, calcium (Ca) 200 ppm, phosphorus (P) 31 ppm, sulphur (S) 64 ppm, magnesium (Mg) 48 ppm, boron (B) 0.5 ppm, iron (Fe) 1 to 5 ppm, manganese (Mn) 0.5 ppm, zinc (Zn) 0.05 ppm, copper (Cu) 0.02 ppm and molybdenum (Mo) 0.01 ppm. Na_2SeO_4 and p-ABA were supplied as foliar spray at 60 ppm and 3000 ppm, respectively. The control samples were treated with water foliar spray, too. All the treatment was performed in pool triplicate, each constituted by 10 plants.

4.1.3 Open field crop

To mimic normal cultivation condition, lettuce plants were grown in open field near FCP company in NPK 11.11.16 soil and then implemented with water - soluble NPK 30.10.10, organic N 7% and calcium nitrate (Ca $(NO_3)_2$) 100 kg/ha. Na_2SeO_4, p-ABA and $ZnSO_4$ were supplied as foliar spray at 60 ppm, 2000 ppm and 4000 ppm, respectively. The control samples were cultivated in same soil with water foliar spray. All the treatments were performed in pool triplicate, each constituted by 10 plants (Figure 6).
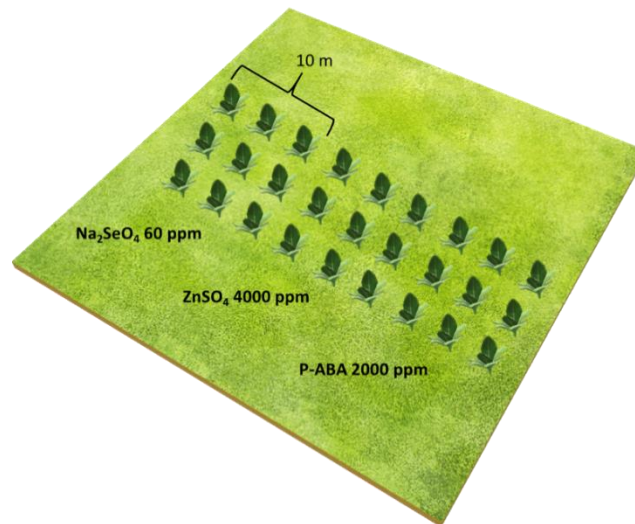
Figure 6. **Scheme showing a graphic of the experiment performed in open field.** Project design of the open field experiment with the distinction of the crop in parcel of ten meters with selected foliar treatments. In addition, a control lettuce parcel was considered in the design experiment.

4.1.4 Sample collection

Plants were harvested and sampled choosing two leaves for each plant section, up, middle and down, grouped for treatment according to the tested spray formulas and growing modality. The sampling was performed in duplicate, due the different following protocols and it was then transported to the laboratory after snap freezing using dry ice under controlled temperature.

4.2 Analysis of microelements by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry): optimization of digestion method on lettuce samples

To detect and quantify "trace element" present at ppm level in plants, high precision, resolution, sensitivity and extremely low detection limits are required. ICP-MS instrument possesses all these features becoming superior to many other techniques (Hansen, de Bang et al. 2013), allowing a simultaneous multi-elemental and trace analyses (Figure 7).
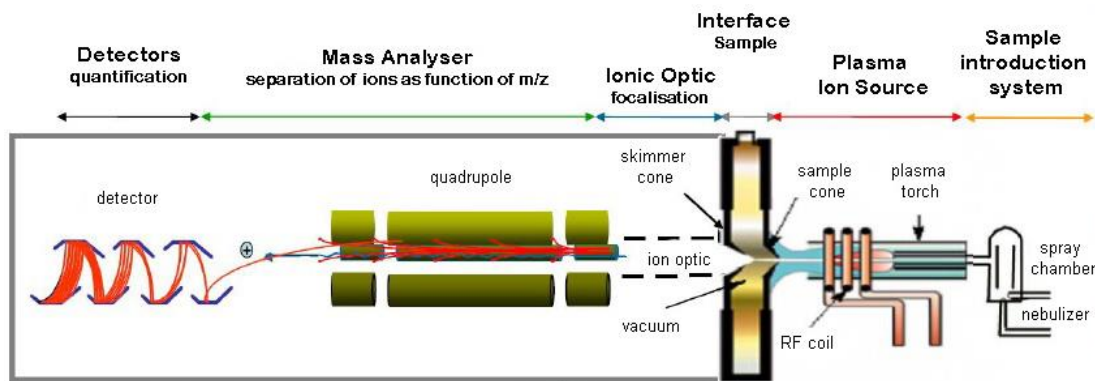
Figure 7. **ICP-MS technology.** Representation of ICP-MS structure and function: from sample injection to the quantification by the detector after mass discrimination.

4.2.1 Sample preparation and digestion

Samples intended to microelement analysis were, first, decontaminated through a wash in a Tween-20 solution (1 g/L) in Milli-Q water (Milli - Q water, Merck Millipore) and three rinses in Milli-Q water to remove dust and terrain-borne contamination and then stored at -80°C before lyophilisation. Freeze-drying was carried out with Freeze Dryer Modulyo (Edwards Vacuum, Milan, Italy) and samples stored in a dry environment at room temperature until digestion (Hansen, de Bang et al. 2013).

Freeze-dried samples were microwave oven digested (Mars 5, CEM, USA) according to different mineralization protocols of closed acid digestion in Polytetrafluoroethylene (PTFE, Teflon®) vessels previously decontaminated with a solution of 0,5 % HNO_3. Ultrapure deionized water (Milli - Q water, Merck Millipore) with resistivity of 18.2 Ω cm^{-1} was used throughout the experiments including for all the dilution and for rinsing the vessels for microwave. All reagents used for microelement determination were of analytical reagent grade. An analytical balance was used to weight the samples and the addition of reagents before and after the microwave processing to evaluate the seal integrity and possible loss of matter.

All the consumable in polypropylene (PP) were decontaminated with a solution of 0,5 % HNO₃ for at least 2 hours up to a maximum of 12 hours to avoid microelement contamination and overestimation (Hansen, de Bang et al. 2013).

4.2.2 Instrument (ICP/MS) settings

Microelements analysis was performed by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) system (Thermo Scientific X Serie II, Fischer Scientific, Hampton, New Hampshire, USA) equipped with a collision/reaction cell technology (CCT) and an ASX 520 autosampler (Cetac Technologies, Omaha, NB, USA).

The digestion method applied was validated by using certified reference material (CRM) from Trace Element in Spinach Leaves (SRM1570a, Sigma Aldrich, Milan, Italy).

CRM 1570A samples and analytical blanks were also prepared in the same way and analyzed to detect any experimental loss or cross-contamination. Precision was evaluated in terms of percent coefficients of variance (CV%), which in turn were obtained by measuring the relative standard deviation of nine CRM analyzed independently.

4.3 Analysis of folate forms by UPLC/MS (Ultra Performance Liquid Chromatography/Mass Spectrometry)

Folate analysis has been an analytical challenge for larger number of structural analogues, lability and low level in natural food samples. For an untargeted metabolomics approach and a sensitive and simultaneous determination of different folates in a particular matrix, UPLC/MS – qTOF was used.

4.3.1 Whole metabolites extraction from lettuce

Lettuce samples collected for folate forms analysis have been immediately frozen at -80°C and pulverized into a fine powder with liquid nitrogen in a A11 grinding mill (IKA, Wilmington, NC, USA) and stored at -80°C until analysis.

With the aim of not wasting significant metabolites and vitamins, we decided to operate a whole metabolites extraction by using a protocol with the use of an *ad hoc* prepared extraction reagent. The extraction reagent, composed by methanol/water (MeOH/H_2O, 80:20) and ascorbic acid 0.1 % (w/v), to protect folates during the procedure, was freshly prepared each time (Delchier, Herbig et al. 2016, Garcia, Garcia-Villalba et al. 2017). An amount of 500 mg of pulverized lettuce was weighted in obscured Pyrex glass flask where 5 volumes of cold extraction reagent were added to each sample and placed in ultrasound bath with ice for 15 minutes. Then, extraction mixtures were centrifugated at 4500g for 10 minutes at 4°C and supernatants were collected and stocked in glass vials until analysis. All the chemicals used for the extraction and analysis were of LC/MS grade purity and were purchased from Waters (Milford, Massachusetts, USA). All manipulations were carried out under subdued light.

4.3.2 Folate forms analysis

Before analysis, samples were diluted 1:2 with H_2O LC/MS grade, filtered with 0,22 μm PVDF filter to be directly analyzed with UPLC/MS Xevo G2-XS Q-TOF (Waters, Milford, Massachusetts, USA). The standard and m/z ratio used for folates detection were: m/z 138 for 4-aminobenzoic acid (p-ABA) $C_7H_7NO_2$, m/z 442 for folic acid $C_{19}H_{19}N_7O_6$, purchased from Sigma reagents (Sigma Aldrich, Milan, Italy), m/z 474 for 5-formyl-5, 6, 7, 8-tetrahydrofolic acid $C_{20}H_{21}N_7O_7$ (Ca), m/z 460 for 5-methyl-5, 6, 7, 8-tetrahydrofolic acid

$C_{20}H_{23}N_7O_6*4H_2O$ (Ca), m/z 457 for 5,10-methenyl-tetrahydrofolic acid $C_{20}H_{22}N_7O_6$ (Cl) purchased from Schrick Laboratories (Schrick, Switzerland). All the standards were injected at 1 ng/µl concentrations.

Ultra-Performance Liquid Chromatography (UPLC) Acquity I – class (Waters) in reverse phase was performed under gradient conditions on an BEH C-18 column (100mm x 2.1mm; 2.7 µm particle size, from Waters) at 30 ° C and an autosampler FTN (Waters) refrigerated at 10 ° C. The mobile phase consisted of 0.1% of formic acid in water (solvent A) and 100 % acetonitrile (solvent B) and was pumped at a flow rate of 0.350 mL/min. The starting condition (99% A+1% B) was isocratic for 1min. The proportion of B was increased linearly to 40% in 9min, to 70% at 12min where it was kept for 1min, followed by an immediately increase to 99% in 30 seconds. Then for the remaining 6min the elution was isocratic for 2 min for then switch back to 99% solvent A in 10 seconds. So, the mobile phase was adjusted to its initial composition and held for 3min for re-equilibration. Five microliters were injected on the column. The UPLC system was coupled to a Xevo G2 – XS mass spectrometer, equipped with electrospray ionization (ESI) source and quadrupole – Time of flight (qTOF). The parameters of the source were the following: 0.8 KV capillary voltage, 30 V cone voltage and 120 ° C source temperature. Nitrogen was used both as nebulizing gas, at a flow rate of 50 L/h, as desolvation gas at a temperature of 500 ° C with a flow of 1000 L/h. The instrument was operated in the ESI positive and negative mode with a collision energy of 35V. and the data were acquired every 0.3 seconds with a range from 50 to 2000 m/z.

4.4 RNA – Seq (RNA – sequencing) analysis of HepG2 treated with vegetal extracts

RNA-Seq is a revolutionary tool to analyse all the transcriptome profiling, in a specific physiological condition, using a deep-sequencing technology. In our study HepG2 cell culture was used as *in vitro* model to test possible action of our biofortified lettuce compare to the control lettuce on its transcriptome, to find differentially expressed genes.

4.4.1 Preparation of dried vegetal extracts

To obtain dried vegetal extracts to test them in vitro on cell lines, about 10 grams of frozen powdered lettuce (control and biofortified lettuce grown in open field, ref.2.1.3) were weighted in darkened tubes and 3 volume of cold extraction reagent were added to each sample to extract all the bioactive metabolites as previously described. The supernatants were collected in glass Pyrex and evaporated in a miVac Duo Speed Trap vacuum pump (Genevac, Ipswich, UK) until to completely reagent evaporation. Dried extracts were stored at -20°C. All manipulations were carried out under subdued light.

4.4.2 HepG2 Cell culture

The human hepatocarcinoma cell line, HepG2, was maintained in RPMI1640 (Sigma Aldrich, Milan, Italy) supplemented with 4mM L-glutamine (Lonza, Basel, Switzerland) 10 % heat-inactivated foetal calf serum (FBS)(Gibco, Thermo Fisher brand, Waltham, Massachusetts, USA), 1% Pen-Strep (100 U/mL), in a humidified 37°C incubator containing 5% CO_2.

4.4.3 Cytotoxicity Assay in HepG2

Cytotoxicity of extracts was assessed using confluent HepG2 cultured as previously described and CellTiter 96©Aqueous cell proliferation Assay (Promega, Madison, WI, USA) containing MTS reagent. Briefly, 200 µl of cells were seeded into two 96-well plates, one for control lettuce extracts and the other for biofortified lettuce, at $50 *10^3$ density and allowed to grow for 24h. The day after, dried extracts were resuspended directly in RPMI, 5% FBS, filtered with 0.22 µm filter and diluted to obtain solution at increasing concentrations: 0 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 250 µg/mL, 300 µg/mL, 350 µg/mL, 400 µg/mL, 500 µg/mL, 700 µg/mL. Culture medium was substituted with supplemented one and cells were incubated with extracts for 24 hours. After incubation, 100µl of medium was removed and 10 µl of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS reagent), was added for each well. After 3 hours of incubation at 37°C in a humidified incubator, cells viability was determined by absorbance at 490 nm using a PowerWave™ XS Microplate Reader (BioTek Instruments, Winooski, Vermont, USA) calculated relatively to cells control.

4.4.4 Vegetal extracts on HePG2 cell culture

HepG2 were maintained in RPMI as previously described. Vegetal extracts, prepared as previously described, were tested at the concentrations of 500 µg/mL. After 24h of incubation with vegetal extracts, cells were harvested by trypsinisation, centrifuged at 1000 rpm (200 g) RT, splitted in aliquots: two resuspended in 1 mL of TRI©Reagent (Sigma-Aldrich, Milan, Italy) for RNA extraction and two in phosphate-buffered saline (PBS) for DNA extraction and stored at -80°C until analysis.

The experiment was performed in triplicate: 3 T75 flask with untreated cells, 3 T75 flask treated with 500 µg/mL of control lettuce and 3 T75 flask with 500 µg/mL of biofortified lettuce.

4.4.5 HepG2 RNA extraction

Total RNA was extracted as follow: for every 1 mL of TRI©Reagent, 200 µL of chloroform were added and mixed for inversion, then the sample were centrifugated 15 minutes at 12000 g at +4°C and supernatants were move to a new tube with 500 µL of cold isopropanol. Waited 10 minutes at room temperature, samples were centrifugated 15 minutes at 12000 g at +4°C, the supernatant was discarded, and the pellet was vortexed after the addition of 500 µL of ethanol 75%. After another centrifugation as before described, ethanol was discarded for inversion and residual RNA was left dry on sterile paper under hood. Finally, 50 µL of RNAasi free water were added for each sample and heated at 60°C for 15 minutes in thermoblock.

RNA quantification was performed with molecular probe Broad range RNA Kit (Applied Biosystem, Foster City, California, USA) on Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, California, USA).

4.4.6 RNA -Seq analysis

RNA-Seq was performed to evaluate the effects, on HepG2 cells, of biofortified and control lettuce extracts. HepG2 RNAs were processed with the TruSeq polyA stranded library preparation kit (Illumina, San Diego, California, USA) for RNA sequencing and analyzed on an Illumina NextSeq 500 platform (Illumina, San Diego, California, USA) generating 1X75bp-reads with a sequencing depth of about 50 million fragments per sample.

Then, as bioinformatics analysis, starting from raw FASTQ files, trimming was used to remove poor quality segments and to connect the sequence fragments. A quality control was also performed. Raw filtered reads were aligned to the Human reference genome GRCh38 using HISAT2. Differential expression values, calculated by fragment per kilobase of transcript per million fragments mapped (FPKM), and comparisons between the different conditions were performed with DESeq2 method. The expression values were expressed as $\log_2$ Fold change and p. value adjusted was provided by the software analysis. The differentially expressed genes were further analyzed using Ingenuity Pathway Analysis (IPA) software (Qiagen, Venlo, Holland). Differential expression values of FOLR1 and MT2A were validated by Real - time qPCR with 7500 Real time PCR System (Applied Biosystems, Foster City, California, USA).

4.4.7 Vegetal extracts on HePG2 cell culture (2)

Control and biofortified lettuce vegetal extracts (Ctrl and Mix grown in growth chamber, ref. 2.1.1) were tested at higher concentration, 2.5 mg/mL, on HepG2 cells. The experiment was performed in triplicate: 3 untreated cells, 3 treated with 2.5 mg/mL of control lettuce and 3 with 2.5 mg/mL of biofortified lettuce. Cells were harvested by trypsinisation, washed with PBS, resuspended in 1 mL of TRI©Reagent (Sigma-Aldrich, Milan, Italy) and RNA extracted as described above. MT2A and FOLR1 were chosen to investigate on gene expression levels that was performed as described above.

4.5 Promoter DNA methylation analysis of HepG2 by bisulfite – NGS (bisulfite - next generation sequencing)

4.5.1 MT2A and FOLR1 demethylation assay

HepG2, were cultured in RPMI1640 as described previously in T75 culture cell flask. When the cells reached 80% confluence, the medium was substituted with a fresh medium with 5 µM L-1, 5-Aza-2' deoxycytidine (Aza) (Sigma-Aldrich, Milan, Italy) for 24 hours and culture cell with fresh RPMI was used as a control. The experiments were performed in triplicate. Cells were harvested by trypsinisation, washed with phosphate-buffered saline, resuspended in 1 mL of TRI©Reagent (Sigma-Aldrich, Milan, Italy) and RNA extracted as described above. MT2A and FOLR1 were chosen to investigate on gene expression levels that was performed as described above.

4.5.2 HepG2 DNA extraction

HepG2 DNA extraction was performed with slightly changes with Wizard© Genomic DNA Purification Kit. Briefly, cells stored in PBS were centrifuge at 13000g for 1 minute, PBS was eliminated, samples were vortexed and 800 µL of Nuclei Lysis Solution were added and mixed by pipetting. Afterwards 3 µL of RNAase Solution were added to the cell nuclei lysate and mixed for inversion. Solutions obtained were incubated for 1 hour at 37°C and cooled to room temperature for 5 minutes. 260 µL of Protein Precipitation Solution were added at room temperature to the samples, that were vortexed for 20 seconds and chilled on ice for 5 before being centrifuged at 13000g for 4 minutes. The pellets, in which there were proteins, were put aside and supernatants were transferred in 1.5 mL new tubes containing 800 µL of room temperature isopropanol, gently mixed by inversion. Tubes were centrifugated at 13000g for 1 minute at room temperature, the supernatants were

removed and 800 μL of room temperature 70% ethanol was added, mixed and centrifuged as before described. Finally, the ethanol was aspirate and pellets were air-dried for 15-30 minutes. DNAs were rehydrated in 100 μL of TE and placed in thermoblock for 1 hour at 65°C. DNA was measured by Qubit™ dsDNA BR Assay Kit on Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, California, USA).

4.5.3 Bisulfite – NGS sequencing

To investigate the level profiling of CpGs in target region, a bisulfite – next generation sequencing was performed by Illumina MiSeq (Illumina, San Diego, California, USA) on selected FOLR1 and MT2A promoter region on HepG2 treated with vegetal extract of control and biofortified lettuce, with bisulfite primer *ad hoc* designed (Figure 8). This part of the study was carried out in collaboration with Genomic Works (Seoul, South Korea). Promoter regions were found on human genome reference UCSC h19 GRch37 in Genome Data Viewer (NCBI), while the nucleotide sequences were found on Ensemble (EMBL-EBI, UK)(Bashtrykov and Jeltsch 2018, Leitao, Beygo et al. 2018)

Gene	Bisulfite primer	Sequences (5'→ 3')
MT2A	Forward	GGGAATGTAAGTAGTATTTTTAAGTTT
	Reverse	ACAATCTACACTAAACATCCCCAAC

Figure 8. **Bisulfite primer.** Bisulfite primer were *ad hoc* designed for MT2A promoter region.

4.6 Gene expression and quantification of exosomes from cell culture treated with vegetal extracts

Exosome characterization, isolation from cell culture and in vitro study of vegetal extracts were carried out in the laboratory of Epigenetic hypertension, at Càtolica Universidad de Santiago de Chile, Chile.

4.6.1 Cell Lines

The endothelial cell line Ea.hy926, derived from the fusion of HUVEC, human umbilical vein cells immortalized with pulmonary cancer cells, was maintained in IMDM (Iscove's Modified Dulbecco Medium) supplemented with 10 % heat-inactivated foetal calf serum (FBS)(Gibco, Thermo Fisher brand, Waltham, Massachusetts, USA), 1% Pen-Strep (100 U/mL). HepG2, was cultured as previously described. All cell lines were cultured in a humidified 37°C incubator containing 5% CO_2 (Figure 9).
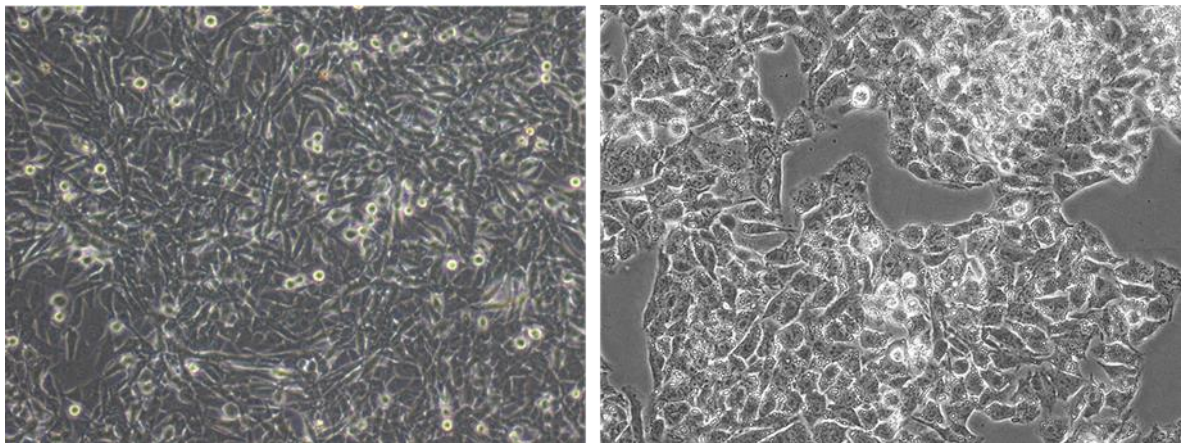
Figure.9 **Ea.hy926 and HepG2 cell culture.** Photograph of Ea.hy926, at the left, and HepG2, at the right, culture cells capture by optical microscopy.

4.6.2 Cytotoxicity Assay in HepG2 and Ea.Hy926 cell Models

Cytotoxicity of extracts was assessed using confluent HepG2 and Ea.Hy926 cell in RPMI and IMDM supplemented with 5% FBS, as previously described. Vegetal extracts concentration tested were: 0 µg/mL, 500 µg/mL, 1 mg/mL, 2 mg/mL, 2.5 mg/mL, 5 mg/mL. Cell viability was assessed using CellTiter 96©Aqueous cell proliferation Assay (Promega, Madison, WI, USA) containing MTS reagent. Absorbance at 490 nm was measured using a PowerWave™

XS Microplate Reader (BioTek Instruments, Winooski, Vermont, USA) and cell viability was calculated relatively to cells control.

4.6.3 Vegetal extracts on cell lines

Ea.hy926 and HepG2 were cultured in 100 mm Petri dishes in IMDM and RPMI, respectively, as described above. To isolate exosomes from culture medium, cells have been subjected to a 24 hours' time of exosome deprivation, that consists of a replacement of culture medium without FBS, necessary to not overestimate the results, since FBS contains exosomes. After FBS deprivation, vegetal extracts were tested on both cell lines at 1 mg/mL as already described. After 24h of vegetal extracts incubation, cells were harvested by trypsinisation, centrifugated at 1000 rpm (200 g), washed with phosphate-buffered saline, resuspended in 1 mL of TRIzol™ Reagent (Thermo Fisher brand, Waltham, Massachusetts, USA) and stored at -80°C. Experiments were carried out with seven replicates for each treatment to have a minimum of 70 mL of culture medium from which isolate exosomes. Culture medium was collected and stored at -30°C until extraction.

4.6.4 Ea.hy and HepG2 RNA extraction

Total Ea.hy926 and HepG2 RNAs were extracted with TRIzol™ Reagent following the procedure already described. RNA concentration was measured with Nanoquant Tecan Infinite V200 Pro (Tecan, Männedorf, Switzerland). DNase (Invitrogen, Carlsbad, California, USA) treatment of Ea.hy926 and HepG2 RNAs was performed at room temperature for 15 minutes and 1 µL of EDTA was added before a passage in thermoblock for 5 minutes at 65°C. Reverse transcription was performed using RT RevertAid H minus (Thermo Fischer, Waltham, Massachusetts, USA) with following condition: 25°C for 10 minutes, 42°C for 60 minutes, 70°C for 10 minutes.

4.6.5 Gene expression analysis by Real - time qPCR

Quantitative real-time PCR was performed with Rotor Gene 2000 (Qiagen, Venlo, Holland) and SYBR Green reaction to evaluate gene expression of FOLR1, MT2A, SLC19A1 (RFC1) and SLC39A13 (ZIP13) with GUSB (β-glucuronidase) as housekeeping gene.

The target and housekeeping genes were amplified with ad hoc primers designed (Table 2). Starting from FASTA mRNA sequence in GenBank (NCBI), a sequence was chosen and for everyone, length, between 18 and 25 bp, ΔG, alignment temperature, self and hetero dimer construction were evaluated by OligoAnalyzer tool (IDT, Coralville, Iowa, USA).

Gene	Primer	Sequences (5'→ 3')
FOLR1	Forward	CTG TCT CCT AGG CCA CTA AA
	Reverse	CCA TGC AAT CCT TGT CTG AG
GUSB	Forward	GAA AAT ACG TGG TTG GAG AGC
	Reverse	CCG AGT GAA GAT CCC CTT T
MT2A	Forward	AGC TAT AAA CAC TGC TTG CC
	Reverse	GGT CAC GGT CAG GGT TGT
SLC19A1 (RFC1)	Forward	CAC CTC GTG TGC TAC CTT T
	Reverse	GAT CTC GTT CGT GAC ATG C
SLC39A13 (ZIP13)	Forward	TGG ACA ACA AGG AAA GCG
	Reverse	AGC AGA TGC AGA AAC ACA T

Table 2. **Primer pairs design *ad hoc* for RT - qPCR.** FOLR1, GUSB, MT2A, RFC1 and ZIP13 primer pairs.

qPCR was carried out as follows: ramp temperature 95°C for 30"; 95°C for 15" followed by 55°C (for RFC1, ZIP13 and GUSB), 61°C (for FOLR1 and MT2A) for 40" (X 40 cycles); 95°C for 15"; 55° for 1'; 95°C for 15" and 60°C for 15". The PCR products were separated on 2% agarose gel and visualize by red safe staining.

4.6.6 Ea.hy926 and HepG2 culture medium exosome extraction by ultracentrifugation

Culture medium supernatant, 70 mL, were centrifugated for 15' at 300g, pellet obtained, cells, were discarded, then the supernatant was centrifugated at 2000 g for 30', pellet that was dead cells was discarded, supernatant was centrifugated at 12000g for 45', cell debris were discarded and supernatant was filtered with 0.22 µm filters and then centrifugated at 120000g for 70', now the pellet contained exosomes and contaminant proteins was washed in PBS with a 100000g centrifugation for 70' and supernatant was discarded, the pellet obtained was resuspended in 1 mL of PBS. The exosome purification procedure was based on differential ultracentrifugation (Thermo Scientific Ultracentrifuge Sorvall™ WX + Series).

4.6.7 Nanoparticles trafficking analysis (NTA) of exosomes

Size distribution and total concentration of exosomes in the supernatants of cultured cells were analysed by nanoparticles trafficking analysis on Nanosight instrument (Nanosight, Amesbury, UK). A laser and a video recording tracks and analyze the particles through light scattering and Brownian motions. Briefly, approximately 1 mL of diluted exosomes was loaded into the sample chamber through an automatic syringe and three videos of either 30 seconds were recorded of each sample. Data analysis was performed with both NTA software (Nanosight, Amesbury, UK).

4.6.8 Exosome mRNA gene expression analysis

Ea.hy926 and HepG2 exosome RNAs were extracted, estimated and reverse transcribed as previously described. Gene expression analysis of selected genes was operated by Rotor Gene 2000 model PCR using SYBER GREEN assay (Biorad, Hercules, California, USA).and

quantified with Nanoquant Tecan Infinite V200 Pro (Tecan, Männedorf, Switzerland) as already described.

4.7 Statistical analysis

Microelements estimation data was calculated starting from ICPs (ion counts per second) and expressed in µg/g on dry weight. Blanks are used for estimating the limit of quantification (LOQ), represented by 3σ.

Folate forms were expressed as relative abundance on area under the peak.

gene expression was expressed as mean of fold change (FC) ± σ according to the $2^{-\Delta\Delta CT}$ method: $\Delta\Delta C_T$ [(($C_{T\ gene\ target} - C_{T\ gene\ housekeeping}$) sample treated $-$ ($C_{T\ gene\ target} - C_{T\ gene\ housekeeping}$) sample untreated)] (Schmittgen and Livak 2008).

All the calculations were performing using GraphPad Prism software version 5 (GraphPad Software, San Diego, USA), the statistical data analysis was performed applying a t-test and were considered statistically significant data with $p < 0.05$.

5. Results

5.1 Evaluation of fertilizer formulas

Preliminary lettuce tests were performed in growth chamber with increasing element concentrations (Table 1) to evaluated possible toxic effects of fertilizer formulas. No visible toxic effects of fertilizer formulas were detected, so that the selected fertilizer formulas were also tested under different cultivation modality (Figure 10).

Figure 10. **Cultivation modality.** Lettuce plants in growth chamber grown in hydroponic modality (a), in soil (b), and in open field (c).

Control and biofortified lettuces showed brilliant colours and excellent organoleptic proprieties in all tested treatments. Plants weight and SPAD index, that determines the relative amount of chlorophyll, were collected (data not shown) during harvest to monitoring growing condition.

5.2 Optimization of digestion protocol for microelements analysis

Microelements analysis by ICP-MS, characterized by great accuracy and sensitive, required several optimization steps. During freeze-drying was evaluated even the possibilities of a weight drop on, deciding to add an extra freeze-dry cycle of 24 hours followed by least 72 hours of drying procedure with copper salts to obtain a stable dry matter weights (Hansen, de Bang et al. 2013).

Microwave digestion parameters, mineralization procedure timing and microelements standard curve were set together. An accurate preparation and setting of calibration solution were necessary to detect microelements in lettuce matrix in the proper range. Standard solutions were prepared from monoelement solutions of ten microelements (Table 3, 4) and was prepared fresh for each experiment and was preferred to a multielement solution, too rich in composition to ICP-MS analysis.

Dilution of standard solutions, in number of 8, were fundamental to evaluate single standard curve calibration, choose the modality of microelement acquisition and calibration of H_2/He levels, that are crucial for the setting of ICP/MS sensibility.

STANDARD SOLUTIONS

SOL A	µL	mg/L	SOL. B	µL	mg/L	SOL. C	µl	µg/L
			SOL A	200		SOL B	250	
Co	200	20	Co		0.4	Co		10
Se	200	20	Se		0.4	Se		10
			Cd	20	2	Cd		50
			Ni	20	2	Ni		50
			Cu	100	10	Cu		250
			B	200	20	B		500
			Mn	200	20	Mn		500
			Al	100	10	Al		250
						Zn	20	2000
						Mg	1000	100000
TOT	400		TOT	840		TOT	1270	
HNO_3	9600		HNO_3	9160		HNO_3	8730	
TOT	10000		TOT	10000		TOT	10000	

Table 3. Standard curve from monoelement solutions.

Measured isotopes: ^{11}B, ^{24}Mg, ^{27}Al, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{78}Se, ^{114}Cd and $_{103}$Rh, were acquire with different modality. Magnesium, manganese, cobalt, nickel, copper and zinc were acquired in KED modality, while aluminium and boron were analysed in CCT modality.

		S1	S2	S3	S4	S5	S6	S7	S8
	HNO$_3$ 2.3%	4950	4900	4850	4800	4600	4200	4000	3000
	final volum	5000	5000	5000	5000	5000	5000	5000	5000
	SOL C µg/L	50	100	150	200	400	800	1000	2000
Co	10	0.10	0.20	0.30	0.40	0.8	1.6	2.0	4.0
Se	10	0.10	0.20	0.30	0.40	0.8	1.6	2.0	4.0
Cd	50	0.50	1.00	1.5	2.0	4.0	8	10	20
Ni	50	0.50	1.00	1.5	2.0	4.0	8	10	20
Cu	250	2.5	5	8	10	20	40	50	100
B	500	5.0	10	15	20	40	80	100	200
Mn	500	5.0	10	15.0	20	40	80	100	200
Zn	2000	20	40	60	80	160	320	400	800
Al	250	2.5	5	7.5	10	20	40	50	100
Mg	100000	1000	2000	3000	4000	8000	16000	20000	40000

Table 4. **Microelement standard solution.** Preparation of microelement standard solutions.

A special attention was given to zinc and selenium modality acquisition. ^{66}Zinc was detected in KED modality, because as we can see in the Figure 11, the standard solution values measured in ICPS (ions count per second) and concentrations calculated corresponded to the prepared solutions (Table 5).
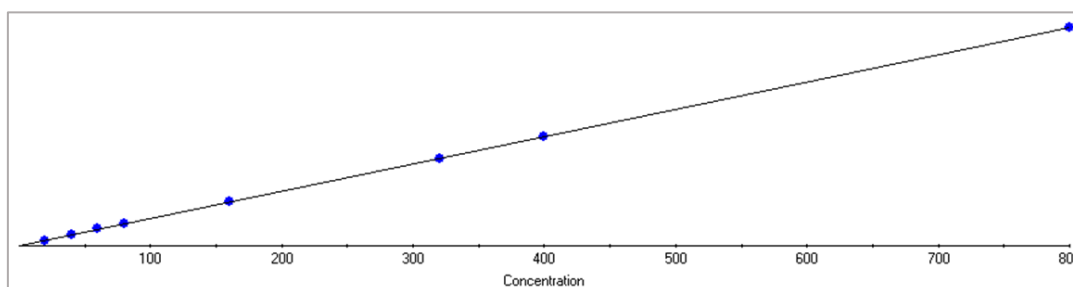
Figure 11. **^{66}Zn standard curve**. Standard curve of ^{66}Zn acquired in KED modality (R^2 = 0.999989)

Standard solutions (Zn µg/L)	ICPS (mean ± σ)	Zn measured (µg/L)
S1 (20)	17044 ± 124	19,685
S2 (40)	34260 ± 138	39,568
S3 (60)	53254 ± 1200	61,506
S4 (80)	69837 ± 203	80,659
S5 (160)	138060 ± 847	159,453
S6 (320)	274767 ± 4672	317,345
S7 (400)	346900 ± 1931	400,655
S8 (800)	693110 ± 3509	800,514

Table 5. **ICP/MS acquisition of Zn standard solutions.** ICPS and concentration of Zn standard solutions measured with KED modality.

[77]Se was first detected in CCT modality with a poor result (Figure 12). Most of the point of the standard solution calculated were out of scale, and the elevate number of ICPS detected show an overestimation of the solutions analyzed (Table 6).
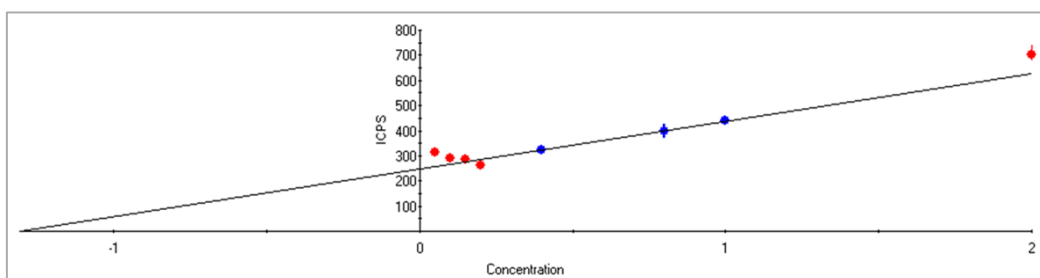
Figure 12. **[77]Se standard curve.** Standard curve of [77]Se acquired in CCT modality (R^2 = 0.999998), in red standard solutions out of scale.

[78]Se, alternately, acquired in KED modality, showed a great correspondence between calculated and prepared standard solution concentrations (Figure 13).
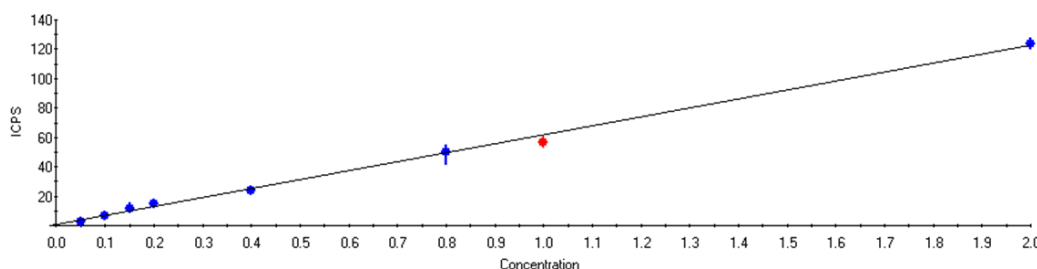
Figure 13. **[78]Se standard curve.** Standard curve of [78]Se acquired in KED modality in red standard solutions out of scale (R^2 = 0.999614).

Standard solutions (Se µg/L)	^{77}Se ICPS (mean ± σ)	^{77}Se measured (µg/L)	^{78}Se ICPS (mean ± σ)	^{78}Se measured (µg/L)
S1 (0.05)	312 ± 11.8	1.643	3 ± 2.1	0.046
S2 (0.1)	291 ± 12.1	1.534	6 ± 0.6	0.106
S3 (0.15)	283 ± 6.4	1.494	12 ± 3.5	0.192
S4 (0.2)	263 ± 10.7	1.388	14 ± 3.5	0.234
S5 (0.4)	324 ± 8.5	1.707	24 ± 2.1	0.389
S6 (0.8)	400 ± 27.8	2.106	50 ± 7.6	0.811
S7 (1)	438 ± 18.1	2.307	57 ± 4.5	0.927
S8 (2)	701 ± 32.4	3.692	123 ± 4.0	2.016

Table 6. **ICP/MS acquisition of Se standard solutions.** Comparison between the ICPS and concentration of standard solutions measured with different modality of acquisition, CCT for ^{77}Se and KED for ^{78}Se.

According to standard protocol of Hansen et al. 2013, maximum temperature, time of digestion (Table 2), addition and H_2O_2 and HNO_3 concentrations were tested to choose the best conditions for digestion of lettuce matrix. Aspect, preferably light coloured and absence of aggregates were evaluated on preliminary mineralised CRM and lettuce samples, during mineralization steps.

Step	Power (W)	% max	Time (min) to raise temperature	Temperature (°C)	Running time (min)
1	400	100	10	80	15
2	400	100	10	120	10
3	400	100	10	160	40
3*	400	100	30	190	40
3**	400	100	30	180	40

Table 7. **Microwave digestion setting.** Different microwave digestion operating conditions: without H_2O_2 (3*), with H_2O_2 and pre-digestion time (3**).

First experiments were focused on H_2O_2 presence or not and on the final volume of digested samples (Table 8). the use of H_2O_2 resulted essential to avoid the presence of flocs, samples not completely mineralized, as well as a minor dilution of the microelements (Figure 14).
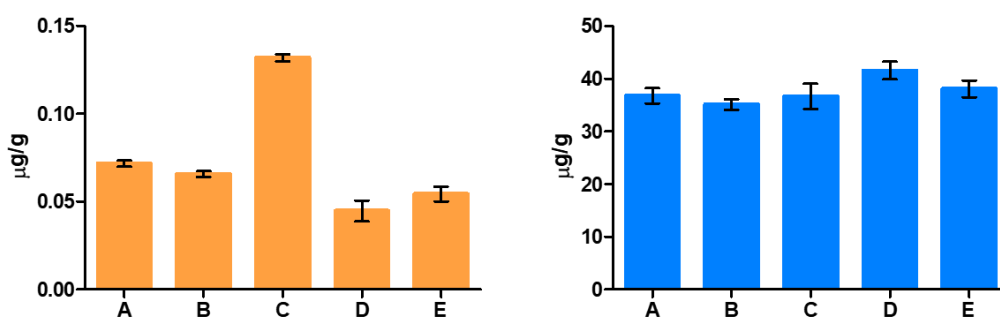
Figure 14. **Quantification of Zn and Se with different digestion protocol.** Selenium (A) and Zinc (B) concentrations in different digestion and ICP-MS analysis conditions. Samples were analysed in triplicate. Zinc and selenium ICP-MS analysed a and b; c, d and e with H_2O_2; 10 mL for a, b, c and 5 mL for d and e.

				Mean ± σ (μg/g)	
Sample	Total volume (mL)	Lettuce weighted (mg)	H_2O_2	Se	Zn
A	10	100	+	0.071 ± 0.003	36.7 ± 2.517
B	10	100	-	0.066 ± 0.004	36.7 ± 4.129
C	10	50	+	0.132 ± 0.003	35.1 ± 1.752
D	5	50	+	0.045 ± 0.010	41.6 ± 2.905
E	5	50	-	0.054 ± 0.007	38.1 ± 2.709

Table 8. **Mineralization procedures.** Selenium and Zinc concentration measured by ICP/MS in different mineralization procedures.

Take into account digestion parameters, previously analysed, CRM mineralization was set (Table 9) and microelements concentrations were compared to the values certified by producer company. Presence of flocs and incorrect values in digested samples without H_2O_2 determined the use of H_2O_2 and the addition of a pre-digestion time to improve CRM mineral composition evaluation.

Trace element	CRM 1570A (µg/g)		
	Certified value	Value found	
		w/o H_2O_2	with H_2O_2
B	37.7 ± 1	23.93 ± 1.32	37.72 ± 1.49
Mg	8900 ± n.d	7421.08 ± 502.58	10684.41 ± 59.59
Al	310 ± 11	208.10 ± 12.82	265.29 ± 3.49
Mn	75.9 ± 1.9	49.09 ± 2.71	68.23 ± 0.38
Co	0.39 ± 0.05	0.26 ± 0.01	0.36 ± 0.004
Ni	2.14 ± 0.10	1.67 ± 0.06	2.13 ± 0.02
Cu	12.2 ± 0.6	8.60 ± 0.24	12.76 ± 0.08
Zn	82 ± 3	63.03 ± 2.05	83.50 ± 0.39
Se	0.117 ± 0.009	0.13 ± 0.02	0.214 ± 0.02
Cd	2.89 ± 0.07	1.84 ± 0.06	2.54 ± 0.03

Table 9. **CRM setting protocols.** Determination of elements compared 1570 CRM certified value to CRM digested with and without H_2O_2

5.3 CRM validation

Validation of digestion parameters and ICP/MS settings was performed by nine CRM independent mineralization protocols then analyzed with ICP/MS. The value of coefficient of variation were all goods (CV < 10%), except for aluminum (Table 10).

	Microelements (µg/g)									
	B	Mg	Al	Mn	Co	Ni	Cu	Zn	Se	Cd
CRM1	38.8	9218.9	207.7	66.4	0.33	1.8	12.0	77.9	0.157	2.45
CRM2	39.3	9417.9	205.3	67.1	0.33	1.9	12.2	79.6	0.141	2.50
CRM3	39.0	9130.4	202.4	65.6	0.32	1.8	12.0	78.9	0.138	2.49
CRM4	38.2	9006.9	214.5	63.6	0.32	1.8	11.8	77.3	0.117	2.43
CRM5	39.7	9256.1	188.0	65.8	0.32	1.8	12.1	79.1	0.145	2.52
CRM6	37.6	8820.0	187.1	62.1	0.30	1.7	11.5	75.6	0.137	2.39
CRM7	38.6	9259.2	245.2	65.4	0.33	1.9	12.0	79.0	0.141	2.52
CRM8	37.5	9010.6	245.3	63.7	0.32	1.9	11.7	77.4	0.126	2.47
CRM9	38.4	8917.0	197.6	63.1	0.31	1.7	11.6	76.4	0.138	2.44
mean	38.6	9115.2	210.3	64.8	0.32	1.8	11.9	77.9	0.138	2.47
sd	0.7	191.2	21.7	1.7	0.01	0.1	0.3	1.3	0.011	0.04
CV	1.9	2.1	10.3	2.6	2.9	4.7	2.1	1.7	8.1	1.8
Expected value	**37.6**	**8900**	**310**	**75.9**	**0.39**	**2.14**	**12.20**	**82.0**	**0.126**	**2.89**
+/-	1.0	-	11	1.9	0.05	0.10	0.60	3.0	0.009	0.07

Table 10. **CRM validation.** Certified Reference Material microelements profiles and validation.

5.4 Microelements ICP-MS analysis of biofortified plants

Preliminary results on microelements profiles in plants supplemented with increased concentrations of fertilizer formulas are shown in Table 11 and Table 12. Microelement profiles resulted stable meanwhile microelements supplemented showed increased levels. Limit of quantification, LOQ, was calculated from three times deviation standard of blank samples (LOQ = 0.06 μg/g).

In Figure 15 the increase of Zn levels according to the increasing addition of foliar fertilizers, in plants grown in soil in growth chamber is shown. Even if the level of Zn, in control plants, is higher than plants treated by 50 ppm of $ZnSO_4$, several independent analyses confirmed the obtained concentration. The level of Se was under LOQ in plants enriched with $ZnSO_4$.

Zn addition (ppm)	Microelements (μg/g)									
	B	Mg	Al	Mn	Co	Ni	Cu	**Zn**	**Se**	Cd
0	40.2	7121	2.4	36.0	0.20	1.06	2.32	**37.7**	< LOQ	1.12
50	40.1	7195	1.8	29.7	0.19	0.82	2.94	**31.3**	< LOQ	0.98
250	38.4	7839	2.2	34.6	0.19	0.77	2.30	**32.1**	< LOQ	0.94
500	36.7	8137	1.9	22.6	0.15	0.75	2.48	**33.9**	< LOQ	0.81
1000	41.3	7850	2.6	31.9	0.21	1.41	3.31	**43.0**	< LOQ	0.93
1500 (500X3)	37.4	7864	1.6	53.9	0.23	1.04	2.51	**49.8**	< LOQ	1.12
3000 (1000X3)	41.9	7891	1.5	58.7	0.26	1.24	2.54	**57.6**	< LOQ	1.19
6000 (2000X3)	40.5	4743.3	1.3	267.7	0.1	0.1	2.1	**90.2**	< LOQ	0.3
12000(4000X3)	41.3	4441.1	1.4	257.5	0.1	0.2	2.0	**110.3**	< LOQ	0.3

Table 11. **ICP-MS analysis of zinc concentration in soil sample.** All the value found were calculated on dry matter.

Se addition (ppm)	Microelements (μg/g)									
	B	Mg	Al	Mn	Co	Ni	Cu	Zn	**Se**	Cd
0	33.3	5568	2.7	32.0	0.25	0.46	3.10	31.7	**0.11**	0.92
2	43.2	7898	1.5	88.2	0.33	0.70	3.54	55.2	**0.20**	1.39
4	37.6	6841	1.7	40.5	0.23	0.89	2.54	41.0	**0.21**	0.99
6	32.0	6992	1.3	29.7	0.23	0.72	2.38	26.5	**0.23**	0.81
15	35.0	6152	1.7	45.4	0.22	0.59	2.56	33.8	**0.34**	1.01
30	31.1	6577	1.8	38.0	0.21	0.65	2.10	32.6	**0.49**	0.89
60	32.6	7011	1.2	41.5	0.26	0.50	2.69	38.2	**0.59**	0.92

Table 12. **ICP-MS analysis of selenium concentration in soil sample.** All the value found were calculated on dry matter.
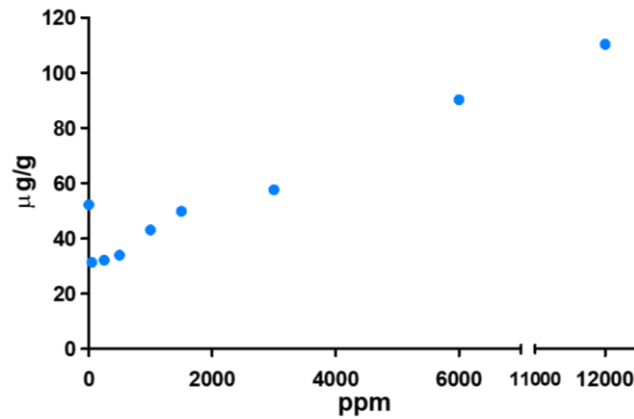
Figure 15. **Zn concentration in soil plant.** Correlation between Zn measured and Zn present in the fertilizer formulas added to plant grown in soil.

In soil plants, grown in growth chamber, supplemented with fertilizers enriched with NA$_2$SeO$_4$, an improvement correlated with the increasing enrichment was found. The latter seem to stretch out plateau with 60 ppm addition, as shown in Figure 16.
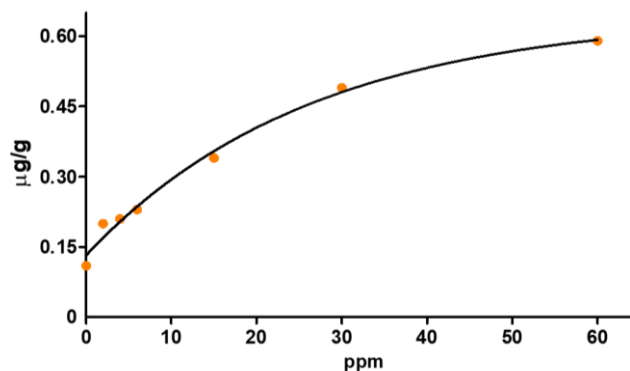
Figure 16. **Se concentration in soil plant.** Correlation between Se measured and Se present in the fertilizer formulas added to plant grown in soil in growth chamber.

In soil plants, treated with p-ABA, microelements profiles were firm (data not shown), in particular Se was undetectable, Se < LOQ, and Zn level were equal to Zn level in plants control.

In plants grown in hydroponic modality, Se was undetectable in control plants, while a great improvement in lettuce treated with 60 ppm foliar spray was found (p = 0.045) (Fig.17).

Trace element (µg/g)	Ctrl hydroponic	Se hydroponic
B	44.79 ± 21.21	59.15 ± 2.25
Mg	4737 ± 1643	6313 ± 155
Al	0.35 ± 0.50	0.91 ± 0.16
Mn	156.92 ± 55.09	129.51 ± 21.92
Co	0.03 ± 0.02	0.015 ± 0.001
Ni	1.22 ± 1.30	0.33 ± 0.10
Cu	6.53 ± 1.79	6.77 ± 1.53
Zn	36.81 ± 5.88	33.02 ± 9.50
Se	< 0.06	**0.163 ± 0.066**
Cd	0.010 ± 0.011	0.022 ± 0.010

Table 13. **Microelement profile.** Microelement profiles of plant grown in hydroponic modality in growth chamber.
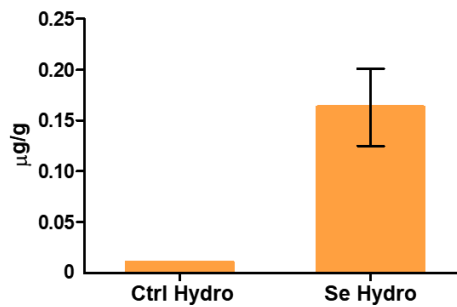
Figure 17. **Se and concentration in lettuce cultured in hydroponic modality.** Comparison between Se level in plant control and plant treated with 60 ppm of sodium selenate.

Moving to open field, lettuce enriched with 60 ppm of sodium selenate showed the highest concentrations as compared to all the other treatments (Table 14). Increase of Se and Zn in biofortified lettuce was significantly higher as compare to controls ($p = 0.03$ and $p = 0.04$, respectively) (Figure 18).

Trace element (μg/g)	Open field		
	Ctrl	Se	Zn
B	39.69 ± 3.65	46.97 ± 1.82	33.14 ± 7.60
Mg	3284 ± 136	3926 ± 40	3131 ± 597
Al	37.60 ± 10.20	64.35 ± 13.49	27.80 ± 6.07
Mn	36.34 ± 3.84	42.05 ± 3.96	34.20 ± 5.63
Co	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.004
Ni	0.61 ± 0.11	0.43 ± 0.06	0.62 ± 0.07
Cu	6.21 ± 0.35	4.80 ± 0.44	5.72 ± 0.35
Zn	25.32 ± 5.54	14.63 ± 2.03	**38.07 ± 2.64**
Se	< 0.06	**1.47 ± 0.26**	< 0.06
Cd	0.59 ± 0.08	0.72 ± 0.03	0.55 ± 0.14

Table 14. **Microelements profiles in open field lettuce.** Trace element measured in lettuce enriched with Mix formulation in growth chamber.
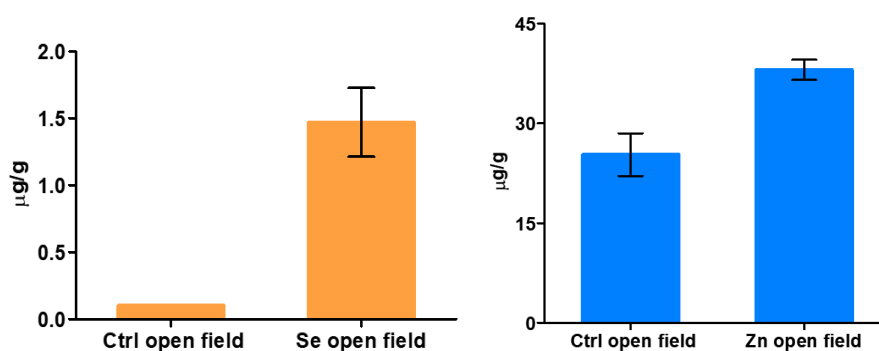
Figure 18. **Se and Zn concentration in lettuce in open field.** Se and Zn levels in plants grown in open field. Se improvement has a $p_{(Se\ vs\ Ctrl)} = 0.03$; while Zn enhance shows a $p_{(Zn\ vs\ Ctrl)} = 0.04$.

Trace element (μg/g)	Ctrl	Mix
B	44.9 ± 8.81	51.6 ± 13.75
Mg	4781 ± 517	5421 ± 1026
Al	1.7 ± 0.88	1.7 ± 0.85
Mn	14.2 ± 3.08	14.8 ± 3.17
Co	0.045 ± 0.01	0.038 ± 0.002
Ni	0.51 ± 0.08	0.40 ± 0.04
Cu	6.8 ± 0.88	6.5 ± 0.69
Zn	78.4 ± 10.14	**116.1 ± 24.20**
Se	<0.060	**0.612 ± 0.17**
Cd	0.123 ± 0.02	0.121 ± 0.02

Table 15. **Trace element in biofortified lettuce**. Microelement profiles measured in lettuce enriched with Mix formulation in growth chamber.

Finally, testing the final fertilizer formula on lettuce in growth chamber, in soil, with ICP/MS analysis a significant increase of Se content comparable to one measured in preliminary tests and in hydroponic modality was observed (Figure 19) (Table 15).
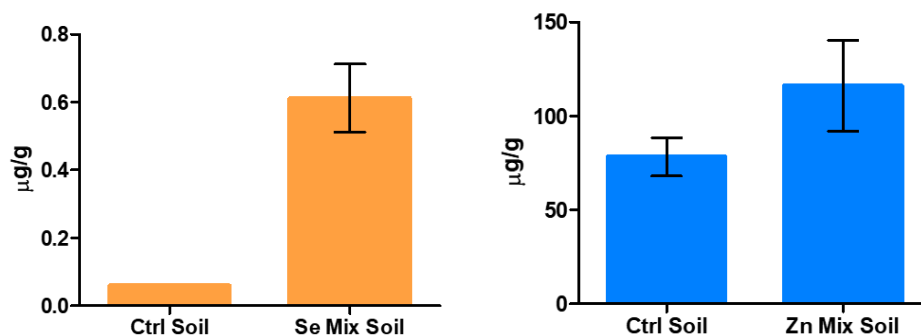
Figure 19. **Se and Zn concentration in lettuce in growth chamber.** Se and Zn levels in plants grown in soil in growth chamber. Se improvement has a $p_{(Se\ vs\ Ctrl)}$ = 0.0313; while Zn enhance shows a $p_{(Zn\ vs\ Ctrl)}$ = 0.2981.

5.5 UPLC/MS analysis of 5-methyltetrahydrofolic acid in lettuce

Only 5-methylTHF, m/z 460 and retention time 3.72 minutes, was visible and found in all samples analysed in positive mode (m/z 463) (Figure 20). In a representative chromatogram is shown the comparison between 5-methylTHF peaks. Hydroponic samples showed, in this regard, the highest 5-methylTHF content. The variation in 5-methylTHF content, measured in lettuce growth in open field, when compared to control lettuce was 2.5 % higher with Zn addition, 23.8 % lower with p-ABA addition and 1.3 % lower with Se addition. In lettuce growth in growth chamber, 5-methylTHFwas 14.5 % higher with Mix treatment, while in lettuce cultivated with hydroponic modality, it was 94.9 % higher with Se addition (p = 0.008) and 123.3 % higher with p-ABA treatment (p = 0.01). Analysis of the distribution and levels of folates in control versus biofortified lettuce showed up to 50-fold enrichment in biofortified lettuce grown in hydroponic modality (Figure 21, 22).
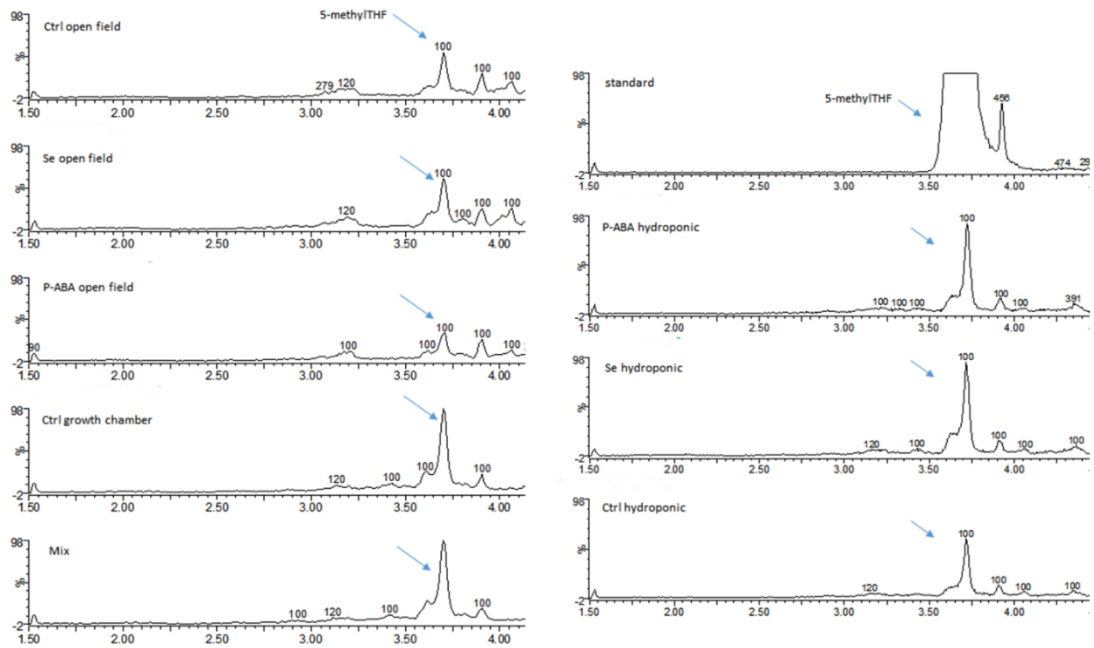
Figure 20. **5-methylTHF chromatograms by UPLC/MS.** Overview of 5-methylTHF cromatograms in different treatment and modality and 5-methylTHF standard (blue arrow).
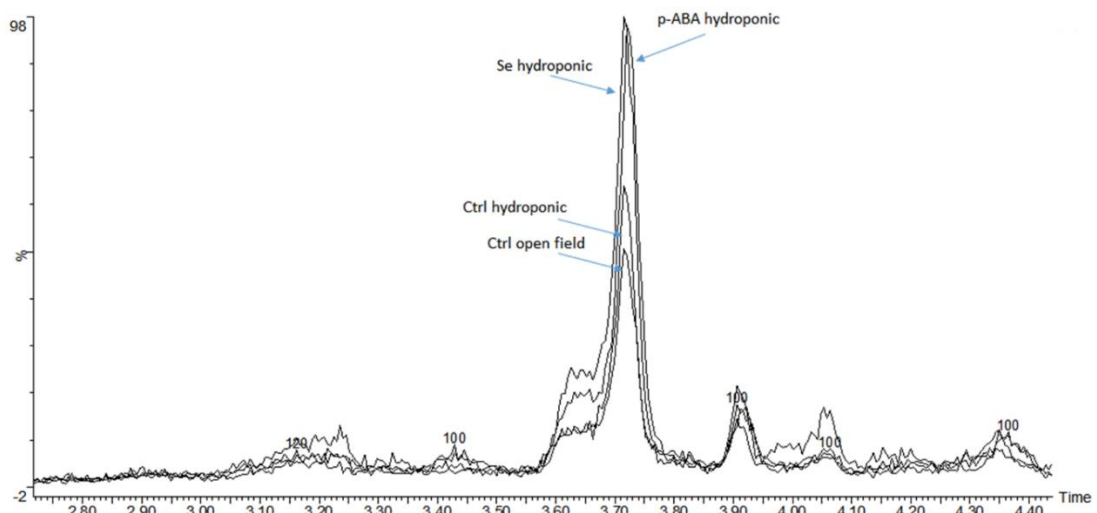
Figure 21. **5-methylTHF cromatogram.** Comparison between the area under the peak of 5-methylTHF in different cultivation modality: hydroponic cultivation and treatment compare to open field cultivation modality.
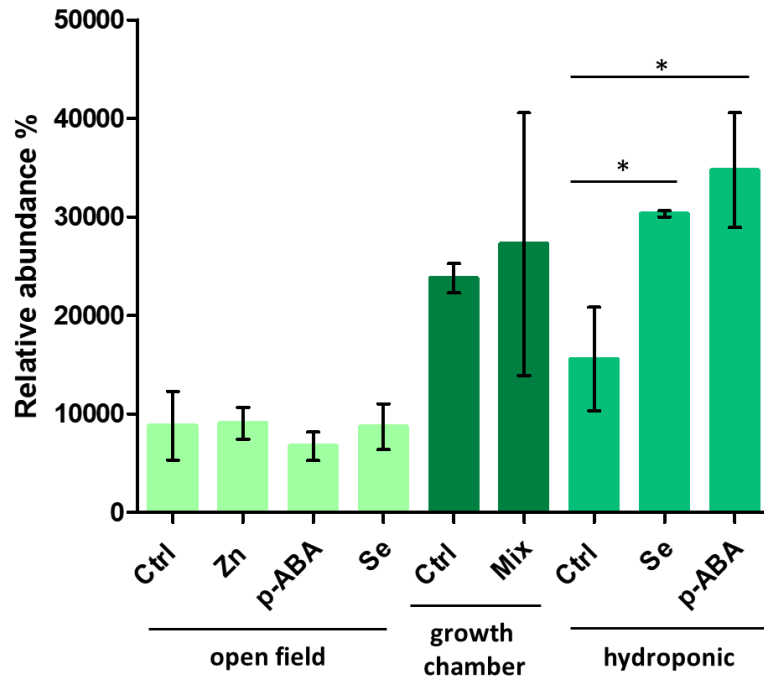
Figure 22. **Relative quantification of 5-methylTHF in lettuce.** Relative abundance of 5-methylTHF in different modality of cultivation. In hydroponic modality, 5-methylTHF improvement has a p $_{(Ctrl\ vs\ Se)}$ = 0.008 and p $_{(Ctrl\ vs\ p-ABA)}$ = 0.01.

5.6 Differentially expressed genes in HepG2 treated with biofortified lettuce extracts by RNA - Seq

Control and biofortified lettuce extracts tested by MTS assay on HepG2 not show differences in cells vitality for all the tested concentrations.

Sequencing of HepG2 mRNA showed the great expression differences between treatments and control, on the contrary few genes were found differentially expressed between the two treatments. In the comparison between HepG2 treated with lettuce biofortified with Mix and control, with a p. value < 0.05, 1323 genes were found downregulated, while 2464 genes were upregulated. In the comparison between HepG2 treated with normal lettuce and control, 1532 genes were downregulated while 2563 were upregulated. In the comparison between the two lettuces, normal versus biofortified, 17 genes were downregulated and 28 were upregulated and statistically significant.

5.7 Analysis of HepG2 differentially expressed mRNAs

RNA Seq comparison data were evaluated by using the IPA software (Qiagen, Venlo, Holland), and the most part of downregulated and upregulates genes were found to be linked and divided in canonical pathways as shown in the Figures. Each pathway is constituted by a number of genes, and IPA analysis shows the percentage of genes implicated in these ones, and more specifically the modification of gene expression as either up- or down-regulated. Some of these canonical pathways are correlated to chromosomal organization, glutathione cycle, antioxidant pathway, mitochondrial function and signaling. Sirtuin pathway includes histone deacetylase, γ glutamyl cycle join in the synthesis of glutathione, pathway of vitamin C, production of ROS in mitochondria and mTOR signaling. A lot of genes involved in different canonical pathways were founded differentially expressed in comparison between HepG2 treated with control or biofortified lettuce and Untreated cells (Figure 23, 24, 25).

Figure 23. **Canonical pathways analysis performed by IPA for the differentially expressed genes between HepG2 treated with control lettuce and Untreated cells.** Differentially expressed genes involved in canonical cellular pathway are represented in red if up-regulated and in green if down-regulated.
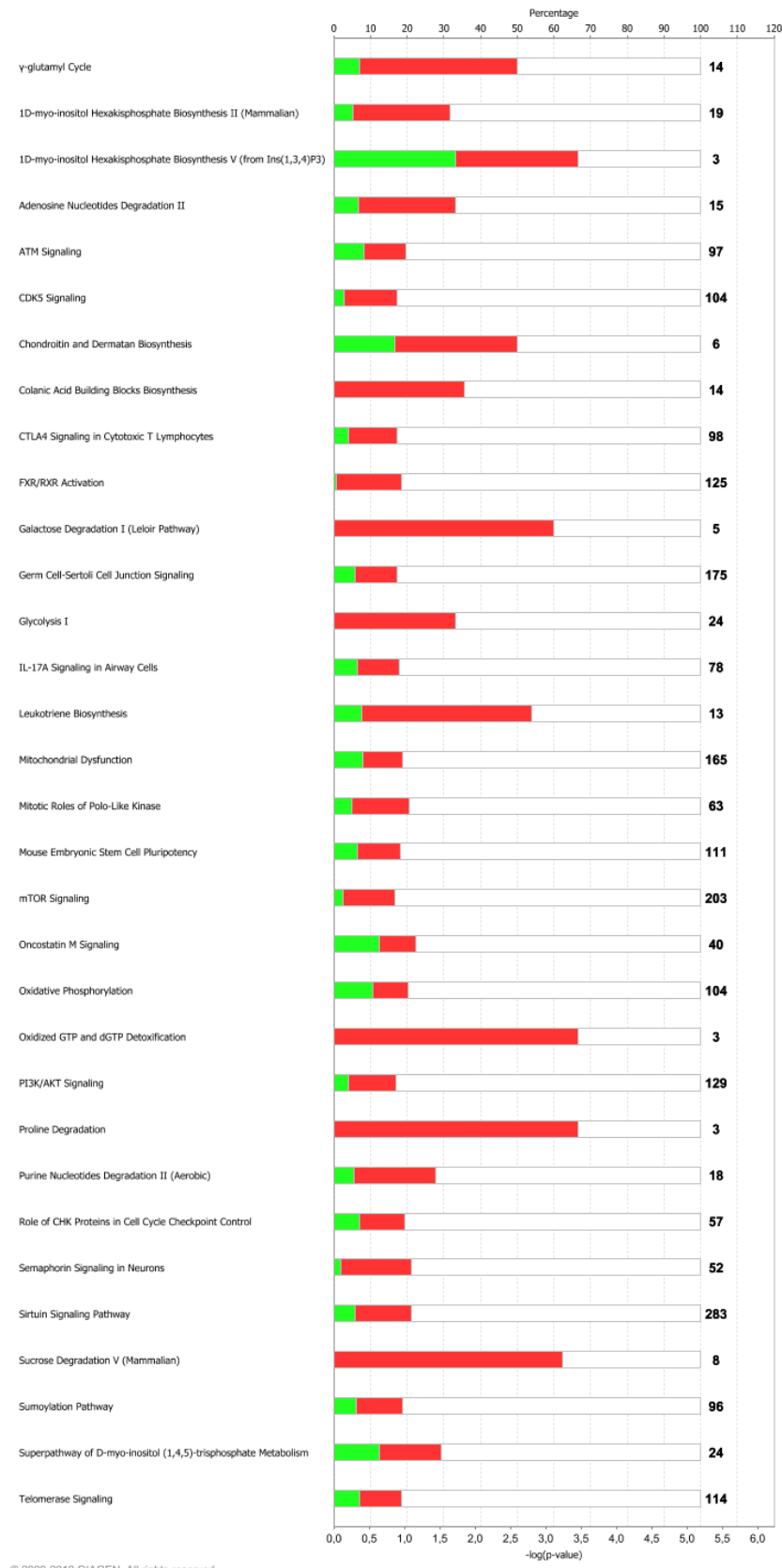
Figure 24. **Canonical pathways analysis performed by IPA for the differentially expressed genes between HepG2 treated with biofortified lettuce and Untreated cells.** Differentially expressed genes involved in canonical cellular pathway are represented in red if up-regulated and in green if down-regulated.
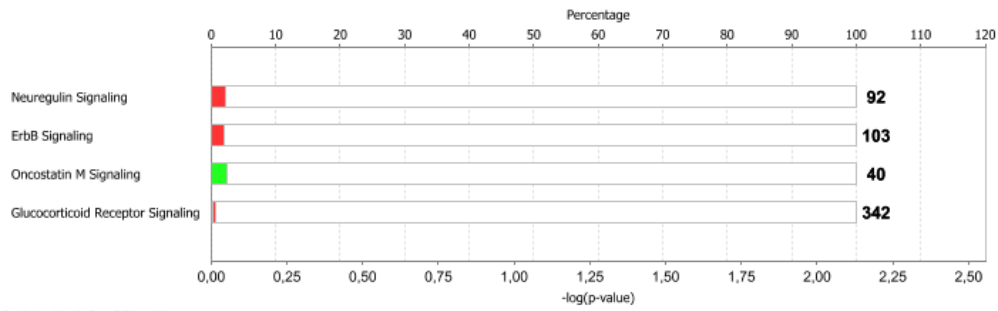
Figure 25. **Canonical pathways analysis performed by IPA for the differentially expressed genes between HepG2 treated with biofortified lettuce and HepG2 treated with control lettuce.** Differentially expressed genes involved in canonical cellular pathway are represented in red if up-regulated and in green if down-regulated.

The lists of genes of interest was obtained through IPA software analysis of RNA-Seq data (Table 16). Genes belonging to folate metabolism found to be differentially expressed in control lettuce versus untreated were MTHFR, SLC19A1, MTHFS, FOLR1, FTCD; genes related to zinc homeostasis are: ZIP13, ZIP3, ZIP5, ZIP6, and MT2A and to antioxidant metabolism are: TXNRD2, SELENOO.

Gene	Ctrl Lettuce vs Untreated		Biofortified vs Untreated	
	$\log_2$FoldChange	padj	$\log_2$FoldChange	padj
MTHFR	1.5	2.108E-23	1.3	2.295E-18
SLC19A1 (RFC1)	1.2	1.689E-29	1.3	2.215E-36
MTHFS	2.4	0.0002	2.8	6.815E-06
FOLR1	1.0	0.4	2.1	0.05
FTCD	1.4	2.890E-37	1.2	1.528E-29
SLC39A13 (ZIP13)	2.0	2.058E-54	1.8	3.705E-41
SLC39A3 (ZIP3)	1.4	4.278E-21	1.5	8.077E-25
SLC39A5 (ZIP5)	1.8	1.859E-67	1.8	2.940E-64
SLC39A6 (ZIP6)	-1.6	8.8957E-144	-1.7	1.0203E-156
TXNRD2	1.4	1.800E-32	1.5	2.165E-36
SELENOO	1.5	1.867E-37	1.6	8.966E-45
MT2A*	0.6	3.348E-05	-0.5	0.0004

Table 16. **Selected differentially expressed genes from RNA-Seq.** Comparison between HepG2 treated with Control lettuce and Untreated cells and comparison between HepG2 treated with Biofortified lettuce and Untreated cells. For MT2A gene, in the comparison between HepG2 treated with Biofortified lettuce and Ctrl lettuce, the $\log_2$FoldChange was -1.1 with a padj = 2.69666E-13.

IPA software allowed to find direct and indirect interaction between differentially expressed genes of our interest. About folate metabolism we can see how different folate forms can affect expression level of receptors, membrane channel and enzymes (Figure 26). Zn homeostasis was tightly regulated by SLC39A family through indirect mechanism and even by direct interaction between, such as SLC39A6 and SLC39A5, found down – regulated and up – regulated, respectively (Figure 27).
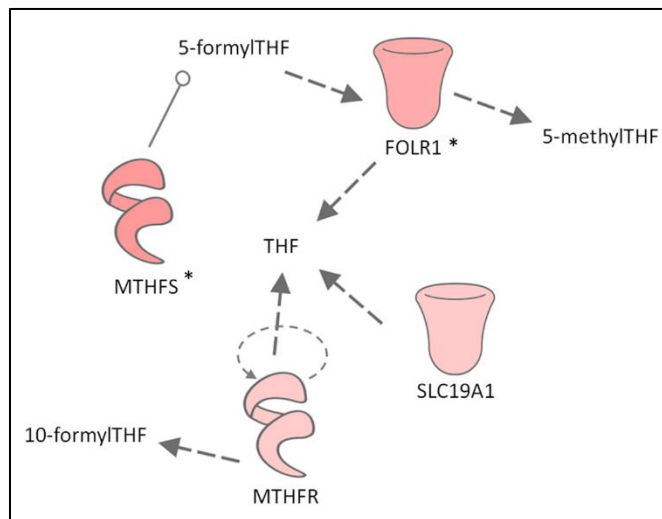
Figure 26. **Folate metabolism pathway by IPA.** Differentially expressed genes involved in one – carbon metabolism found in the comparison between biofortified and Ctrl lettuce versus untreated. FOLR1 was founded up - regulated only in Biofortified lettuce vs Untreated.
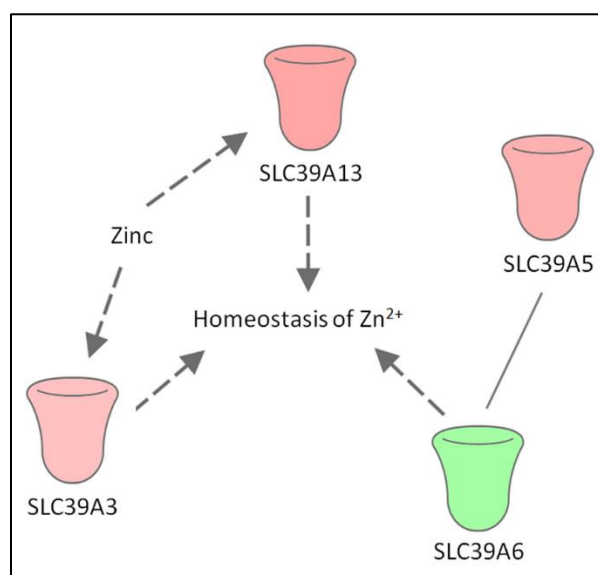
Figure 27. **Zn homeostasis pathway by IPA.** Differentially expressed genes involved in the homeostasis of Zn. Correlation founded through IPA analysis. in the comparison between biofortified and Ctrl lettuce versus untreated.

For down- regulated MT2A genes was possible only to find a predicted interaction related to the $Log_2FoldChange$ measured in the comparison between biofortified versus Ctrl lettuce. In orange there is predicted gene up-regulated MT3, metallothionein 3, that probably lead to MT2A inhibition. In blue there is the predicted down – regulated p53 gene by MT2A protein. Zn^{2+} and Cr^{6+} showed up – regulation and down – regulation, respectively (Figure 28).
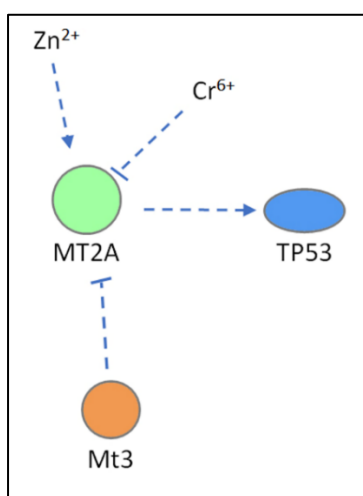
Figure 28. **MT2A predicted pathway by IPA.** Differentially expressed genes found correlated to predicted MT2A pathway in Biofortified lettuce versus Ctrl lettuce.

5.8 Gene expression analysis of HepG2 cells treated with biofortified vegetal extracts.

FOLR1 and MT2A genes were selected to validate RNA – Seq data. HepG2 treated with 500 µg/mL of biofortified and control lettuce data obtained by Real Time qPCR confirmed the trend found in mRNA sequencing on the same genes. FOLR1 gene was found up – regulated in HepG2 treated with Ctrl lettuce and Mix lettuce extracts, fold change were 1.1 and 1.2, respectively. MT2A gene was found down – regulated in HepG2 treated with Mix lettuce extracts (fold change = 0.5) (Figure 29).
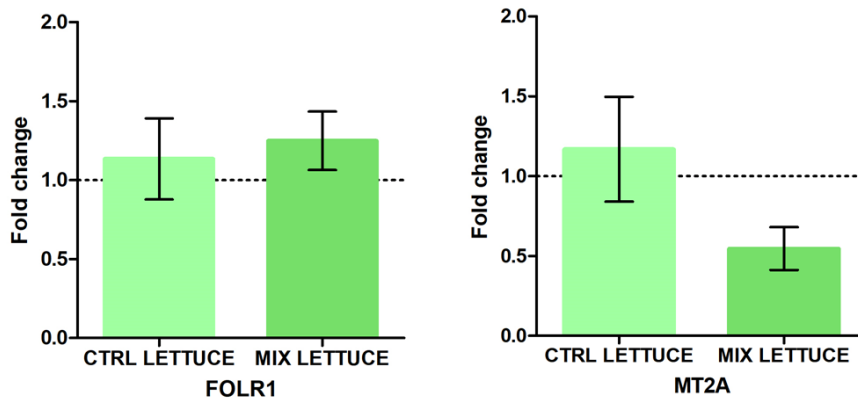
Figure 29. **HepG2 MT2A, FOLR1 gene expression analysis.** FOLR1 and MT2A gene expression analysis by real – time qPCR in HepG2 treated with Ctrl and Mix lettuce extracts 500 µg/mL.

In HepG2 treated with Ctrl and Mix lettuce extracts 2.5mg/mL, MT2A gene was found more down – regulated in HepG2 treated with Ctrl lettuce (fold change = 0.4) than HepG2 treated with Mix lettuce extracts (fold change = 0.8), while FOLR1 gene was found down – regulated at the same level in HepG2 treated with Ctrl lettuce (fold change = 0.5) and Mix lettuce extracts (fold change = 0.4) (Figure 30).
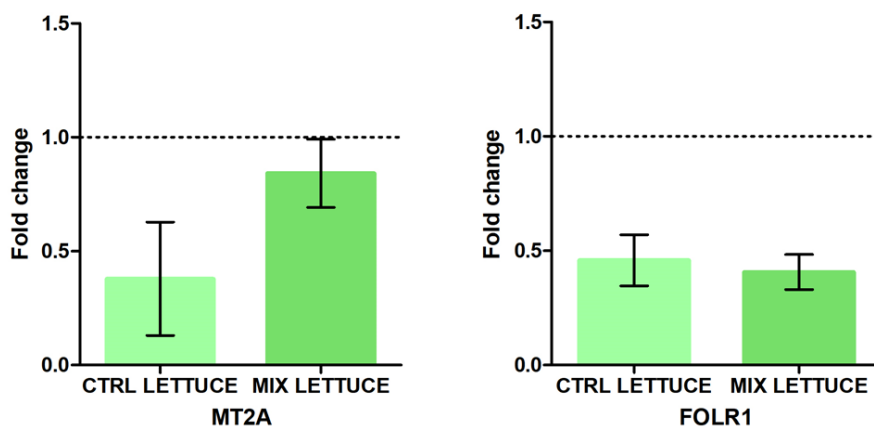
Figure 30. **HepG2 MT2A, FOLR1 gene expression analysis.** FOLR1 and MT2A gene expression analysis by real – time qPCR in HepG2 treated with Ctrl and Mix lettuce extracts 2.5 mg/mL.

5.9 Demethylation assay in HepG2

After the treatment with 5 µM Azacytidine (AZA), FOLR1 and MT2A gene expression were found up – regulated compare to untreated HepG2 cells with a fold change of 2.3 and 1.6, respectively (Figure 31).
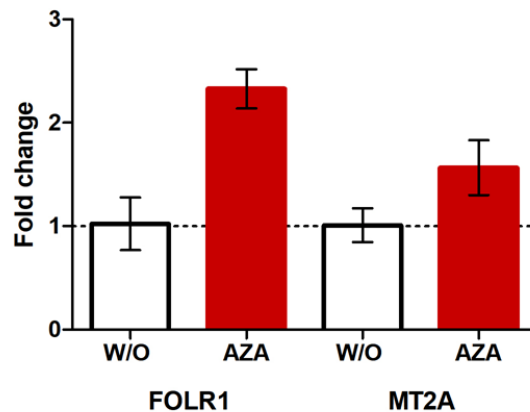
Figure 31. **FOLR1 and MT2A demethylation assay.** FOLR1 and MT2A gene expression analysis by Real Time qPCR in HepG2 treated with 5 µM AZA.

In the following promoter DNA methylation analysis on HepG2 by bisulfite – next generation sequencing (Bisulfite-NGS), none of 54 CpGs analysed in MT2A promoter region of all treatment (AZA, Ctrl lettuce and Mix lettuce) was found differentially methylated. All the 54 CpGs in MT2A selected promoter region were unmethylated (data not shown). FOLR1 promoter region analysis was impossible to be perform for technical problems in bisulfite primer operation.

5.10 Gene expression analysis of MT2A, FOLR1, ZIP13, RFC1 in Ea.hy926 and HepG2

Ea.hy926 and HepG2 treatment with lettuce extracts were performed in the laboratory of Epigenetic hypertension, at Càtolica Universidad de Santiago de Chile, Chile.

In Ea.hy926 treated with Ctrl lettuce and Mix lettuce extracts 1mg/mL, MT2A gene was found strongly up – regulated with Mix extracts compare to Ctrl lettuce extract with a fold change of 6.7 vs 3.0 (p = 0.04), FOLR1 gene, instead, was found more down – regulated in cells treated with Ctrl lettuce extract compare to ones treated with Mix extract, also down – regulated (fold change = 0.5 and fold change = 0.7). ZIP13 gene was found down – regulated more in cells treated with Mix lettuce extracts (fold change = 0.4) that one's treated with Ctrl lettuce extract (fold change = 0.7) (p = 0.01). RFC1 gene was found slightly up – regulated in cells treated with Mix lettuce extract (fold change = 1.2) compare to cells treated with Ctrl lettuce extract (fold change = 0.9) (Figure 32).
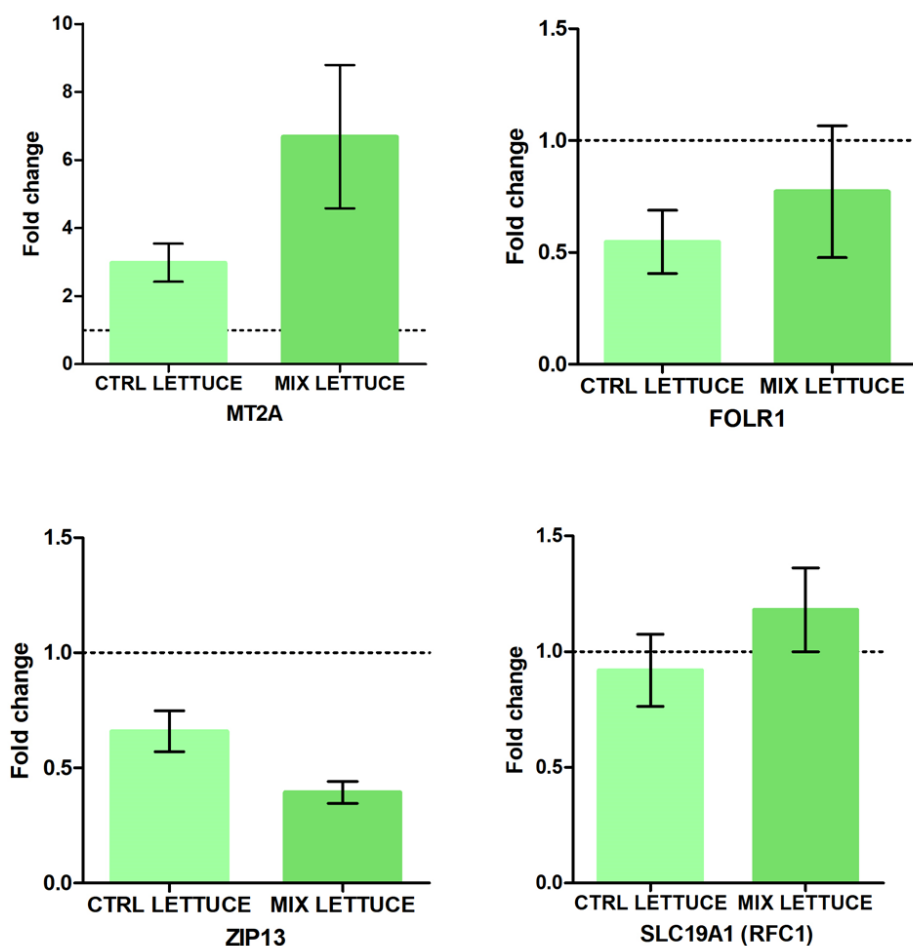
Figure 32. **Ea.hy926 MT2A, FOLR1, ZIP13 and RFC1 gene expression analysis.** MT2A, FOLR1, ZIP13 and RFC1 gene expression analysis by real – time qPCR in Ea.hy926 treated with Ctrl and Mix lettuce extracts 1 mg/mL.

In HepG2 treated with Ctrl lettuce and Mix lettuce extracts 1mg/mL, MT2A gene was found slightly down – regulated in cells treated with Ctrl lettuce extract (fold change = 0.7) compare to HepG2 treated with Ctrl lettuce extract (fold change = 1.0) (p = 0.01), did not show differences in gene expression as compared to untreated HepG2 cells. FOLR1 gene, instead, was found strongly up – regulated in cells treated with Mix lettuce extract (fold change = 3.5) compare to ones treated with Ctrl extract (fold change = 0.7) (p = 0.02). ZIP13 gene was found down – regulated in the same way with both treatment (fold change = 0.4 and 0.5) Instead RFC1 gene, even if showed little difference in expression between the two treatments (fold change = 0.8 and fold change = 1.1), it was statistically significant, p = 0.004 (Figure 33).
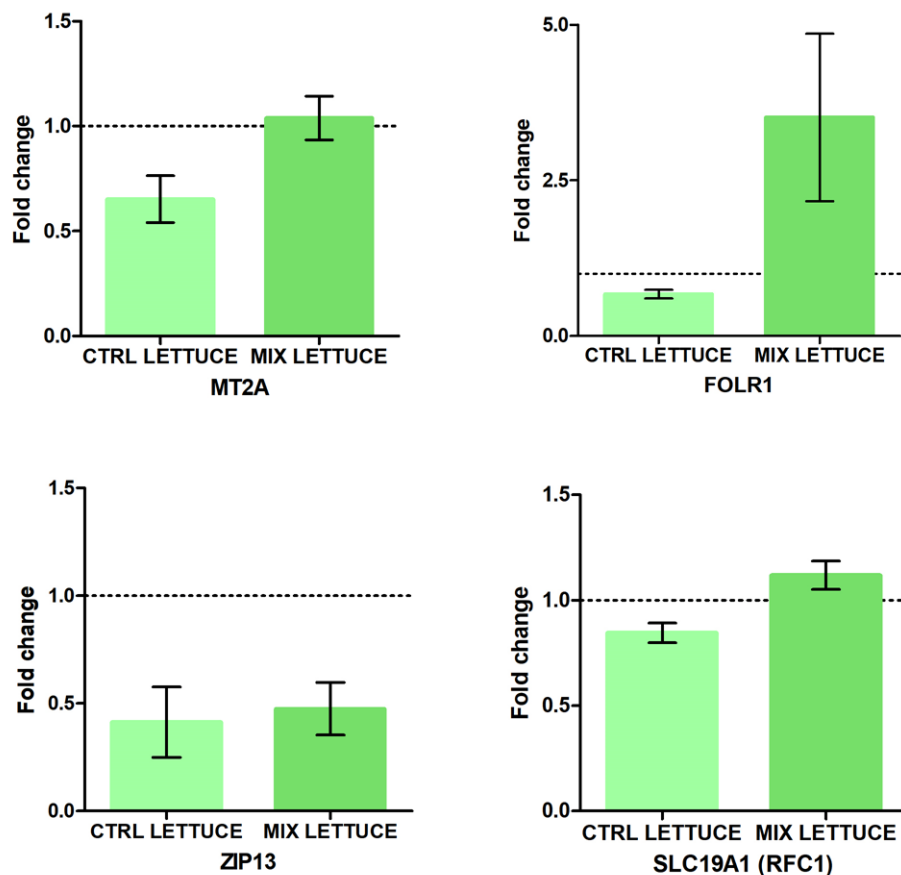
Figure 33. **HepG2 MT2A, FOLR1, ZIP13 and RFC1 gene expression analysis.** MT2A, FOLR1, ZIP13 and RFC1 gene expression analysis by real – time qPCR in HepG2 treated with Ctrl and Mix lettuce extracts 1 mg/mL.

5.11 Exosome analysis of Ea.hy926 and HepG2 treated with lettuce extracts

Ea.hy926 exosomes analysis was carried out after the treatments with Ctrl and Mix lettuce extracts 2.5mg/mL. NTA analysis allowed us to measure diameters and concentrations between the different treatments. As show in the Table 17, measured parameters show that Ctrl lettuce and Mix lettuce extract improve exosomes concentration of an order of magnitude, while the values of diameters, a mean value obtained by the triplicate measure by NTA software (Figure 34) confirm exosomes extraction by ultracentrifugation.

HepG2 exosomes analysis was impossible to be perform for poor availability of exosomes collected by ultracentrifugation.
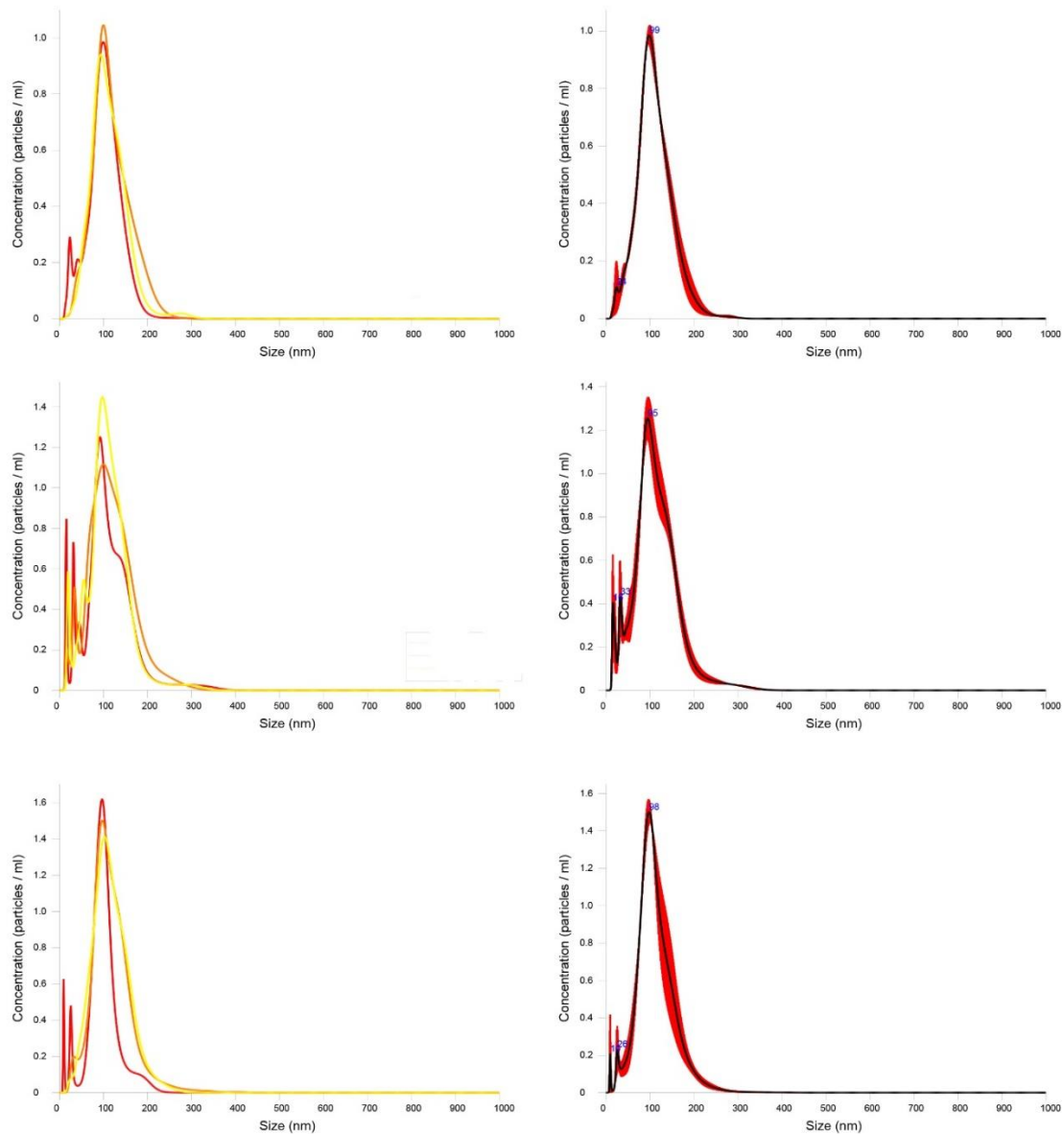
Figure 34. **Exosome NTA analysis.** Exosome diameters measurements, performed in triplicate, obtained by NTA. In order Ea.hy926 exosomes of Untreated cells, Ctrl lettuce treated Ea.hy926 exosomes and Mix lettuce treated Ea.hy926 exosomes.

Ea.hy exosomes	Mean +/- Standard Error	
	diameter (nm)	concentration (particles/ml)
w/o	110.3 +/- 4.5	8.13e+008 +/- 3.74e+007
Ctrl Lettuce	113.4 +/- 2.2	1.18e+009 +/- 6.90e+007
Mix Lettuce	111.0 +/- 5.8	1.14e+009 +/- 1.38e+008

Table 17. **Ea.hy926 exosomes features.** Ea.hy926 exosomes diameters and concentration.

5.11.1 Ea.hy926 exosome RNA qPCR

Ea.hy926 exosome RNA was quantified and MT2A, FOLR1, ZIP13, RFC1 and GUSB gene expression level were performed by real time qPCR. MT2A gene was detected only in exosome of Ea.Hy926 treated with Mix lettuce extract (Figure 35) and GUSB gene were detected, with different levels expression, in all the conditions: exosome of Untreated Ea.hy926, exosome of Ea.hy926 treated with Ctrl lettuce and Mix lettuce extracts (Figure 36). FOLR1, ZIP13, RFC1 genes were not amplificated.
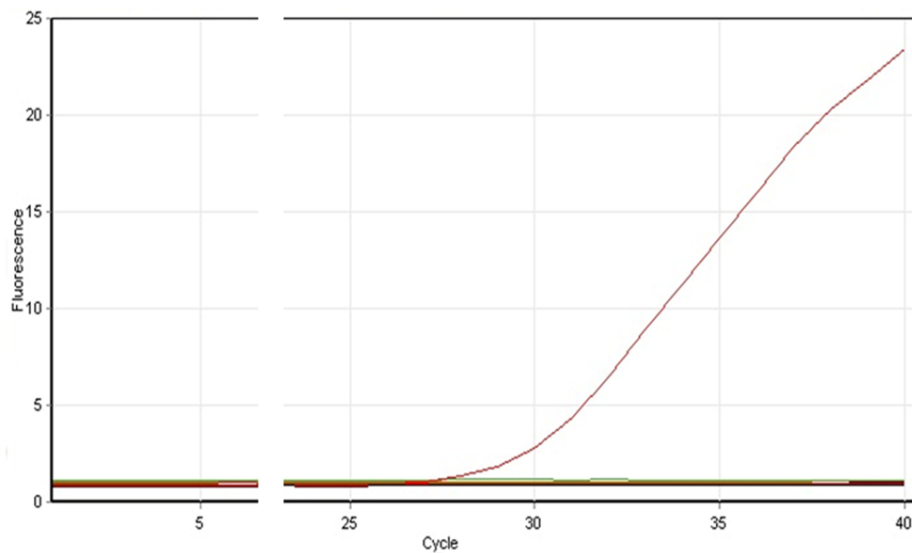
Figure 35. **Exosome MT2A gene expression.** Gene expression level of MT2A gene in Ea.hy926 exosomes treated with Mix lettuce extract.
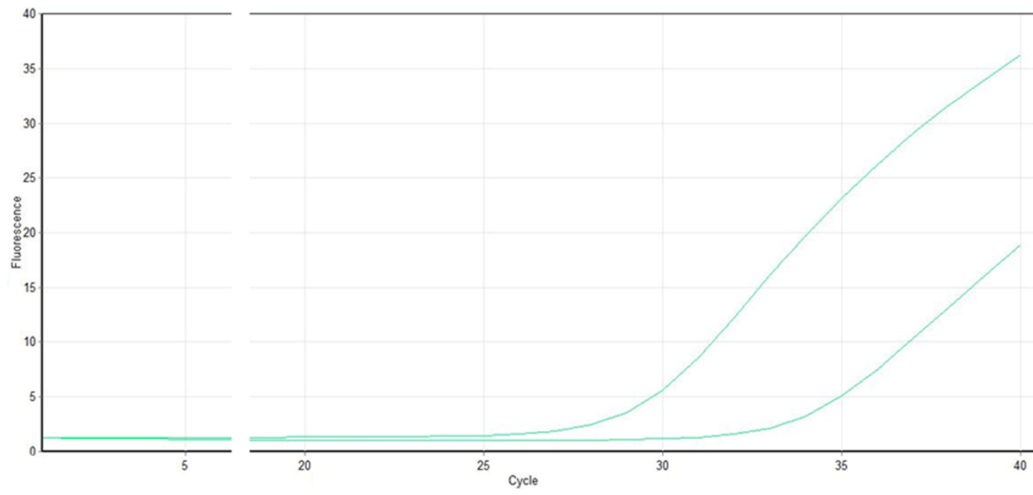
Figure 36. **Exosome GUSB gene expression.** Gene expression level of GUSB gene in Ea.hy926 exosomes.

6. Discussion

The study aimed at investigating possible modifications in epigenetic signatures on *in vitro* cell culture models tested with biofortified lettuce extracts by using a novel fertilizer formula added with microelements and a natural precursor of folate.

6.1 Evaluation of the microelements improvement in biofortified lettuce

Selenium, zinc and p-ABA are key elements in human and plant nutrition for their fundamental role in several metabolic pathways (Rayman, Infante et al. 2008). Zinc and selenium are trace elements with a vital function for both human and plant metabolism being enzyme cofactors in several biochemical reactions. P-ABA, as a natural precursor of THF and hydrophobic weak acid has a simple diffusion as well as can stimulate folate biosynthesis in plant.

During biofortification, metal content must be properly balanced and controlled to avoid intake of high doses that can lead to toxic effects.

The first step of the study was to confirm the absorption of microelements by lettuce and to evaluate the health status of the plants. Considering the content of microelements at a trace level an ICP/MS method was set up and optimized for this purpose and used to quantify several trace elements in plant extracts samples.

In metal composition with ICP/MS monitoring, the choice of the appropriate digestion and analysis were crucial steps due to the complexity and variability of vegetal matrix and because they influence the accuracy of the analysis as a whole. ICP-MS possesses in fact several characteristics that makes it well suited for the analysis of plant tissue, being its main analytical advantage is the detection of fingerprints of multiple elements.

Nevertheless ICP-MS has extremely low detection limits at a ng level (ng/L), selenium ions detection exhibited an overestimation problem probably caused by polyatomic ions interactions. Selenium is difficult to be accurately quantified at trace levels because it has a high first ionization potential (IP= 9.75 eV), which means that it is poorly ionized in plasma and therefore its signal intensity is low, thus giving a signal suppression in complex matrix samples. All the Se isotopes suffer from multiple spectral interferences. For these reasons we tried first, to acquire Se in collision cell technology (CCT) modality, that was used to remove or at least reduce polyatomic interferences. However there were problems of overestimation so instrument configuration was switched to kinetic energetic discrimination (KED) modality for Se acquisition. This configuration allows to discriminate slower atoms from faster ones even with the same atomic mass.

The analysis of CRM was then crucial to evaluate the quality of the obtained results. Preliminary results on lettuce sample and CRM showed that the use of H_2O_2 combined with HNO_3 was necessary to obtain the complete matrix mineralization. These conditions allowed the application of lower digestion temperature.

For what concern CRM quantification, the results obtained for the optimized protocol showed values in close agreement with the provided reference values.

So, the proposed HNO_3-H_2O_2 digestion procedure provides great accuracy for the digestion of a complex matrix and a safer, faster, and more economical method.

6.1.1 Fertilizer formulas improves Se and Zn in soil, hydroponic and open field modality.

Agronomic biofortification was effective in terms of microelements absorption for both Zn and Se in all the cultivation modality tested. First, trials performed in soil by using increasing concentration of the trace elements did not show any toxic effects on plants. In particular, the maximum absorption level was obtained with 60 ppm of Na_2SeO_4 and with 12000 ppm of $ZnSO_4$. In growth chamber, Se and Zn enrichment showed comparable values in hydroponic and soil cultivation modality. Interestingly, the open field cultivation showed the highest levels of Se in lettuce, probably for the exposition of the plants to the direct sun light, thus improving plant metabolism. Selenomethionine is in fact the most predominant form of detectable Se in plants, while selenocysteine is the most common form detected in animal tissues (Zayed, Lytle et al. 1998). Higher animals are unable to synthesize selenomethionine and only selenocysteine was detected in rats supplemented with Se as selenite. In mammals, ingested selenomethionine is absorbed in the small intestine via the Na^+-dependent neutral amino acid transfer system (Tapiero, Townsend et al. 2003). Starting from these results, it would be very intriguing to assess Se and Zn bioavailability of this biofortified lettuce since physiological effects that include also the conversion of the microelements into the active compounds must be taken into account.

6.2 The 5 – methylTHF is the main folate forms in biofortified lettuce

Identification and relative quantification of folate forms performed by UPLC/MS analysis revealed unexpected results. The highest improvement in 5 – methylTHF content was measured in lettuce grown in hydroponic modality supplemented whether with 60 ppm of Na_2SeO_4 or with 3000 ppm of p-ABA. These could signify that all the natural precursor of folate was used by the plant to synthesize folate that was stored in lettuce leaves. In the lettuce grown in growth chamber in soil, treated with Mix fertilizer formula there was an improvement of 5 – methylTHF, even if it was not statistically significant. Conversely, the lettuce cultivated in open field did not show any differences in 5 - methylTHF levels among the distinct fertilizer formulas. The surprisingly increase of 5 – methylTHF in plants supplemented with Na_2SeO_4 could have different possible explanations. First of all, the selenate treatment by foliar spray promotes selenium absorption through the leaves; selenium is in part weakly metabolized into selenoaminoacids and the selenate stored in shoots (i.e. stems and leaves) representing more than 90% of the total shoot selenium (de Souza, Pilon-Smits et al. 1998).
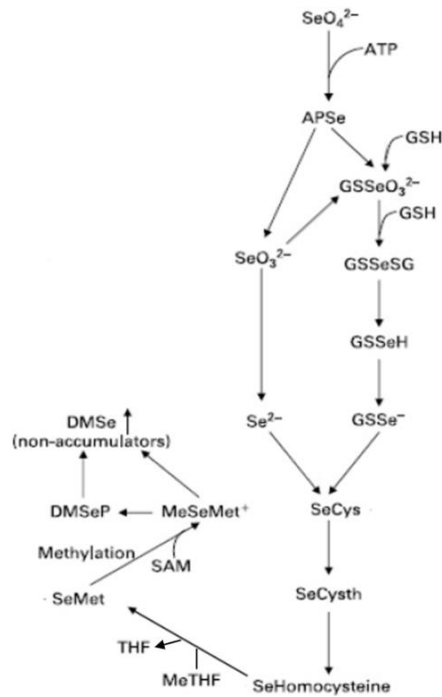
Figure 37. **Se metabolism in plants.** Selenium and folate metabolisms correlation in plants.

In short, selenate forms undergo several transformations, entering the metabolic pathway of glutathione (Figure 37). Although selenite and selenate are less absorbed than selenomethionine and organically bound selenium from foods, they are equally or even more effective in raising glutathione peroxidase activity, which is an indicator widely used to estimate Se bioavailability (Lintschinger, Fuchs et al. 2000). The high 5 − methylTHF content in lettuce supplemented with Na_2SeO_4 could be explained by the improvement of Se metabolism that is linked to folate synthesis (Figure 37). A hypothesis is that Se increases GPx activity and ultimately promotes the increase of methionine and by regulatory compensatory effect on homocysteine trans-sulfuration pathway, thereby increasing the 5-methyl THF for SAdoMet synthesis.

We observed that the 5 – methylTHF basal levels are influenced by the distinct cultivation modality as detected in the control lettuces, and this can indicate that even the cultivation modality affects *per se* the synthesis, of folates.

The controlled system of growth chamber is the best condition to improve and control metabolites synthesis and accumulation (Figure 38). Clearly, in open field there are several uncontrolled variabilities that can affect the plant growth and elements absorption from the soil including the strong competition among nutrients at root level that can affect in different ways metabolisms and the metabolites storage in leaves.
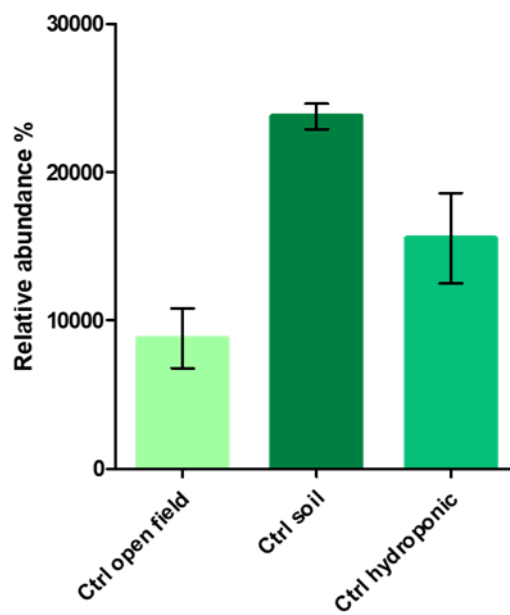
Figure 38. **Relative abundance of 5 – methylTHF in lettuce.** Basal level of relative abundance of 5 – methylTHF in the comparison between different cultivation modality.

6.3 Modification in the transcriptome of HepG2 treated with lettuce extracts

In vitro models allow to test nutrients and microelements of biofortified lettuce on hepatic and endothelial cell lines, and to study possible synergic or additive effects of antioxidants

and vitamins contained in lettuce. The choice to test a whole metabolite extract rather than single elements on different cell models derived from recent studies that observed that the overlapping and complementary effects of metabolites and vitamins provide a better protective effect on health than single ones (Eberhardt, Lee et al. 2000).

RNA – Seq profiling of transcriptome of HepG2 treated with lettuce extracts showed differentially expressed genes as response of the cells to the treatment. The great number, more than 12000, of either up- or down-regulated genes, in HepG2 cells treated by lettuce extracts, revealed a clear effect on transcriptomic profile potentially affecting several metabolic pathways and not only folate metabolism and pathways related to Zn and Se that were indeed clearly influenced by lettuce extracts exposure.

Induction of FOLR1 and RFC1 gene expression, both by control and biofortified lettuce extracts, suggested the increased internalization of the different folate forms within HepG2 cells.

The inhibition of FOLR1 gene expression in HepG2 treated with higher concentration of lettuce extract, 2 mg/mL, can be explained by a negative regulation of FOLR1 caused by excessive folate concentrations.

The up – regulation of MTHFR, a key enzyme in the one-carbon metabolism, responsible for the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methylTHF (Friso, Choi et al. 2002), confirms the metabolization by HepG2 of folates, accumulated in the lettuce. The effects of lettuce extract treatment on folate metabolism are of particular interest since the perturbation of this pathway are related to aberrant cell growth and to increased cancer risk, due to imbalance in DNA precursors, uracil mis-incorporation, and chromosome breakage (Pieroth, Paver et al. 2018).

MT2A showed significant differences in gene expression when comparing HepG2 treated with control lettuce and treated with biofortified lettuce. MT2A gene is widespread expressed and has scavenger activity on heavy metal ions and ROS. MTs expression is regulated also through epigenetic mechanisms, which to date are not fully understood (Hattori, Naito et al. 2016). Moreover, inhibitors of histone 21 deacetylase (HDAC) and DNA methyltransferase (DNMT) synergistically activate MT2A expression thus suggesting that, the cross-linking of HDAC1–DNMT1 complexes to chromatin might be involved in the inhibition of MTs transcription (Kimura and Kambe 2016).

Recent studies have also indicated a strong relationship between MT and p53, a critical component in damage repair and apoptotic cell death regulation. It has been suggested that the metal binding properties of MT can affect the activity of p53, a zinc-dependent protein, in agreement with our data obtained from RNA sequencing analysed with IPA software. The zinc ions are essential for the conformational stability and the activation of the p53 protein that activates proapoptotic signals when DNA damage is severe (Ostrakhovitch, Olsson et al. 2006).

6.4 Evaluation of MT2A promoter DNA methylation

The up-regulation of MT2A and FOLR1 gene expression in demethylation assay suggested a role for DNA methylation in the epigenetic regulation of these two genes. In fact, 5-azacytidine is a chemical analog for the nucleoside cytidine and severely inhibits the action of the DNA methyltransferase enzymes on CpGs. The DNA methylation of CpG islands localized in the promoter regions of specific genes is involved in their transcriptional regulation (Phillips 2008). The MT2A promoter region that we analyzed resulted completely

unmethylated suggesting that the regulation probably occurs in another promoter region or at another regulation level. Moreover potential artifacts could derive from the use of immortalized cell lines, which present aberrant methylation patterns in culture (Weber, Hellmann et al. 2007).

6.5 "Mirroring effect" in exosomes cargo of Ea.hy926 treated with biofortified lettuce extract

Ea.hy926 cell line was chosen as endothelial model to study the effects of biofortified lettuce on exosome cargo composition. Microelements have been studied in Ea.Hy926 cells: Zn has protective role in atherosclerosis, while Se, incorporated in SeP (selenium - proteins) mediates protection process against oxidative damage (Steinbrenner, Bilgic et al. 2006). Lettuce extracts were tested on HepG2 and Ea.hy926 with an intermediate concentration, 1 mg/mL, since previous experiments with higher concentration of lettuce extract, 2.5 mg/mL, lead to a down – regulation of FOLR1 gene expression. Effects on microelements and folates related genes were investigated in both cell lines.

Major effects were measured in cell lines after treatments with 1 mg/mL of biofortified lettuce extracts. In Ea.Hy926 cells the remarkable upregulation of MT2A gene expression could reflect the cellular response to the increased Zn concentration, since metallothioneins chelate free ions. Meanwhile the upregulation of FOLR1, specifically expressed in hepatic cells, in HepG2 treated with biofortified lettuce, reflects the increased folate levels in the cell culture medium.

The effects of biofortified lettuce on cellular metabolism were further investigate taking into account the cargo of microvesicles secreted by the cells. Since exosome can transfer mRNA horizontally, i-e- between neighbouring cells, we decided to investigate exosome mRNA cargo.

Interestingly, we observed that only the exosomes derived from Ea.hy926 treated with biofortified lettuce, carry on MT2A transcript, showing a "mirror effect" as observed in the exosome secreting cells. The "mirror effect" of the exosomes has been previously described in cardiovascular disease and cancer (Ailawadi, Wang et al. 2015, M, Bayraktar et al. 2017) and it would be interesting to further investigate these effects in our *in vitro* model.

7. Conclusions and perspective

A novel fertilizer formula was successfully set up to effectively increase the content of Se, Zn and the active form of folate 5-methylTHF in lettuce, a widely utilized food plant as a key component of human nutrition. The beneficial effects of food plants for a healthy diet are well-known, though the precise molecular effects underlying this largely described association from epidemiological approaches is still to be clearly defined. It is known that nutrients affect gene expression through epigenetic mechanisms especially by modulating DNA methylation. Moreover, deficiencies of microelements and vitamins, among which folate is one of the most important in this regard, may impair this major epigenetic feature of DNA eventually leading to aberrant signatures linked to main disease development. The present study showed an effective food plant agronomical biofortification formulas to improve Se and Zn levels and 5methylTHF as the active form of folate. Results from the present study show that open field is the best cultivation modality to improve microelements accumulation in lettuce while hydroponic modality, a strictly controlled system, is the best cultivation modality to improve both microelements and 5-methylTHF content in lettuce. Interestingly, fertilizer formula enriched only in Na_2SeO_4 induces accumulation of 5methylTHF in lettuce similar to that obtained with the treatment containing p-ABA. This may open up to simple method of agronomical biofortification by the addition of only Se to increase the form of folate that is necessary for methylation reactions including that of DNA.

Extracts of biofortified lettuce extracts alter gene expression of an ample number of genes in studies *in vitro* using HepG2 and Ea.hy926 cell culture models highlighting modulation of several different cellular metabolic pathways.

Bisulfite – NGS analysis on specific promoter region of MT2A, opened up for a deeper investigation to better understand how methylation impacts gene expression through food plants. Moreover, the effect seen on modification of exosomal cargo by cells exposed to biofortified lettuce, show a possible cell-to-cell mechanism due to biofortified lettuce extracts exposure potentially useful to explain the beneficial effects of food plant in the whole organism besides the specific tissue where the absorption occurs.

Biofortification using a novel Se, Zn and p-ABA-enriched formula as developed and tested in the present study is effective to ameliorate the content of such microelements and 5methylTHF in lettuce and lead to gene expression and gene specific DNA methylation modulation in HepG2 and Ea.hy926 cell culture models. Future perspectives include specific investigation of biofortified lettuce nutritional and microelements bioavailability and specific epigenetic modulation in humans for possible correction of widespread microelements and vitamin deficiencies with the purpose of reducing the risk of several major chronic diseases related to nutritional deficiencies.

8. Bibliography

Ailawadi, S., X. Wang, H. Gu and G. C. Fan (2015). "Pathologic function and therapeutic potential of exosomes in cardiovascular disease." Biochim Biophys Acta **1852**(1): 1-11.

Allis, C. D. and T. Jenuwein (2016). "The molecular hallmarks of epigenetic control." Nature Reviews Genetics **17**(8): 487-500.

Arcot, J. and A. Shrestha (2005). "Folate: methods of analysis." Trends in Food Science & Technology **16**(6-7): 253-266.

Barros, E. R. and C. A. Carvajal (2017). "Urinary Exosomes and Their Cargo: Potential Biomarkers for Mineralocorticoid Arterial Hypertension?" Front Endocrinol (Lausanne) **8**: 230.

Bashtrykov, P. and A. Jeltsch (2018). "DNA Methylation Analysis by Bisulfite Conversion Coupled to Double Multiplexed Amplicon-Based Next-Generation Sequencing (NGS)." Methods Mol Biol **1767**: 367-382.

Bekaert, S., S. Storozhenko, P. Mehrshahi, M. J. Bennett, W. Lambert, J. F. Gregory, K. Schubert, J. Hugenholtz, D. Van Der Straeten and A. D. Hanson (2008). "Folate biofortification in food plants." Trends in Plant Science **13**(1): 28-35.

Berg, J. M. and Y. Shi (1996). "The Galvanization of Biology: A Growing Appreciation for the Roles of Zinc." Science **271**(5252): 1081-1085.

Bin, B. H., J. Seo and S. T. Kim (2018). "Function, Structure, and Transport Aspects of ZIP and ZnT Zinc Transporters in Immune Cells." J Immunol Res **2018**: 9365747.

Blancquaert, D., S. Storozhenko, J. Van Daele, C. Stove, R. G. F. Visser, W. Lambert and D. Van Der Straeten (2013). "Enhancing pterin and para-aminobenzoate content is not sufficient to successfully biofortify potato tubers and Arabidopsis thaliana plants with folate." Journal of Experimental Botany **64**(12): 3899-3909.

Bocker, M. T., I. Hellwig, A. Breiling, V. Eckstein, A. D. Ho and F. Lyko (2011). "Genome-wide promoter DNA methylation dynamics of human hematopoietic progenitor cells during differentiation and aging." Blood **117**(19): e182-189.

Bouis, H. E. and R. M. Welch (2010). "Biofortification—A Sustainable Agricultural Strategy for Reducing Micronutrient Malnutrition in the Global South." Crop Science **50**(Supplement_1): S-20.

Cakmak, I. (2007). "Enrichment of cereal grains with zinc: Agronomic or genetic biofortification?" Plant and Soil **302**(1-2): 1-17.

Choi, S.-W. and S. Friso (2010). "Epigenetics: A New Bridge between Nutrition and Health." Advances in Nutrition **1**(1): 8-16.

Cousins, R. J., R. K. Blanchard, J. B. Moore, L. Cui, C. L. Green, J. P. Liuzzi, J. Cao and J. A. Bobo (2003). "Regulation of zinc metabolism and genomic outcomes." J Nutr **133**(5 Suppl 1): 1521S-1526S.

Crider, K. S., T. P. Yang, R. J. Berry and L. B. Bailey (2012). "Folate and DNA Methylation: A Review of Molecular Mechanisms and the Evidence for Folate's Role." Advances in Nutrition **3**(1): 21-38.

Crosby, D., G. (1963). "The Organic Constituents of Food. I. Lettuce."

Daniel, M. and T. O. Tollefsbol (2015). "Epigenetic linkage of aging, cancer and nutrition." Journal of Experimental Biology **218**(1): 59-70.

de Souza, M. P., E. A. Pilon-Smits, C. M. Lytle, S. Hwang, J. Tai, T. S. Honma, L. Yeh and N. Terry (1998). "Rate-limiting steps in selenium assimilation and volatilization by indian mustard." Plant Physiol **117**(4): 1487-1494.

Delchier, N., A. L. Herbig, M. Rychlik and C. M. G. C. Renard (2016). "Folates in Fruits and Vegetables: Contents, Processing, and Stability." Comprehensive Reviews in Food Science and Food Safety **15**(3): 506-528.

Eberhardt, M. V., C. Y. Lee and R. H. Liu (2000). "Antioxidant activity of fresh apples." Nature **405**(6789): 903-904.

Ehrlich, M. (2006). "Cancer-Linked DNA Hypomethylation and Its Relationship to Hypermethylation." **310**: 251-274.

Fairweather-Tait, S. J., R. Collings and R. Hurst (1997).

Fenech, M. and L. R. Ferguson (2001). "Vitamins/minerals and genomic stability in humans." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis **475**(1-2): 1-6.

Friso, S., C. A. Carvajal, C. E. Fardella and O. Olivieri (2015). "Epigenetics and arterial hypertension: the challenge of emerging evidence." Transl Res **165**(1): 154-165.

Friso, S. and S. W. Choi (2002). "Gene-nutrient interactions and DNA methylation." J Nutr **132**(8 Suppl): 2382S-2387S.

Friso, S., S. W. Choi, D. Girelli, J. B. Mason, G. G. Dolnikowski, P. J. Bagley, O. Olivieri, P. F. Jacques, I. H. Rosenberg, R. Corrocher and J. Selhub (2002). "A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status." Proceedings of the National Academy of Sciences **99**(8): 5606-5611.

Funes-Collado, V., A. Morell-Garcia, R. Rubio and J. F. Lopez-Sanchez (2013). "Study of selenocompounds from selenium-enriched culture of edible sprouts." Food Chem **141**(4): 3738-3743.

Garcia, C. J., R. Garcia-Villalba, M. I. Gil and F. A. Tomas-Barberan (2017). "LC-MS Untargeted Metabolomics To Explain the Signal Metabolites Inducing Browning in Fresh-Cut Lettuce." J Agric Food Chem **65**(22): 4526-4535.

Gilbert, S. F. (2012). "Commentary: 'The Epigenotype' by C.H. Waddington." International Journal of Epidemiology **41**(1): 20-23.

Gupta, M. and S. Gupta (2016). "An Overview of Selenium Uptake, Metabolism, and Toxicity in Plants." Front Plant Sci **7**: 2074.

Hainaut, P. and K. Mann (2001). "Zinc Binding and Redox Control of p53 Structure and Function." Antioxidants & Redox Signaling **3**(4): 611-623.

Hall, I., C.A. and Cuppett, S.L. (1997). "Structure-activeties of natural antioxidants." **Antioxidant methodology: In vivo and in vitro concepts.**: 141-172. .

Hansen, T. H., T. C. de Bang, K. H. Laursen, P. Pedas, S. Husted and J. K. Schjoerring (2013). "Multielement Plant Tissue Analysis Using ICP Spectrometry." **953**: 121-141.

Hanson, A. D. and J. F. Gregory (2011). "Folate Biosynthesis, Turnover, and Transport in Plants." Annual Review of Plant Biology **62**(1): 105-125.

Hardy, T. M. and T. O. Tollefsbol (2011). "Epigenetic diet: impact on the epigenome and cancer." Epigenomics **3**(4): 503-518.

Hartikainen, H. (2005). "Biogeochemistry of selenium and its impact on food chain quality and human health." Journal of Trace Elements in Medicine and Biology **18**(4): 309-318.

Hattori, Y., M. Naito, M. Satoh, M. Nakatochi, H. Naito, M. Kato, S. Takagi, T. Matsunaga, T. Seiki, T. Sasakabe, S. Suma, S. Kawai, R. Okada, A. Hishida, N. Hamajima and K. Wakai (2016). "Metallothionein MT2A A-5G Polymorphism as a Risk Factor for Chronic Kidney Disease and Diabetes: Cross-Sectional and Cohort Studies." Toxicol Sci **152**(1): 181-193.

Hooper, J. J., S. D. James, D. C. Jones, D. M. Lee and J. M. Gal (2008). "The influence of training with heavy rugby balls on selected spin pass variables in youth rugby union players." J Sci Med Sport **11**(2): 209-213.

Hou, Z. and L. H. Matherly (2014). "Biology of the major facilitative folate transporters SLC19A1 and SLC46A1." Curr Top Membr **73**: 175-204.

Kelly, P., J. McPartlin, M. Goggins, D. G. Weir and J. M. Scott (1997). "Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements." The American Journal of Clinical Nutrition **65**(6): 1790-1795.

Kimura, T. and T. Kambe (2016). "The Functions of Metallothionein and ZIP and ZnT Transporters: An Overview and Perspective." International Journal of Molecular Sciences **17**(3): 336.

Lee, K. W., H. J. Lee and C. Y. Lee (2004). "Vitamins, phytochemicals, diets, and their implementation in cancer chemoprevention." Crit Rev Food Sci Nutr **44**(6): 437-452.

Leitao, E., J. Beygo, M. Zeschnigk, L. Klein-Hitpass, M. Bargull, S. Rahmann and B. Horsthemke (2018). "Locus-Specific DNA Methylation Analysis by Targeted Deep Bisulfite Sequencing." Methods Mol Biol **1767**: 351-366.

Li, Y. and T. O. Tollefsbol (2011). "DNA Methylation Detection: Bisulfite Genomic Sequencing Analysis." **791**: 11-21.

Lintschinger, J., N. Fuchs, J. Moser, D. Kuehnelt and W. Goessler (2000). "Selenium-enriched sprouts. A raw material for fortified cereal-based diets." J Agric Food Chem **48**(11): 5362-5368.

Lucock, M. (2000). "Folic Acid: Nutritional Biochemistry, Molecular Biology, and Role in Disease Processes." Molecular Genetics and Metabolism **71**(1-2): 121-138.

M, H. R., E. Bayraktar, K. H. G, M. F. Abd-Ellah, P. Amero, A. Chavez-Reyes and C. Rodriguez-Aguayo (2017). "Exosomes: From Garbage Bins to Promising Therapeutic Targets." Int J Mol Sci **18**(3).

Manzocco, L., M. Foschia, N. Tomasi, M. Maifreni, L. Dalla Costa, M. Marino, G. Cortella and S. Cesco (2011). "Influence of hydroponic and soil cultivation on quality and shelf life of ready-to-eat lamb's lettuce (Valerianella locusta L. Laterr)." Journal of the Science of Food and Agriculture **91**(8): 1373-1380.

Meeran, S. M., A. Ahmed and T. O. Tollefsbol (2010). "Epigenetic targets of bioactive dietary components for cancer prevention and therapy." Clinical Epigenetics **1**(3-4): 101-116.

Meplan, C., M. J. Richard and P. Hainaut (2000). "Metalloregulation of the tumor suppressor protein p53: zinc mediates the renaturation of p53 after exposure to metal chelators in vitro and in intact cells." Oncogene **19**(46): 5227-5236.

Mercken, E. M., S. D. Crosby, D. W. Lamming, L. JeBailey, S. Krzysik-Walker, D. T. Villareal, M. Capri, C. Franceschi, Y. Zhang, K. Becker, D. M. Sabatini, R. de Cabo and L. Fontana (2013). "Calorie restriction in humans inhibits the PI3K/AKT pathway and induces a younger transcription profile." Aging Cell **12**(4): 645-651.

Notaro, S., D. Reimer, H. Fiegl, G. Schmid, A. Wiedemair, J. Rossler, C. Marth and A. G. Zeimet (2016). "Evaluation of folate receptor 1 (FOLR1) mRNA expression, its specific promoter methylation and global DNA hypomethylation in type I and type II ovarian cancers." BMC Cancer **16**: 589.

Ong, T. P., F. S. Moreno and S. A. Ross (2011). "Targeting the Epigenome with Bioactive Food Components for Cancer Prevention." Journal of Nutrigenetics and Nutrigenomics **4**(5): 275-292.

Ostrakhovitch, E. A., P.-E. Olsson, S. Jiang and M. G. Cherian (2006). "Interaction of metallothionein with tumor suppressor p53 protein." FEBS Letters **580**(5): 1235-1238.

Phillips, T. (2008). "The role of methylation in gene expression.".

Pieroth, R., S. Paver, S. Day and C. Lammersfeld (2018). "Folate and Its Impact on Cancer Risk." Curr Nutr Rep **7**(3): 70-84.

Powell, S. R. (2000). "The antioxidant properties of zinc." J Nutr **130**(5S Suppl): 1447S-1454S.

Quinlivan, E. P., A. D. Hanson and J. F. Gregory (2006). "The analysis of folate and its metabolic precursors in biological samples." Analytical Biochemistry **348**(2): 163-184.

Rayman, M. P., H. G. Infante and M. Sargent (2008). "Food-chain selenium and human health: spotlight on speciation." British Journal of Nutrition **100**(02).

Rimm, E. B., A. Ascherio, E. Giovannucci, D. Spiegelman, M. J. Stampfer and W. C. Willett (1996). "Vegetable, fruit, and cereal fiber intake and risk of coronary heart disease among men." Jama-Journal of the American Medical Association **275**(6): 447-451.

Ríos, J. J., M. A. Rosales, B. Blasco, L. M. Cervilla, L. Romero and J. M. Ruiz (2008). "Biofortification of Se and induction of the antioxidant capacity in lettuce plants." Scientia Horticulturae **116**(3): 248-255.

Saif, I., Y. Kasmi, K. Allali and M. M. Ennaji (2018). "Prediction of DNA methylation in the promoter of gene suppressor tumor." Gene **651**: 166-173.

Saini, R. K., S. H. Nile and Y.-S. Keum (2016). "Folates: Chemistry, analysis, occurrence, biofortification and bioavailability." Food Research International **89**: 1-13.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T) method." Nat Protoc **3**(6): 1101-1108.

Selhub, J. and I. H. Rosenberg (2016). "Excessive folic acid intake and relation to adverse health outcome." Biochimie **126**: 71-78.

Sharif, R., P. Thomas, P. Zalewski and M. Fenech (2012). "The role of zinc in genomic stability." Mutat Res **733**(1-2): 111-121.

Smrkolj, P., L. Pograjc, C. Hlastanribi and V. Stibilj (2005). "Selenium content in selected Slovenian foodstuffs and estimated daily intakes of selenium." Food Chemistry **90**(4): 691-697.

Steinbrenner, H., E. Bilgic, L. Alili, H. Sies and P. Brenneisen (2006). "Selenoprotein P protects endothelial cells from oxidative damage by stimulation of glutathione peroxidase expression and activity." Free Radic Res **40**(9): 936-943.

Steinmetz, K. A. and J. D. Potter (1996). "Vegetables, fruit, and cancer prevention: A review." Journal of the American Dietetic Association **96**(10): 1027-1039.

Suzuki, M. M. and A. Bird (2008). "DNA methylation landscapes: provocative insights from epigenomics." Nat Rev Genet **9**(6): 465-476.

Szeto, Y. T., T. C. Kwok and I. F. Benzie (2004). "Effects of a long-term vegetarian diet on biomarkers of antioxidant status and cardiovascular disease risk." Nutrition **20**(10): 863-866.

Tapiero, H., D. M. Townsend and K. D. Tew (2003). "The antioxidant role of selenium and seleno-compounds." Biomed Pharmacother **57**(3-4): 134-144.

Tomasi, N., R. Pinton, S. Gottardi, T. Mimmo, M. Scampicchio and S. Cesco (2015). "Selenium fortification of hydroponically grown corn salad (Valerianella locusta)." Crop and Pasture Science **66**(11): 1128.

Udali, S., P. Guarini, S. Moruzzi, S. W. Choi and S. Friso (2013). "Cardiovascular epigenetics: from DNA methylation to microRNAs." Mol Aspects Med **34**(4): 883-901.

Weber, M., I. Hellmann, M. B. Stadler, L. Ramos, S. Paabo, M. Rebhan and D. Schubeler (2007). "Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome." Nat Genet **39**(4): 457-466.

Witthöft, C. M., K. Forssén, L. Johannesson and M. Jägerstad (2016). "Folates - food sources, analyses, retention and bioavailability." Näringsforskning **43**(1): 138-146.

Zalewska, M., J. Trefon and H. Milnerowicz (2014). "The role of metallothionein interactions with other proteins." Proteomics **14**(11): 1343-1356.

Zayed, A., C. M. Lytle and N. Terry (1998). "Accumulation and volatilization of different chemical species of selenium by plants." Planta **206**(2): 284-292.

9. Publications

Friso, S., Udali S., **De Santis, D.**, Choi, S.-W. One-carbon metabolism and epigenetics. <u>Molecular Aspects of Medicine</u>, Volum **54**, April 2017, Pages 28–36, 2016.

Udali, S., **De Santis, D.**, Ruzzenente, A., Moruzzi, S., Mazzi, F., Beschin, G., Tammen, S.A., Campagnaro, T., Pattini, P., Olivieri, O., Guglielmi, A., Choi, S.-W., Friso, S. DNA methylation and hydroxymethylation in primary colon cancer and synchronous hepatic metastasis. <u>Frontiers in Genetics</u>, Volum **8**, Issue JAN, 9 January 2018.

Udali, S., Castagna, A., Corbella, M., Ruzzenente, A., Moruzzi, S., Mazzi, F., Campagnaro, T., **De Santis, D.**, Franceschi, A., Pattini, P., Gottardo, R., Olivieri, O., Perbellini, L., Guglielmi, A., Choi, S.-W., Girelli, D., Friso, S. Hepcidin and DNA promoter methylation in hepatocellular carcinoma. <u>European Journal of Clinical Investigation</u>, Volum **48**, Issue 2, February 2018.