REVIEW

Claimed effects, outcome variables and methods of measurement for health claims proposed under European Community Regulation 1924/2006 in the framework of protection against oxidative damage and cardiovascular health

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D. Martini [a], S. Rossi [a], B. Biasini [a], I. Zavaroni [b,c], G. Bedogni [d], M. Musci [e], C. Pruneti [f], G. Passeri [g], M. Ventura [h], S. Di Nuzzo [i], D. Galli [j], P. Mirandola [j], M. Vitale [j], A. Dei Cas [b,c], R.C. Bonadonna [b,c], D. Del Rio [a,*]

[a] The Laboratory of Phytochemicals in Physiology, Department of Food and Drug, University of Parma, Parma, Italy
[b] Department of Medicine and Surgery, Division of Endocrinology, University of Parma, Italy
[c] Azienda Ospedaliera Universitaria of Parma, Parma, Italy
[d] Clinical Epidemiology Unit, Liver Research Center, Basovizza, Trieste, Italy
[e] Department of Food and Drug, University of Parma, Parma, Italy
[f] Department of Medicine and Surgery, Clinical Psychology Unit, University of Parma, Medical School Building, Parma, Italy
[g] Department of Medicine and Surgery, Building Clinica Medica Generale, University of Parma, Parma, Italy
[h] Department of Chemistry, Life Sciences and Environmental Sustainability, Laboratory of Probiogenomics, University of Parma, Parma, Italy
[i] Department of Medicine and Surgery, Section of Dermatology, University of Parma, Parma, Italy
[j] Department of Medicine and Surgery, Sport and Exercise Medicine Centre (SEM), University of Parma, Parma, Italy

Abstract *Background and aims:* The high number of negative opinions from the European Food Safety Authority (EFSA) to the requests for authorization of health claims is largely due to the design of human intervention studies, including the inappropriate choice of outcome variables (OVs) and of their methods of measurement (MMs). The present manuscript reports the results of an investigation aimed to collect, collate and critically analyse the information in relation to claimed effects, OVs and MMs, in the context of protection against oxidative damage and cardiovascular health compliant with Regulation 1924/2006.

Acronyms: 8-OHdG, 8-hydroxy-2-deoxy-guanosine; 8-oxo-dG, 8-oxo-2-deoxyguanosine; ABPM, Ambulatory blood pressure monitoring; ADP, Adenosine diphosphate; AHA, American Heart Association; AIx, Augmentation Index; AUC, Area under the curve; BP, Blood pressure; CAD, Coronary artery disease; CAT, Catalase; CHD, Coronary heart disease; CL-HPLC, Chemiluminescence-based high pressure liquid chromatography; CM, Chylomicrons; CVD, Cardiovascular disease; DBP, Diastolic blood pressure; DNP, Dinitrophenyl; DNPH, Dinitrophenylhydrazine; ECD, Electrochemical detection; EFSA, European Food Safety Authority; ELISA, Enzyme-linked immunosorbent assay; Endo III, Endonuclease III; F2-IsoPs, F2-isoprostanes; FL, Fluorescence; FMD, Flow-mediated dilation; FPG, Formamidopyrimidine DNA glycosilase; G1P, Glycerol-1-phosphate; GC, Gas chromatography; GSH, Glutathione; GSH-Px, Glutathione peroxidase; GSSG, Glutathione disulfide; HDL, High density lipoprotein; HDL-C, HDL cholesterol; HO, Heme oxygenase; HPLC, High performance liquid chromatography; IDL, Intermediate density lipoprotein; IsoPs, Isoprostanes; LC-MS/MS, Liquid chromatography tandem mass spectrometry; LDL, Low density lipoprotein; LDL-C, Low density lipoprotein cholesterol; LTA, Light transmission aggregometry; MDA, Malondialdehyde; MM, Method of measurement; MS, Mass spectrometry; Ox-LDL, Oxidized LDL; OV, Outcome variable; PC, Phosphatidylcholine; PCOOH, Phosphatidylcholine hydroperoxide; PGI2, prostacyclin 2; P-sel, P-selectin; RCT, randomized controlled trial; ROS, Reactive oxygen species; RXNO, Nitroso/nitrosyl species; SAC, Systemic arterial compliance; SBP, Systolic blood pressure; SBs, Strand breaks; SOD, Superoxide dismutase; TBA, Thiobarbituric acid; TC, Total cholesterol; TG, Triglycerides; TRL, Triglycerides rich lipoproteins; TXA2, Thromboxane A2; UV, Ultraviolet; VLDL, Very-low-density lipoprotein.

* Corresponding author. The Laboratory of Phytochemicals in Physiology, Department of Food and Drug, University of Parma, Medical School, Building C, Via Volturno 39, 43125 Parma, Italy.
    E-mail address: daniele.delrio@unipr.it (D. Del Rio).

Methods and results: Claimed effects, OVs and the related MMs were collected from EFSA Guidance documents and applications for authorization of health claims under Articles 13.5 and 14. The OVs and their MMs were evaluated only if the claimed effect was sufficiently defined and was considered beneficial by EFSA.

The collection, collation and critical analysis of the relevant scientific literature consisted in the definition of the keywords, the PubMed search strategies and the creation of databases of references. The critical analysis of the OVs and their MMs was performed on the basis of the literature review and was aimed at defining the appropriateness of OVs and MMs in the context of the specific claimed effects.

Conclusions: The information provided in this document could serve to EFSA for the development of further guidance on the scientific requirements for health claims, as well as to the stakeholders for the proper design of human intervention studies aimed to substantiate such health claims.

Contents

1. Introduction

The concept of "functional food" grounds its roots in Japan during the 80's and rapidly expanded to other countries. Starting from the 90's, within European Community, a growing number of functional foods has been labelled and publicized, demonstrating an increased interest from stakeholders about the possibility of reporting health claims on them. This situation has entailed the need of an international regulation in this context, to guarantee a high level of protection of consumers' interest. Within the European Community, the use of nutrition and health claims made on foods is regulated by the Regulation (EC) 1924/2006 [1]. The consumers may perceive food promoted with claims as having advantage in comparison to similar or other foods, providing a direct influence on food choice with a consequence on total intake of certain nutrients or other substances. In order to address these potential undesirable effects, it is suitable to impose specific restrictions on the products bearing claims. In the context of scientific substantiation of health claims, appropriate criteria have been established in the PASSCLAIM [2] concerning the process for the assessment of scientific support for claims on foods.

Nevertheless, to date a huge number of unacceptable requests of authorisation to apply health claims on food has been proposed from stakeholders to the European Food Safety Authority (EFSA). The main concerns include the insufficient characterization of food/food constituent(s), the lack of beneficial physiological effect of the proposed claimed effect and, above all, the quality of the studies provided for the scientific substantiation of the claims. The most critical points involve the design and the strength of the studies provided in the application, including the proper choice of outcome variables and their methods of measurement. These points, together with others like a proper study design and an adequate sample size, represent the main pillars in the quality assessment of controlled intervention studies.

In this framework, a project has been developed with the aim of improving the quality of applications provided by stakeholders to EFSA, through an appropriate choice of outcome variables (OVs) and methods of measurement (MMs). It consists of six reports that deals with six main areas, as mentioned in the Guidance documents adopted by the Panel on Dietetic Products, Nutrition and Allergies. Such areas are: (i) protection against oxidative damage and cardiovascular health [3], (ii) post-prandial blood glucose responses/blood glucose control and weight management [4], (iii) bone, joints, oral and skin health [5], (iv) neurological and physiological functions [6], (v) gut and immune functions [7] and, (vi) physical performance [8].

The present manuscript gathers information in the context of protection against oxidative damage and cardiovascular health, reporting the results obtained from the collection, collation and critical analysis of the information in relation to claimed effects, OVs and MMs.

2. Strategy

The manuscript refers to OVs and MMs collected from the relative Guidance document (EFSA 2011), from the applications for authorization of health claims under Articles 13.5 and 14 of Regulation 1924/2006 related to oxidative damage and cardiovascular health (ec.europa.eu/nuh-claims/), as well as from comments received during public consultations. The OVs and their MMs were considered only if the food/food constituent(s) was sufficiently characterized and the claimed effect, suitably defined, provided a beneficial physiological effect. Following this decision tree, 11 claimed effects with 32 OVs were evaluated under Article 13.5, whereas 4 disease risk reduction claims and 1 claimed effect referred to children development were selected under the Article 14. For each OV, all the MMs proposed in the scientific opinions and/or in the Guidance documents were included in the evaluation. If no methods were proposed or any proposed method was considered inappropriate, also the best or the most widely used method was included. Subsequently, different databases of references were created on PubMed based on the keywords defined from each OV, in order to permit a specific critical analysis of the OVs and the MMs. The critical evaluation for each OV and MM was performed following a review of the literature deriving from the so obtained databases. Each OV and related MM was ranked in one of the following categories: (i) appropriate alone; (ii) appropriate only in combination with other OVs or MMs; (iii) not appropriate *per se*; (iv) not appropriate in relation to the specific claimed effect proposed by the applicant(s), (v) not appropriate alone, but useful as supportive evidence for the scientific substantiation of the claimed effect. In general, the index at the beginning of this paper lists the OVs and the respective MMs from the most appropriate to the least, when a ranking was applicable. The flow chart shown in Fig. 1 summarizes the strategy used for collection, collation and critical analysis of OVs and MMs.

3. Critical evaluation of outcome variables and methods of measurement

3.1. Claims falling under Art. 13(5)

3.1.1. Protection of cells against oxidative damage

3.1.1.1. Antioxidant enzymes The harmful effects of free radicals and peroxides are controlled *in vivo* by a wide spectrum of anti-oxidative defence mechanisms, including vitamins (e.g. E and C), carotenoids, other metabolites (e.g. glutathione) and antioxidant enzymes. These compounds represent a first line of defence against oxidative stress, acting through the removal of key reactive oxygen species (ROS), and play an important role in the human antioxidant network protecting cells and biomolecules from excessive oxidative damage. The main enzymes in this line of defence include superoxide dismutase (SOD, catalysing dismutation of the superoxide anion into H_2O_2), catalase (CAT, detoxifying H_2O_2) and glutathione peroxidase (GSH-Px, removing H_2O_2 and converting lipid peroxyl radicals to nontoxic alcohols) [9]. Low levels of active antioxidant enzymes promote oxidative stress, with increased endogenous formation of malondialdehyde (MDA) and isoprostanes (IsoPs) and trigger inflammatory processes, leading to endothelial dysfunction.

To evaluate the appropriateness of antioxidant enzymes as OV of protection of cells against oxidative damage, database #1 was generated (see Table 1).

Antioxidant enzymes have been measured as biological indicators of many pathological conditions, including hyperlipidaemia, atherosclerosis and diabetes. However, the antioxidant defence system is a complex network, with interactions and synergistic mechanisms that must be taken into account for a critical evaluation. In detail, when a single measure of antioxidant status is considered in isolation, it is often difficult to critically evaluate whether the food/food component up-regulates the antioxidant enzyme (to increase body's antioxidant protection), or it acts as a pro-oxidant agent (causing oxidative stress itself and forcing the body to up-regulate the antioxidant enzyme to protect itself against the pro-oxidative component).

In addition, antioxidant enzymes exhibit a wide inter-individual variability, which should be considered when they are measured to substantiate a possible health benefit linked to the intake of a specific food or food component.

On the basis of current literature, induction of antioxidant enzymes does not appear to be sufficient for the substantiation of health claims in the context of protection of cells against oxidative damage. However, antioxidant enzymes can provide a mechanistic explanation of the processes involved in this context, so they can be included as an additional variable in the framework of *in vivo* studies demonstrating protection of bio-molecules and cells from oxidative damage.

3.1.1.1.1. Enzymatic assays The most important parameter determining the biological impact of the antioxidant enzymes is their activity. The measurement of the activity of the antioxidant enzymes is traditionally performed by enzymatic assays and native gels [10]. The activity assay requires 10-fold more protein than the gel assays but has the advantage of giving a quantitative result; on the contrary, the native gel requires a lower amount of protein, but has the drawback of providing only qualitative results.

SOD activity can be measured by both activity assays and activity gels. In SOD assays, a xanthine-oxidase system is traditionally used to generate superoxide anions and a chromagen reduction is used as an indicator of $O_2^{\bullet-}$ production. SOD will compete with the chromagen for $O_2^{\bullet-}$ and the percent inhibition of chromagen reduction is a measure of the amount of SOD present.

CAT activity is instead commonly evaluated by a spectrophotometric procedure measuring peroxide removal, while GSH-Px can be measured using H_2O_2 and cumene hydroperoxide or tert-butyl hydroperoxide as the substrate.

Besides enzymatic assays, standardized immunohisto-chemical and immunofluorescence techniques are
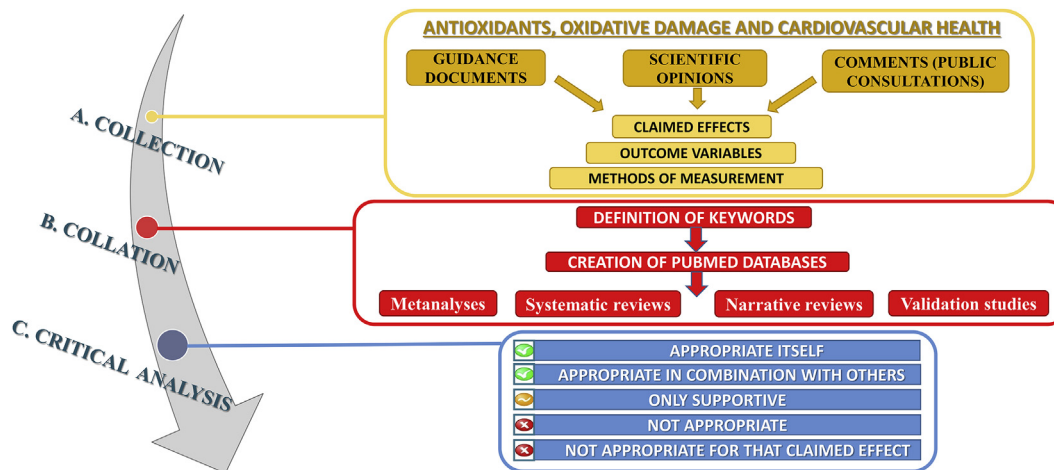
Figure 1   Strategy used for collection, collation and critical analysis of outcome measures and methods of measurement.

Table 1 Strategies used for retrieving the literature pertinent with outcome variables and methods of measurement under investigation.

DB Number	Syntax	Total Articles	Narrative reviews	Systematic reviews/metanalyses	Validation studies	Outcome variables
1	("superoxide dismutase"[mesh] OR "catalase"[mesh] OR "glutathione peroxidase"[mesh]) AND "humans"[mesh] AND "english"[language]	18574	1404	71	10	Antioxidant enzymes
2	"heme oxygenase"[all fields] AND "humans"[mesh] AND "english"[language]	4021	749	23	3	HO
3	"glutathione"[mesh] AND "humans"[mesh] AND "english"[language]	14911	1356	42	18	GSH
4	("dna breaks, single-stranded"[mesh] OR "dna breaks, double-stranded"[mesh]) AND "humans"[mesh] and "english"[language]	2969	458	10	8	DNA SBs
5	(("oxidized"[all fields] AND "dna base"[all fields]) OR ("dna-formamidopyrimidine glycosylase"[mesh] OR "ap endonuclease"[mesh])) AND "humans"[mesh] AND "english"[language]	372	36	1	4	Oxidized DNA damage
6	"isoprostanes"[mesh] AND "humans"[mesh] AND "english"[language]	1074	137	6	6	F2-IsoPs
7	"oxidized"[all fields] AND "cholesterol, ldl"[mesh] AND "humans"[mesh] AND "english"[language]	473	56	1	0	Oxidized LDL
8	"lipid peroxides"[mesh] AND "phosphatidylcholines"[mesh] AND "humans"[mesh] AND "english"[language]	60	3	0	0	PCOOH
9	"malondialdehyde"[mesh] AND "humans"[mesh] AND "english"[language]	7711	124	14	10	MDA
10	"amino acids"[mesh] AND "oxidized"[all fields] AND "humans"[mesh] AND "english"[language]	2124	184	0	4	Oxidative changes of amino acids in proteins
11	"protein carbonylation"[mesh] AND "humans"[mesh] AND "english"[language]	765	55	3	2	Protein oxidation by-products (e.g. protein carbonyls)
12	"cholesterol"[mesh] AND "humans"[mesh] AND "english"[language]	76129	7938	1068	125	- LDL-C - HDL-C - TC
13	"triglycerides"[mesh] AND "humans"[mesh] AND "english"[language]	35668	2350	365	71	- Fasting TG - Postprandial TG
14	"blood pressure"[mesh] AND "english"[language] AND "humans"[mesh]	134321	10609	1709	529	- Point SBP - Point DBP - 24 h SBP - 24 h DBP - AUC BP
15	"blood pressure"[mesh] AND "vascular stiffness"[mesh] AND "english"[language] AND "humans"[mesh]	747	46	9	7	Central SBP
16	"vasodilation"[mesh] AND "humans"[mesh] AND "english"[language]	12442	1970	93	27	- Endothelium dependent vasodilation - Endothelium independent vasodilation
17	"compliance"[mesh] AND "arteries"[mesh] AND "humans"[mesh] AND "english"[language]	1037	97	5	9	SAC
18	("nitrosylated"[all fields] OR "nitrosated"[all fields]) AND "humans"[mesh] AND "english"[language]	512	97	2	3	RXNO
19	("platelet aggregation"[mesh] AND ("agonists"[all fields] OR "adp"[all fields] OR "arachidonic acid"[all fields] OR collagen[all fields] OR "thrombin receptor activating peptide"[all fields])) AND "humans"[mesh] AND "english"[language]	9646	365	14	6	Percent of inhibition in platelet aggregation
20	"thromboxane a2"[mesh] AND "humans"[mesh] AND "english"[language]	2471	354	7	2	TXA2 Generation
21	"p-selectin"[mesh] AND "humans"[mesh] AND "english"[language]	3711	186	8	3	P-Sel
22	"homocysteine"[mesh] AND "humans"[mesh] AND "english"[language]	9251	1193	174	30	Homocysteine

Table 1 (*continued*)

DB Number	Syntax	Total Articles	Narrative reviews	Systematic reviews/metanalyses	Validation studies	Outcome variables
23	"venous reflux"[all fields] AND "ultrasonography, doppler"[mesh] AND "humans"[mesh] AND "english"[language]	207	8	2	0	Venous reflux
24	"popliteal vein"[mesh] AND "ultrasonography, doppler"[mesh] AND "humans"[mesh] AND "english"[language]	197	9	1	2	Popliteal vein diameter
25	"saphenous vein"[mesh] AND "ultrasonography, doppler"[mesh] AND "humans"[mesh] AND "english"[language]	642	30	8	3	Great saphenous diameter
26	"pulse wave analysis"[mesh] AND "vascular stiffness"[mesh] AND "english"[language] AND "humans"[mesh]	530	22	11	6	Arterial stiffness

AUC: Area under the curve; BP: Blood pressure; DBP: Diastolic blood pressure; DNA: Deoxyribonucleic acid; F2-IsoPs: F2-isoprostanes; GSH: Glutathione; HDL-C: High density lipoprotein cholesterol; HO: Heme oxygenase; LDL-C: Low density lipoprotein cholesterol; MDA: Malondialdehyde; Ox-LDL: Oxidized LDL; PCOOH: Phosphatidylcholine hydroperoxide; P-sel: P-selectin; RXNO: Nitroso/nitrosyl species; SAC: Systemic arterial compliance; SBP: Systolic blood pressure; SBs: Strand breaks; TC: Total cholesterol; TG: Triglicerydes; TXA2: Thromboxane A2.

commonly used to determine endogenous antioxidants in most laboratories. The antibodies used in these applications are commercially available and can be used on both fresh or fixed tissues and cells. Immunohistochemical analysis is another method for determining cell-specific antioxidant expression levels. However, it does not measure the activity of the enzyme and, because the protein can be expressed while remaining inactive (especially in disease conditions), immunohistochemical analysis is not the ideal method for measuring the antioxidant defence status.

On the basis of the current evidence, the enzymatic assay seems to be appropriate for measuring antioxidant enzymes.

3.1.1.2. Heme oxygenase Heme oxygenase (HO) is the rate-limiting enzyme in the metabolism of heme, catalysing the degradation of heme to biliverdin (rapidly converted to bilirubin), with the concurrent release of iron and CO. The system consists of two isozymes: HO-1(inducible) and HO-2 (constitutive).

There is a growing interest in the role of HO in diabetes, inflammation, heart disease, hypertension, neurological disorders, and other diseases.

To evaluate the appropriateness of HO as OV of protection of cells against oxidative damage, the literature deriving from database #2 was critically evaluated (see Table 1). HO is the enzyme responsible for physiologic heme degradation into equimolar amounts of CO and biliverdin, and releases free iron. The degradation of heme is considered a critical step in cellular defence, because of the pro-oxidant heme removal and the increased production of bilirubin and CO. It has been suggested that the inducible form HO-1 may represent a non-specific response to oxidative stress conferring protection against oxidative stress [11]. Moreover, the increase of HO-1 activity is correlated with the increase of CuZn SOD activity, suggesting a synergistic antioxidant action of the two enzymes.

On the basis of current data, the induction of HO does not appear appropriate for substantiation of health claims in the context of protection of cells against oxidative damage. However, it can provide a mechanistic explanation of the processes involved in this context, so it can be included as an additional variable in the framework of *in vivo* studies demonstrating protection of biomolecules and cells from oxidative damage.

3.1.1.2.1. Chromatographic techniques The interest in the function of the HO system has increased over the last few years, and measuring HO activity represents an important tool in understanding the functional significance of the enzyme.

The quantification of the enzyme activity is typically based on the detection of biliverdin or bilirubin and has been performed by applying spectrophotometric, HPLC and radiochemical methods [12].

The adequacy of measuring the rate of bilirubin production depends upon the presence of an excess of biliverdin reductase, needed to convert biliverdin to bilirubin. Despite several variants of the method have been proposed, the spectrophotometric approach has been generally criticized for its poor sensitivity and specificity, explained mostly by spectral interferences.

To by-pass these limitations, HPLC methods have been proposed, resulting in more sensitive and specific measures of the enzyme activity. With these methods, HO activity is generally calculated considering the rate of formation of bilirubin equivalents, by detecting both bilirubin and biliverdin at 405 nm using visible absorbance spectrometry.

However, this approach allows measuring one single product of HO activity, failing to detect iron or CO. In addition, the main disadvantage of the "measurement of products" approach is the impossibility to distinguish HO-1 (the inducible form) and HO-2 (the constitutive form), so the method clearly lacks specificity.

On the basis of the current evidence, and considering the drawbacks of the most commonly applied methods, measurement of HO-1 activity by chromatographic techniques, preferably HPLC-based, should be coupled with supplementary data related to protein and mRNA levels of the two different isozymes.

3.1.1.3. Glutathione Glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is a tri-peptide present in relatively high

levels in mammalian cells (1–10 mM). Micromolar concentrations are typically detected in plasma, while very high concentrations are present in erythrocytes.

GSH plays an important role in protecting cells and tissues against oxidative stress, by maintaining the intracellular redox balance and removing toxic compounds like free radicals and peroxides. The synthesis of GSH from its constituent amino acids involves the actions of glutamate cysteine ligase, the expression of which is mediated by the antioxidant response elements and by GSH synthetase. In order to counteract oxidative damage, one of the main mechanisms is the transactivation of genes encoding enzymes involved in GSH metabolism and synthesis.

In cells, GSH is free or bound to proteins. Free GSH is present mainly in its reduced form, which can be converted to the oxidized form (GSSG) in conditions of high oxidative stress, and can then be reverted to the reduced form by the action of reductase. Bound GSH is covalently bound to proteins through glutathionylation. The reduced form is generally predominating over the oxidized form, considering that GSSG is present at very low levels in physiological conditions. In pathological conditions (e.g. cardiovascular diseases, diabetes mellitus, rheumatoid arthritis, Alzheimer's diseases), as well as in physiological ageing, a higher proportion of GSH is present in its oxidized form, resulting in a lower cellular GSH/GSSG ratio. For this reason, in addition to GSH, the GSH/GSSG ratio is often measured and considered an indicator of the overall redox state of the cell [13].

To evaluate the appropriateness of GSH as OV of protection of cells against oxidative damage, the literature obtained from database #3 was critically evaluated (see Table 1).

GSH levels reported in the literature may exhibit a 10-fold variation and errors may arise during GSH determination (e.g. GSH autoxidation or haemolysis causing overestimation in view of the very high GSH levels in erythrocytes). In addition, as already observed for other outcome variables of antioxidant status (i.e. antioxidant enzymes), when a single measure of antioxidant status is considered in isolation, it is often difficult to critically evaluate if a given food/food component is really able to up-regulate the endogenous antioxidant machinery thus conferring antioxidant protection to the body. Actually, the effect could be pro-oxidant to some extent, causing oxidative stress itself and forcing the body to up-regulate endogenous antioxidant responses as a reaction toward the increased oxidative stress. On the basis of these considerations, the use of GSH alone does not appear appropriate for the substantiation of health claims in the context of protection of cells against oxidative damage. However, as observed for other outcome variables (e.g. antioxidant enzymes, HO), it can provide a mechanistic explanation of the processes involved in this context and it could be included for the evaluation process, in addition to *in vivo* studies demonstrating increased protection of biomolecules and cells from oxidative damage.

A similar conclusion can be made for the GSH/GSSG ratio. This ratio reflects changes in the cellular redox status and is not a direct biomarker of oxidative damage, as it has been reported to decrease without a parallel increase of oxidized lipids or proteins.

3.1.1.3.1. Chromatographic techniques Several analytical methods for the measurement of GSH are available. The measurement of GSH in biological samples requires caution to prevent artifacts or data misinterpretation. An important limitation of many methods is the artefactual auto-oxidation of GSH to GSSG, which occur mostly during sample deproteination by strong acids. Non-separative methods, generally based on spectrophotometric or fluorimetric measurements of products formed in the reaction of GSH with thiol-reacting molecules, were the first to be developed for the measurement of GSH. In spite of their simplicity and low cost, these assays often lack specificity, sensitivity and reproducibility.

For these reason, new methods have been proposed with increased specificity, mostly owing to reduced artefactual auto-oxidation of GSH. Progress has been made by applying separation steps through HPLC prior to detection. HPLC with fluorescence (FL), ultraviolet (UV) and electrochemical detection (ECD) are widely used because of their convenience, sensitivity, and selectivity [13]. However, most of these methods require sample pre-treatment for the exact measurement of GSH and GSSG level. This is especially important in view of the fact that GSH concentration is 500-fold higher in erythrocytes than in plasma and that haemolysis may cause overestimation in plasma samples. HPLC coupled with ECD has some advantages, allowing the simultaneous determination of GSH and GSSG without prior treatment or derivatization. More recently, mass spectrometric (MS) detection has been proposed for the quantification of blood GSH and GSSG contents, once again without derivatization steps, owing to the sensitivity and selectivity of the method [14]. The comparison between methods is often difficult, especially because of the high variations of GSH levels reported in the literature (partially due to errors arising from different phases of GSH determination) and because GSH and GSSG can be expressed relative to different parameters (e.g. tissue weight, cell number).

However, on the basis of current evidence, HPLC methods employing electrochemical or MS detectors can be properly used for the measurement of GSSG and GSH in biological fluids.

3.1.2. Protection against generic DNA damage
3.1.2.1. DNA strand breaks DNA strand breaks (SBs) are recognized as single SBs or double SBs resulting from any DNA lesion due to exposure to endogenous and/or exogenous (e.g. ionizing radiations) DNA damaging agents, including incomplete DNA repair process. Both types of DNA SBs may cause an alteration in DNA properties and may induce anomalies during DNA replication and translation. For these reasons, DNA repair is essential for the maintenance of cell functioning and survival.

To evaluate the appropriateness of spontaneous DNA SBs as OV of generic DNA damage, the literature deriving from database #4 was critically evaluated (see Table 1).

DNA integrity is clearly crucial for the maintenance of normal cell function. DNA is the storehouse of genetic information in living cell, and its integrity and stability are essential to life.

It is widely recognised that DNA damage, mainly in the event of double SBs, plays a major role in mutagenesis, carcinogenesis (e.g. in chromosomal rearrangements and deletions, and in mitotic recombination in somatic cells), and ageing. SBs may produced by several exogenous agents, such as ionizing radiation, but also occurs spontaneously during cellular processes. Oxidative DNA damage is mostly caused by ROS generated during metabolic endogenous processes makes a significant contribution to SB formation. To repair this damage, eukaryotic cells have evolved a variety of repair mechanisms, collectively known as non-homologous DNA end joining.

As DNA SBs may be induced by several factors, not only by oxidative processes, this outcome variable cannot be considered appropriate for the substantiation of health claims in the context of protection against oxidative damage to DNA. However, as spontaneous DNA SBs are associated with an altered cell function spontaneous DNA SBs are appropriate for the substantiation of health claims in the context of protection against generic DNA damage.

3.1.2.1.1. Comet Assay The Comet Assay is a very simple, fast and cheap technique for the detection of oxidative and non-oxidative DNA damage in single eukaryotic cells. In its traditional version, the alkaline Comet Assay detects DNA migration caused by SBs, alkaline labile sites, as well as repair sites. Individual cells are embedded in a thin agarose gel on a microscope slide. A lysis step removes all cellular proteins from the cells. DNA is then allowed to unwind under alkaline/ neutral conditions, and subjected to electrophoresis during which damaged DNA migrates away from the nucleus. The DNA is then stained with a DNA-specific fluorescent dye, and the gel is read for the amount of fluorescence in head and tail. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage. The term "comet" refers to the image of DNA migration obtained through the electrophoresis gel, which often resembles a comet. The traditional Comet Assay can be appropriately applied to measure spontaneous DNA SBs. However, owing to the lack of specificity for oxidative damage, it is not appropriate for evaluating *in vivo* oxidative damage to DNA. The same consideration can be made for other variants of the Comet Assay, which determine resistance against oxidative modification using *ex vivo* pro-oxidant challenges (e.g. H_2O_2). On the other hand, specific oxidized bases can be readily detected using the Comet Assay by incorporating in the assay (after cell lysis) a step in which DNA is incubated with a lesion-specific enzyme that converts the altered bases (purines or pyrimidines) to SBs. The most common enzymes employed for this purpose are formamidopyrimidine DNA glycosilase (FPG), which recognizes and removes oxidatively damaged purines (e.g., 8-oxo-7,8-dihydroguanine) and endonuclease III (Endo III) for pyrimidines. This assay directly reflects DNA oxidative damage within cells (e.g. circulating lymphocytes), differently from the traditional method. Although absolute values cannot be obtained from the assay, quantitative assessments are possible if appropriate controls are included in the analysis.

Differently from *in vitro* studies, where the Comet Assay has been extensively and successfully used, experience with *in vivo* studies is limited. However, current data show that this methodology also works *in vivo*, although a high inter-laboratory variability has been reported when using lesion-specific enzymes [15].

Validation studies have shown that the Comet Assay using lesion-specific enzymes has high intra-laboratory reproducibility. On the other hand, it is still difficult to make comparisons between different laboratories as there is no accepted standard protocol and there are considerable differences on how protocols are implemented by different research groups. Nevertheless, in the framework of a randomized controlled trial (RCT), the high intra-laboratory reproducibility guarantees the consistency of the results, making the Comet Assay appropriate, if performed with Endo III and FPG, for the evaluation of DNA damage of oxidative nature (see Section 3.1.3.1). On the other hand, the traditional Comet Assay can be appropriately used for the evaluation of the non-oxidative DNA damage represented by spontaneous DNA SBs.

3.1.3. Protection against oxidative damage to DNA

3.1.3.1. Oxidized DNA bases Oxidized DNA bases are one of the main products of oxidative DNA damage. Although more than 20 base lesions have been identified, the most studied lesion is 8-oxo-2-deoxyguanosine (8-oxo-dG), which is produced by keto−enol tautomerism of 8-hydroxy-2-deoxy-guanosine (8-OHdG).

8-oxo-dG has been widely studied because it is produced *in vivo* and can be measured in cells following the hydrolysis of DNA to its bases. In particular, urinary 8-OHdG has been frequently chosen to evaluate oxidative damage because it is a non-invasive and technically less demanding variable.

To evaluate the appropriateness of oxidized DNA bases as OV of oxidized damage to DNA, the literature deriving from database #5 was critically evaluated (see Table 1).

The production of 8-OHdG is a function of both oxidation and excision repair of DNA, and can result from the oxidation of free bases or nucleotides or from oxidation of other nucleic acids.

The measurement of 8-OHdG is convenient, but the fact that the analyte can originate from different processes not directly reflecting DNA oxidation within cells, should be carefully considered [16]. For instance, when the role of agents that increase the repair activity is investigated, an increase of 8-OHdG may be mistaken for an increased oxidative damage. In conclusion, the use of 8-OHdG as biomarker of oxidative stress should be considered with caution and limited to support results of

direct measurements of oxidative damage to DNA. Therefore, 8-OHdG, and oxidized DNA bases in general, are not appropriate to be used alone for the substantiation of health claims in the context of protection against oxidative damage to DNA. However, they could be used as supportive evidence.

3.1.3.1.1. Chromatographic techniques The measurement of 8-OHdG in blood, tissues and urine is commonly used as surrogate marker of oxidative damage to DNA. The most widely used methods of quantitative analysis are HPLC-ECD, gas chromatography(GC)-MS, and liquid chromatography (LC)-MS/MS.

To analyse 8-OHdG in tissues and lymphocytes, 8-OHdG has to be released from the nuclear DNA into a soluble compound with enzymes (e.g. P1 nuclease and alkaline or acid phosphatase) before it can be quantified by HPLC or LC-MS/MS. GC-MS is indeed generally performed with samples hydrolysed to bases by incubation with formic acid and derivatized by trimethylsilylation. HPLC has also been used to determine free 8-OHdG in plasma and urine without going through enzymatic digestion. However, the HPLC procedure often includes complex extraction and separation steps for 8-OHdG isolation. From many years, HPLC-ECD is the most frequently used method for the measurement of 8-OHdG in tissues, lymphocytes and plasma. It has the ability to measure several oxidized products at the same time. Amperometric detection is less sensitive than colorimetric detection, and is not appropriate for measuring low levels of damage [17]. Among the other methods, GC-MS has generally high coefficients of variation and LC-MS/MS shows poor linearity. On the basis of the current evidence, HPLC appears the most appropriate method for the analysis of 8-OHdG.

3.1.3.1.2. Comet Assay Please refer to 3.1.2.1.1 "Comet Assay".

3.1.4. Protection against oxidative damage to lipids

3.1.4.1. F2-isoprostanes F2-isoprostanes (F2-IsoPs) are a relatively newly discovered class of molecules, originating from oxidation of polyunsaturated fatty acids. They include a series of prostaglandin F2a-like compounds produced *in vivo* by non-enzymatic peroxidation of arachidonic acid, esterified in phospholipids and subsequently hydrolysed to a free acid form by platelet-activating factor acetylhydrolase [18]. F2-IsoPs are released from the cell membrane into circulation by phospholipases and can be quantified in all human tissues and biological fluids, including plasma and urine. A high level of F2-IsoPs has been observed in a number of conditions associated with increased oxidative stress, including diabetes and neurodegenerative and pulmonary disease.

Compared to other markers of oxidative damage, F2-IsoPs are very specific and chemically stable end-products of polyunsaturated fatty acid peroxidation, and, for this reason, are the most well studied markers of oxidative stress [19]. Among the F2-IsoPs isomers, 8-iso-15(S)-prostaglandin F2α has very high concentration in biological fluids and is the most commonly evaluated.

To evaluate the appropriateness of F2-IsoPs as OV of oxidized damage to lipids, the literature deriving from database #6 was critically evaluated (see Table 1).

F2-IsoPs are advantageous over other markers of lipid peroxidation due to their stability and detectability in a variety of human tissues and biological fluids (e.g. plasma and urine). In detail, F2-IsoPs can be measured as free IsoPs in biological fluids, as esterified forms in specific target sites, and as major urinary metabolites. It has been reported that F2-IsoPs levels have some diurnal variations, possibly due to variations in the oxidative stress, so it essential to take this factor into account in human studies (e.g. sampling many time-points during the day or night) [20]. On the basis of the current evidence, quantification of F2-IsoPs in urine is considered the most accurate and robust measurement of their presence within the body and it has the advantage of being a non-invasive method of assessment.

The accuracy and robustness of measuring F2-IsoPs support their appropriateness in the substantiation of health claims in the context of protection against oxidative damage to lipids.

3.1.4.1.1. Chromatographic techniques Many chromatographic-based techniques have been used to separate and detect F2-IsoPs and their metabolites in biological fluids, such as plasma and urine.

The procedures used for measuring F2-IsoPs by GC/MS are usually very laborious due to the derivatization step before GC separation [21]. In detail, IsoPs are typically converted to pentafluorobenzyl esters by treatment with pentafluorobenzyl bromide. This step makes the technique time-consuming and can lead to artifacts, but the approach is nonetheless highly specific and sensitive.

LC combined with mass spectrometry (HPLC-MS or HPLC/MS-MS) is becoming increasingly employed by many laboratories, being suitable for poorly volatile compounds without the requirement of a derivatization step [22]. This methodology makes sample preparation easier, reducing artifacts and increasing analytical throughput.

On the basis of the current evidence, the analysis of F2-IsoPs in 24-h urinary samples by HPLC-MS or GC/MS is the most appropriate method for evaluating lipid damage in biological fluids. However, the method requires quite expensive instruments.

3.1.4.1.2. Immunological techniques F2-IsoPs can be measured using immunoassays, which have expanded IsoPs research due to their low cost and relative ease of use. Immunological techniques that allow a very rapid analysis of biological samples have been developed to facilitate routine cost-effective analyses of several bio-markers. Although immunoassay methods are easier and cheaper, there is controversy about their specificity and correlation with the accepted chromatographic methodology [22]. In detail, a lack of specificity, owing mostly to cross-reactions with other prostanoids, has been observed and should be taken into account when these assays are used to support the substantiation of a health claim related to lipid damage.

Studies comparing immunological methods to GC/MS have shown at least 30-fold higher levels with

immunoassays, with no clear correlation with GC/MS [21]. Part of this difference may be because immunoassay methods, while being able to discriminate against most isomers, are unable to prevent cross-reactivity with all or some of their metabolites.

On the basis of these considerations, more studies are required comparing immunoassay methods to MS or more specific and accurate enzyme-linked immunosorbent assay (ELISA) kits need to be developed. Until then, the use of immunological techniques in measuring F2-IsoPs remains questionable.

3.1.4.2. Oxidized low-density lipoprotein

The term "Oxidized low-density lipoprotein" (ox-LDL) generally refers to a particle derived from circulating LDL that may have undergone oxidation toward one or both its main components, namely apoprotein and lipids. This oxidative modification may originate directly from radical molecules or through the action of lipid peroxidation end-products like aldehydes. Ox-LDL appear implicated in atherogenesis, but the exact mechanisms involved in the process still need to be unravelled [23]. In general, their presence at high levels seem to contribute to a wide range of atherosclerosis steps, from early lesion formation to plaque rupture. They have been reported to exert direct cytotoxic effects on endothelial cells, to increase chemotactic properties of monocytes, to trigger increased expression of growth factors, to modulate transformation of macrophages to foam cells via scavenger-receptors, to stimulate platelet adhesion and aggregation, and to enhance proliferation of various cell types, such as endothelial cells and monocytes [24].

To evaluate the appropriateness of ox-LDL as OV of oxidized damage to lipids, the literature deriving from database #7 was critically evaluated (see Table 1).

LDL oxidation has been recognized as playing a pivotal role in the initiation and progression of atherosclerosis. However, despite the pathophysiological rationale, the oxidative modification hypothesis of atherosclerosis is still debated.

For instance, foam cell formation can occur also in the presence of native LDL, and advanced human endothelial lesions may contain high concentrations of antioxidants, such as vitamin E and vitamin C. This fact is inconsistent with the hypothesis that the oxidation of LDL occurs in case of complete depletion of antioxidants.

Human studies evaluating the association of ox-LDL with atherosclerosis or cardiovascular events have been highly conflicting, and many clinical trials failed to prove the oxidative theory of atherosclerosis.

Despite the absence of a convincing link between the plasma levels of ox-LDL and atherosclerosis onset or development, in the framework of the function claims, ox-LDL appears to be an appropriate outcome variable for the substantiation of health claims related to the reduction of oxidative damage to lipids.

3.1.4.2.1. Immunological techniques

The assessment of human circulating ox-LDL has become common after the introduction of several ELISA procedures, based on sandwich and competitive assays, partly because of the availability of commercial kits.

Extensively ox-LDL may have a very short half-life in human plasma, because they are rapidly cleared from the circulation via scavenger receptors. However, small but significant amounts of ox-LDL are detectable in normal plasma using specific monoclonal antibodies. However, some studies reported that slight variations in the procedure were associated with substantial differences in the results [25].

On the basis of the current evidence, immunological methods can be applied for measuring ox-LDL, but great care should be used when evaluating the characteristics of a given assay, considering that even small variations in sensitivity and specificity may have profound effects on the obtained results.

3.1.4.3. Phosphatidylcholine hydroperoxides

Phosphatidylcholine hydroperoxide (PCOOH) may be a sensitive and specific index of lipid peroxidation in vivo. In detail, PCOOH is a primary oxidation product of phosphatidylcholine (PC), a major component of phospholipids in plasma and cell membranes. Accumulation of PCOOH in blood plasma has been observed in atherosclerosis. Quantitative analysis of plasma PCOOH is an important step in evaluating the biochemical processes leading to oxidative injury.

To evaluate the appropriateness of PCOOH as OV of oxidized damage to lipids, the literature deriving from database #8 was critically evaluated (see Table 1).

Plasma PCOOH can be used as a general indicator of lipid peroxidation and increased levels of PCOOH may reflect in vivo oxidative stress or oxidative damage to organs. Therefore, quantitative determination of lipid peroxides like PCOOH in plasma represents an important step in the evaluation of the biochemical processes leading to oxidative damage.

When evaluating lipid damage, the effect of the antioxidant systems in plasma should also be taken into account, considering that it has been reported that GSH-Px can directly reduce the concentrations of phospholipid hydroperoxide [26]. On the basis of the current evidence, PCOOH in blood or tissue is a sensitive and specific index of lipid peroxidation in vivo and an appropriate marker of lipid peroxidation to be used for the substantiation of health claims in the context of protection against oxidative damage to lipids. However, the combined analysis of PCOOH and F2-IsoPs (markers of polyunsaturated fatty acid oxidation) is preferable.

3.1.4.3.1. HPLC

Increased interest in lipid peroxidation has accelerated the development of techniques for the quantification of lipid peroxides in biological fluids. To this aim, many methods have been developed, mainly using liquid chromatography, which separates molecules in solution using their different affinity between a liquid mobile phase and a solid stationary phase. One of the most precise and accurate methods for the detection of lipid hydroperoxides is a chemiluminescence-based high-performance liquid chromatography assay (CL-HPLC). CL-HPLC

systems were developed and applied for the hydroperoxide-specific determination of PCOOH in biological tissues, such as human blood plasma. This approach involves the separation of PC from total plasma lipids with normal-phase silica gel HPLC and post-column detection of the hydroperoxide-dependent chemiluminescence of PCOOH. The method in use appear to be highly sensitive and specific for lipid hydroperoxides [27].

On the basis of current evidence, CL-HPLC appears to be a reliable and appropriate technique for measuring PCOOH in blood or tissues.

3.1.4.4. Malondialdehyde MDA is one of the main aldehydes that can be formed as secondary products of lipid peroxidation. In detail, the main source of MDA in biological samples is the peroxidation of polyunsaturated fatty acids with two or more methylene-interrupted double bonds. Several hypotheses describing the formation of MDA *in vivo* have been proposed. MDA has been reported to be able to generate cross-links in DNA and proteins with severe biological effects, potentially contributing to the pathogenesis of several chronic diseases, including cancer and atherosclerosis [28].

To evaluate the appropriateness of MDA as OV of oxidized damage to lipids, the literature deriving from database #9 was critically evaluated (see Table 1).

For many years, MDA has been used as a biomarker of lipid oxidation. However, its validity has been widely criticized for problems with post sampling formation and for the lack of specificity, both from biological and methodological perspectives [28,29]. As the biological perspective is concerned, MDA is not just a marker of lipid peroxidation.

Despite quite sensitive methods have been developed to assess MDA, the wide variability of values reported in the literature suggest that it should be considered a "relative" rather than an "absolute" marker of lipid oxidation, and that it should be applied to compare groups only within the same study and with the same method. On the basis of current evidence, similarly to other outcome variables (e.g. lipid peroxides, conjugated dienes), MDA is not a reliable marker of *in vivo* lipid peroxidation and therefore is not appropriate to be used alone for the substantiation of health claims in the context of protection against oxidative damage to lipids. However, concentrations of MDA in blood or tissue could be used as supportive evidence when appropriate techniques are used for analysis.

3.1.4.4.1. Chromatographic techniques Since the 60s, several methods have been developed to assess MDA, in order to quantify the level of oxidative stress *in vivo* and *in vitro*. Due to their simplicity and low cost, these methods are still being used in many laboratories worldwide.

Among these methods, the simplest and most frequently used is the thiobarbituric acid (TBA) assay, developed on the basis of MDA derivatization with TBA. However, the measured absorbance or fluorescence corresponds to a range of products (i.e. thiobarbituric acid reactive substances) in addition to MDA, because several other molecules react with TBA and absorb or fluoresce at similar wavelengths. The result is often an overestimation of MDA [28].

On the other hand, the use of HPLC or GC in repeated-measure studies appears to be appropriate for the detection and quantification of MDA derivatives, due to high specificity and sensitivity. A number of HPLC methods have been developed based either on direct measurement of MDA or MDA-adducts or detection from UV/Vis and FL instruments. Several methods have been proposed for quantifying both free MDA (unbound, detected without any hydrolytic sample treatment) or total MDA (bound to matrix molecules, requiring a hydrolytic step). The chromatographic approach is more advisable, as it allows a significant improvement of the specificity, including a step where the MDA-TBA adduct is quantified without confounding [30].

On the basis of current evidence, in spite MDA is not a reliable *in vivo* marker of lipid peroxidation, chromatographic techniques are appropriate for the measurement of MDA.

3.1.5. Protection against oxidative damage to proteins

3.1.5.1. Oxidative changes of amino acids in proteins Proteins represent one of the major targets for oxidative damage, a fact that is attributable to their abundance in cells as well as to their rapid rates of reaction with many radicals. Protein oxidation can be defined as the covalent modification of a protein induced directly by ROS as well as by reactions with secondary by-products of oxidative stress, leading to many functional consequences [31]. Amino acids in proteins are highly susceptible to oxidation by one or more ROS that may be present as pollutants in the atmosphere, generated as by-products of normal metabolic processes, or formed during exposure to X-, λ-, or UV-radiations.

Considering that many mechanisms might be involved in the induction of protein oxidation and because all of the amino acyl-side chains can be oxidatively modified, many products of oxidative modification of amino acids have been reported in the literature. However, cysteine and methionine, both containing susceptible sulphur atoms, are by far the most sensitive to oxidation from all types of ROS. The main products of oxidative modifications of these amino acids are disulphide bonds, mixed disulphides, thyil radicals (for cysteine), and methionine sulfoxide [31,32].

To evaluate the appropriateness of oxidative changes of amino acids in proteins as OV of oxidized damage to proteins, the literature deriving from database #10 was critically evaluated (see Table 1).

The ability to quantify distinct amino acid oxidation products in plasma or serum is a valuable tool for exploring the roles of different oxidation pathways in the pathogenesis of atherosclerosis and other diseases [31,33].

The products of oxidative modifications to proteins are relatively stable and can serve as suitable markers for measuring the oxidative damage to proteins or, better, the balance between oxidative and anti-oxidative processes involving proteins [31]. Therefore, on the basis of the current evidence, the direct measurements of oxidative damage to proteins *in vivo*, such as the measurement of

oxidative changes of amino acids, is appropriate for the substantiation of health claims in the context of protection against oxidative damage to proteins.

3.1.5.1.1. Chromatographic techniques Many methods have been developed for the detection and quantification of oxidative changes of amino acids in proteins [31]. These methods can be classified on the basis of the molecular target, which can be the loss of the parent material, the appearance of intermediate species, or the products of oxidation reactions. The common approach to detect and quantify changes in parent amino acid residues involves the hydrolysis of proteins to their constituent amino acids and their derivatization with o-phthaldialdehyde, followed by analysis via reversed-phase HPLC. This approach allows the simultaneous quantification of many protein oxidation products, like methionine sulfoxide [34]. For the measurement of cysteine residues, the detection of the thiol group of protein-bound residues is typically performed by HPLC, preferably coupled with MS. The combination of the physical separation capabilities of HPLC with the mass analysis capabilities of MS allows an increased specificity.

On the basis of current evidence, the use of chromatographic techniques with MS detection is appropriate for the *in vivo* measurement of oxidative changes of amino acids in proteins [31].

The main advantage of this approach is that it allows the separation of the molecules of interest from other substances, especially in plasma. However, it is important to take into account the artefacts that may occur during sample preparation and analysis, which can influence the accuracy of the measurement.

3.1.5.2. Protein oxidation by-products (e.g. protein carbonyls)
Oxidative damages to proteins, lipids, or DNA are all deleterious and may occur concomitantly [35]. However, proteins are possibly the most immediate vehicle for inflicting oxidative damage to cells, because they are more often catalysts rather than stoichiometric mediators. CO groups (aldehydes and ketones) are produced on protein side chains when they are oxidized. Protein carbonyls are therefore the biomarker that is generally used to estimate protein oxidation. These moieties are chemically stable, a useful feature for both sample storage and analytical detection [36]. Protein carbonyl derivatives can also be generated through oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains, leading to formation of peptides in which the N-terminal amino acid is blocked by an α-ketoacyl derivative [35]. Lysine, arginine, proline, and histidine are the amino acid residues most prone to form carbonyl derivatives.

To evaluate the appropriateness of protein oxidation by-products as OV of oxidized damage to proteins, the literature deriving from database #11 was critically evaluated (see Table 1).

Protein carbonyls are the most commonly measured products of protein oxidation in biological samples. However, they can be better regarded as a measure of the balance between oxidative and anti-oxidative processes involving proteins [35,36]. Their use as a measure of

oxidative damage may help identifying individuals at risk of developing diseases associated with oxidative damage to proteins [37]. However, there is a considerable variability in the basal levels of protein carbonyls in some tissues, also depending on how the carbonyl assay is performed, and the specificity is related to the tissue/sample used in the analysis [35,36]. Even if this bias would not be relevant in the framework of a RCT, many interfering factors, changing irrespectively of oxidative stress, could invalidate the use of protein carbonyls. Therefore, based on current data, the measurement of protein oxidation by-products, such as protein carbonyls, should be used only in combination with direct markers of oxidative damage to proteins *in vivo* for the substantiation of health claims in the context of improved protection against oxidative.

3.1.5.2.1. ELISA methods In the late 90s, an ELISA method using an anti-dinitrophenyl (DNP) antibody was developed for measuring total protein CO groups. In its original version, the protein sample reacts with dinitrophenylhydrazine (DNPH) and is then adsorbed to the wells of an ELISA plate before reacting with anti-DNP antibody [38].

A limit of the ELISA test is that it requires expensive and specialized equipment. Moreover, similarly to the spectrophotometric assay, it does not provide any information on the extent of oxidative damage to a particular protein in a complex mixture. The ELISA test has however some important advantages, such as the requirement of very small amounts (about 60 μg) of protein (similar to HPLC), compared with the higher amounts (10 mg) required from the colorimetric assay. This has facilitated the use of the ELISA method when only limited amounts of protein were viable for analysis. In addition, the ELISA test is easier to use, less labour-intensive, and handles more samples per day than the colorimetric assay. Moreover, some studies have shown a good correlation with the classical colorimetric assay [34,35].

In conclusion, ELISA methods appear to be appropriate for measuring protein oxidation by-products.

3.1.5.2.2. Colorimetric procedures Many assays are currently available for the detection of protein carbonyls (e.g. carbonyl derivatives of proline, arginine and lysine), the conventional one being a colorimetric assay involving DNPH derivatization of carbonyl groups, which leads to the formation of a stable DNP hydrazone product [35,36]. Considering that the DNP group itself absorbs UV light, the total carbonyl content of a protein or mixture of proteins can be obtained by a spectrophotometric assay quantifying the hydrazones at 370 nm [34]. This assay does not require expensive or specialized equipment, has been shown to be very sensitive for the quantification of carbonyl, and is useful to quantify carbonyl content in mixtures of proteins, such as plasma; however, it is unreliable in protein extracts that contain high amounts of chromophores that absorbs at 370 nm (e.g. haemoglobin).

However, this essay does not provide any information on the extent of oxidative damage to a particular protein in a complex mixture. In addition, this method requires a

relatively high amount of proteins, is time-consuming and has high variability owing to many washing steps. Moreover, the quantitative derivatization requires a large amount of reagents.

The spectrophotometric DNPH assay can be coupled to protein fractionation by HPLC. This method provides a more quantitative information than the simple spectrophotometric assay and is a highly sensitive technique [37]. Its drawback lies in the protein mixture fractionation made by HPLC, which cannot completely separate proteins of close molecular weights [35,36].

The spectrophotometric DNPH assay coupled to protein fractionation by HPLC is far more sensitive than the spectrophotometric method alone, implying smaller quantities of the sample for the quantification of carbonyl content [39,40].

In conclusion, the colorimetric procedures do not appear to be appropriate for measuring protein oxidation by-products to be used for the substantiation of health claims related to the protection of proteins against oxidative damage.

3.1.6. Improvement of blood lipid profile

3.1.6.1. Low density lipoprotein-cholesterol   As other fat-like substances, cholesterol needs specific proteins (lipoproteins) to be carried in the blood stream. Lipoproteins are usually classified on the basis of their density. In detail, from the lowest to the highest density, lipoproteins are classified as chylomicrons (CM), very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL).

The lipoproteins have different affinities for and behave differently with the transported cholesterol. A LDL-C particle is a microscopic micelle whose outer rim is made of lipoprotein and whose centre is made of cholesterol. Elevated levels of LDL-cholesterol (LDL-C) in the blood are associated with an increased risk of atherosclerosis and cardiovascular diseases (CVD) mostly because: 1) LDL-C tend to deposit into the walls of arteries; 2) The white blood cells try to digest LDL-C inside the arterial wall and in doing so they oxidize it and make it more toxic; 3) LDL-C attract other inflammatory cells inside arterial wall, creating persistent inflammation [41–50].

To evaluate the appropriateness of LDL-C as OV of blood lipid profile, the literature deriving from database #12 was critically evaluated (see Table 1).

LDL-C usually represents up to 60–70% of total serum cholesterol. The main protein found in LDL is apolipoprotein-B which contains a binding site that causes LDL to be deposited in the extracellular matrix of many tissues. LDL-C infiltration, accumulation and oxidation in the intima of endothelial vessels is one of the early and main features in the atherosclerotic process [45,46,48–51]. LDL oxidation is a prerequisite for macrophage uptake via unregulated macrophage scavenger receptors to form foam cells which accumulate in the plaque lipid core [52]. LDL particle size may also be important when assessing risk but its evaluation is not currently endorsed by clinical guidelines [53]. In conclusion, the measurement of blood LDL-C concentration

appears to be appropriate for the substantiation of health claims in the context of improved lipid profile.

3.1.6.1.1. Enzymatic assays   A number of methods are available for the determination of LDL-C. The enzymatic assay involves the dissolution of non-LDL lipoprotein particles (HDL, VLDL and CM) but leaves LDL particles intact. The degradation of the dissolved cholesterol fraction is then carried out using two enzymes (cholesterol esterase and cholesterol oxidase). The remaining LDL particles are then solubilized and the soluble cholesterol and cholesterol esters are oxidized by cholesterol oxidase to produce hydrogen peroxide (H_2O_2). There are two types of probes for the enzymatic determination of H_2O_2: chromogenic and fluorogenic. In general, chromogenic probes are less sensitive than fluorogenic probes. In some studies, LDL-C has been calculated using Friedewald formula (LDL-C = Total cholesterol - HDL-C - triglycerides/2.2 if concentrations are given in mmol/L, or LDL-C = Total cholesterol - HDL-C - triglycerides/5) if given in mg/dL [54]. The use of this formula is associated with some problems: 1) methodological errors may accumulate because the formula requires three separate analyses of total cholesterol (TC), triglycerides (TG), and HDL-C; 2) a constant cholesterol/TG ratio in VLDL is assumed, which is not always true 3) blood must be obtained under fasting conditions, which is sometimes unpractical. For these reasons, many guidelines discourage the use of Friedewald formula [55,56]. On the basis of current evidence, the enzymatic assay seems to be appropriate for measuring blood LDL-C and can be used for the substantiation of health claims in the context of improved blood lipid profile and of reduced risk of coronary heart disease (CHD) (see Section 3.2.1.2).

3.1.6.2. High density lipoprotein-cholesterol   HDL contain cholesterol, phospholipids and TG. HDL has been extensively investigated because it extracts cholesterol from tissues and delivers it to the liver, where it may be converted into bile acids and excreted (reverse cholesterol transport).

To evaluate the appropriateness of HDL-C as OV of blood lipid profile, the literature deriving from database #12 was critically evaluated (see Table 1).

HDL-C seems to exert cardioprotective effects, mainly by promoting reverse cholesterol transport, a process by which excess cholesterol is delivered from peripheral tissues back to the liver. Specifically, cholesterol efflux from macrophages to HDL-C might play an important role in protecting from the development and progression of atherosclerosis [47,57]. HDL composition can be modified by oxidative mechanisms, which reduce the protein's ability to promote reverse cholesterol transport. In addition, recent studies have suggested to include the evaluation of HDL function, together with HDL levels, to fully define the cardioprotective potential of this class of lipoproteins [43–46,49,57–61]. In conclusions, in the framework of function claims, HDL-C can be considered an appropriate outcome variable for the substantiation of health claims in the context of improved lipid profile.

3.1.6.2.1. Enzymatic assays   The concentration of HDL-C may be determined enzymatically with an assay that

quantifies both cholesterol esters and free cholesterol [62]. Most of the available assays are of high quality, but the method used should be validated against the available reference methods. During the procedure, serum samples react with polyethylene glycol and all the VLDL and LDL are precipitated, while HDL remains in the supernatant. Cholesterol esters may be hydrolysed via cholesterol esterase into fatty acids and free cholesterol, making it possible to detect separately the two types of molecules in the presence and absence of cholesterol esterase. Cholesterol is then oxidized by cholesterol oxidase into the ketone cholest-4-ene-3-one, producing hydrogen peroxide. In the presence of peroxidase, the generated hydrogen peroxide reacts to form a photometrically measurable product. There are two types of probes for the enzymatic determination of H_2O_2: chromogenic and fluorogenic. The intensity of absorbance or fluorescence is proportional to cholesterol concentration and is easily measured. In general, chromogenic probes are less sensitive than fluorogenic ones. On the basis of the current evidence, enzymatic assays are appropriate to measure serum/plasma HDL-C concentration to be used for the substantiation of health claims in the context of improved blood lipid profile and of reduced risk of CHD (see Section 3.2.1.3).

3.1.6.3. Fasting triglycerides In the human body, TG are primarily stored in adipocytes. TG are mobilized from adipocytes into the bloodstream, usually as non-esterified fatty acids and glycerol, under enzymatic, hormonal and neural control. TG are hydrophobic and travel in the bloodstream primarily by means of lipoproteins (triglyceride-rich lipoproteins, TRL), which are secreted by the liver (VLDL) and by the gut (CM). TG levels can be intended as three different outcome variables: fasting TG (obtained after an overnight fast of 8−14 h); post-prandial TG (obtained at a specific time point within up to 8−14 h from the performance of a standardized fat tolerance test; non-fasting TG (obtained at any time point within 8−14 h after any normal meal). However, current guidelines refer only to fasting state. The Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults defines as normal fasting TG levels <150 mg/dL and as borderline-high and high the ranges of 150−199 mg/dL and 200−499 mg/dL, respectively. A more detailed classification has been proposed by the American Heart Association (AHA) that presently considers serum TG levels ≤ 100 mg/dL as "optimal" and ≤150 mg/dl as "acceptable" [63]. High TG levels are often associated with low HDL-C and high levels of small dense LDL and TRL remnants, a condition usually referred to as "atherogenic dyslipidaemia".

To evaluate the appropriateness of fasting TG as OV of blood lipid profile, the literature deriving from database #13 was critically evaluated (see Table 1).

Overnight fasting represents the traditional and most widely used method to assess TG levels within blood lipid profile and to diagnose hypertriglyceridemia for two main reasons: 1) the lower variability of TG measurements in the fasting state vs. the postprandial state; 2) the almost

exclusive use of the Friedewald equation to estimate LDL-C (LDL = TC - HDL - TG/5.0 mg/dL), which requires fasting TG concentration. On the other hand, the correlation between fasting and post-prandial TG concentrations is high, as individuals with low fasting TG levels generally do not have an amplified and delayed response of TG after a fat-tolerance test (FTT), conversely to those presenting levels higher than 180 mg/dL. The analysis of fasting TG concentration after an overnight fast (from 8 to 14 h) is recommended to avoid the variability associated with meal.

In conclusions, in the framework of function claims, the reduction of fasting TG levels is considered beneficial and fasting TG can be considered an appropriate outcome variable for the substantiation of health claims in the context of improved blood lipid profile.

3.1.6.3.1. Enzymatic assays TG are generally measured using accurate and cheap enzymatic techniques [64]. Such techniques are based on a lipase-induced enzymatic hydrolysis of TG to glycerol and free fatty acids. The glycerol released by this chemical reaction is measured by a coupled enzymatic reaction system. In the first analytical step, TG are hydrolysed to glycerol and free fatty acids by lipoprotein lipase. Glycerol is then phosphorylated by adenosine triphosphate forming glycerol-1-phosphate (G1P) and adenosine diphosphate (ADP). G1P is then oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and H_2O_2. H_2O_2 is then coupled with 4-aminoantipyrine and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine by means of peroxidase to produce a quinoneimine dye that shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to the triglyceride content of the sample. There are two types of probes for the enzymatic determination of H_2O_2: chromogenic and fluorogenic probes. In general, chromogenic probes are less sensitive than fluorogenic probes. Because all enzymatic assays measure TG based on glycerol concentration, the presence of endogenous glycerol will cause TG overestimation. The use of a glycerol blank (i.e. glycerol-blanking system) within the TG analysis is convenient and allows to overcome this problem. However, the measure of free glycerol concentration may be useful when a patient has a high level of endogenous lipoprotein lipase activity in response to treatment with heparin. On the basis of the current evidence, the enzymatic assay is appropriate for measuring blood concentration of fasting and postprandial TG (see Section 3.1.6.4) to be used for the substantiation of health claims in the context of improved blood lipid profile and of reduced risk of CHD (see Section 3.2.1.5).

3.1.6.4. Post prandial triglycerides Unlike fasting TG, there is no consensus on the definition of a normal postprandial TG range. However, when their concentration becomes high, non-fasting TG may play a role in plaque formation. The rise of blood TG is characterized by an early peak, which occurs from 10 to 30 min after the consumption of a meal containing fat, and by a subsequent peak that occurs 3−4 h after the meal. The first peak has been explained as produced by the secretion of lipids consumed in an earlier

meal suggesting the presence of an enterocyte TG storage pool for and a relation between the release of CM and the cephalic phase of food intake [65].

To evaluate the appropriateness of post-prandial TG as OV of blood lipid profile, the literature deriving from database #13 was critically evaluated (see Table 1).

Measurement of fasting TG is the traditional method to assess plasma TG levels. However, compared to fasting, a post-prandial or non-fasting measurement may be more informative, being representative of body capacity during post-prandial state. It should be considered that the intake of multiple meals during the day not only maintains TG levels constantly above fasting concentration for the major part of the 24-h period, but also affects TG response dependent from the last and previous meal(s). The ingestion of a high-fat meal results indeed in a rise in circulating TG levels and in the production of TRL (CM and, secondarily, VLDL). In the post-prandial phase, these plasma lipoproteins and their subclasses undergo variations in concentration and composition and are metabolized to TG-rich remnant lipoproteins, which can overcome the endothelial cells and reach the sub-endothelial space. Here, they can promote early atherogenesis, contributing to the formation of foam cells. High post-prandial circulating TG levels seem to be associated with atherogenic small LDL particles and with pro-thrombotic and pro-inflammatory biomarkers, such as factor VII and C-reactive protein. Because it is still debating how to appropriately perform FTT, a standardized and accurate methodology should be identified for post-prandial plasma to assess TG and TRL levels [66–68]. In conclusion, the measurement of post-prandial blood TG concentration seems to be an appropriate outcome variable to use in combination with other biomarkers (e.g. HDL-C and LDL-C) for the substantiation of health claims in the context of post-prandial lipid profile.

3.1.6.4.1. Enzymatic assays Please refer to Section 3.1.6.3.1.

3.1.7. Maintenance/reduction of blood pressure

3.1.7.1. Point systolic blood pressure   The heart is a pump of the "two-stroke" variety, with a filling and an emptying phase. Because both the left and right hearts perform their work in a cyclic fashion, flow is pulsatile. Blood pressure (BP) is measured in millimetres of mercury (mmHg). The mean BP in the large systemic arteries is approximately 95 mmHg. This is a single time-averaged value but BP varies between from a systolic maximum (SBP, $\leq$ 140 mmHg) corresponding to the contraction of the ventricle to a diastolic minimum, (DBP, $\leq$ 90 mmHg) corresponding to the relaxation of the ventricle. Pulse pressure is the difference between SBP and DBP. The arterial pressure wave has an initial rapid rising phase followed by an early systolic peak, followed by a second late systolic bulge. The maximum SBP may occur either during the early or late wave, DBP is the minimum pressure at the end of the runoff period [69–73].

To evaluate the appropriateness of point SBP as OV of BP, the literature deriving from database #14 was critically evaluated (see Table 1).

SBP increases progressively with age, often leading to increases in pulse pressure and thus to isolated systolic hypertension. This consideration led to rethink the role of DSP, that has been considered for years the most important component of BP. BP homeostasis is complex and several factors can influence SBP levels and BP in general [69,71,74]. BP increases with ageing as blood vessels become stiffer. Emotions (as in the case of white coat effects, also called doctor's office effect), exercise and increased body temperature are known factors increasing point BP [75,76]. BP levels increase during the day and decrease during the night, as a result of circadian fluctuations. For the above reasons, the single measurement of point BP may provide limited information compared to the extended measurements (i.e. 24 h ambulatory blood pressure monitoring, ABPM). Nevertheless, point SBP appears to be appropriate for the substantiation of health claims in the context of maintenance/reduction of BP, even though studies must be designed to account for intra-individual variability and SBP must be measured according to standardized protocols.

3.1.7.1.1. Office blood pressure Office BP should be assessed using a manual sphygmomanometer in both arms as a 10% difference in right and left arm readings exists. BP measurement requires that: 1) the arm is supported, with upper arm at heart level and feet on the floor (back supported, legs uncrossed); 2) BP is measured after at least 5 min resting; 3) BP should not be assessed when under stress, within 30 min of caffeine or tobacco consumption, or following exercise. For study purposes, due to the "white coat effect" and physiological fluctuations, BP should be measured every 2 min by the same investigator until two measurements, both systolic and diastolic, differ no more than 5 mmHg. The average of three readings (systolic and diastolic) should be calculated. To date, concerns about the accuracy of automated Office BP readings have been raised, particularly for their use in clinical studies [69,77–79].

In conclusion, the (mercury) sphygmomanometer is considered the "gold standard" device for the assessment of office BP to be used for the substantiation of health claims when pre-specified standard protocols are used.

3.1.7.1.2. Home blood pressure   Affordable and validated automated BP devices are commercially available for home BP self-measurement. These devices should be checked for accuracy at least every 1–2 years. Although home BP monitors may be inaccurate, depending on the accuracy threshold used, home BP measurement data usually correlate well with daytime BP ambulatory values [76–81]. It has been argued that the ease of use of the electronic devices (aneroid sphygmomanometers) and their relative insensitivity to observer bias, can balance any inherent inaccuracy of the sphygmomanometer method [82]. Standardization of home BP measurements are currently lacking. On the basis of the current evidence, home BP does not appear to be appropriate for measuring point BP to be used in the substantiation of health claims.

3.1.7.2. 24 h systolic blood pressure   As mentioned above, arterial pressure wave has an initial rapid rising phase followed by an early systolic peak, followed by a second

late systolic bulge. The mean 24-h systolic BP gives a better approximation of the "true" BP [83].

To evaluate the appropriateness of 24 h SBP as OV of BP, the literature deriving from database #14 was critically evaluated (see Table 1).

As mentioned for point SBP (see Section 3.1.7.1), an increase in SBP may lead to an increase in differential BP when DBP declines.

Repeated 24 h ABPM systems provide detailed and useful information about the circadian rhythm of BP: daytime and night-time BP profiles, day–night BP differences, morning BP surge and nocturnal dipping. Specifically, the ABPM is the sole tool to identify the absence of a nocturnal fall in BP, known as "non-dipping" status [79,84]. In addition, ABPM enables the identification of the white-coat phenomenon or of masked hypertension [75,76,85].

Finally, as several factors, including emotions, exercise, and body temperature may influence point BP levels, the isolated measurement of BP could provide limited information with respect to monitoring systems, which may also be used when high variability in point SBP is registered between measurements during the same or different visits [86]. In conclusion, the measurement of 24-h SBP appears to be appropriate to measure BP for the substantiation of health claims in the context of maintenance/reduction of BP.

3.1.7.2.1. 24 h ambulatory blood pressure monitoring ABPM is a non-invasive method that measure BP over a 24-h period, thus recording BP variability not recovered by single measurements [83]. ABPM devices consist of a BP cuff that is worn on the non-dominant arm and which is attached to a recorder worn on a belt. The ABPM device registers BP periodically (usually at 15- or 30-min intervals) during 24 h, including sleep, with a total number of readings between 50 and 100. BP data are stored in the device and subsequently downloaded into device-specific computer software. ABPM allows the recording of average daytime, night-time, and 24-h blood pressures (calculated as the arithmetic mean of all measurements and/or by the mean of hourly averages in the respective periods) which are the most commonly used variables [87]. ABPM devices are accurate enough for routine clinical use in most individuals. On the basis of current evidence, 24-h ambulatory BP seems to be appropriate for measuring mean 24-h BP to be used for the substantiation of health claims in the context of maintenance/reduction of BP.

3.1.7.3. Area under blood pressure curve Arterial pressure is one of the most important and widely assessed cardiovascular risk factors [69–73,88,89]. The area under blood pressure curve (AUC BP) allows estimating the within-subject changes in BP from baseline. Specifically, repeated measures of SBP or DBP are taken during a time period and graphically plotted. By using the trapezoidal rule, a software can calculate the integral of the curve that is called AUC. The AUC of both SBP and DBP can be calculated.

To evaluate the appropriateness of AUC BP as OV of BP, the literature deriving from database #14 was critically evaluated (see Table 1).

AUC BP represents a time-weighted measure of BP and can accommodate the short-term fluctuations of an individual's change pattern, thus correcting for short-term measurement variability [90]. The area under the blood pressure curve can play a useful and complementary role in the evaluation of 24-h ABPM, allowing to take into account both the magnitude and the temporal persistence of BP variations. One limitation of the AUC measure is that it is strictly dependent on the baseline threshold considered [87]. In conclusion, the measurement of AUC BP as a biomarker of BP appears to be appropriate for the substantiation of health claims in the context of maintenance/reduction of BP, but studies need to be well designed.

3.1.7.3.1. Office blood pressure Please refer to Section 3.1.7.1.1.

3.1.7.3.2. Home blood pressure Please refer to Section 3.1.7.1.2.

3.1.7.4. Central systolic blood pressure Central BP is the pressure in the aorta, which not only serves as a conduit during systole but also acts as a reservoir for blood during diastole. The pressure generated by left ventricle ejection causes a pressure pulse wave through the arterial tree, which is reflected mainly in distal arteries, and interacts with the incident waveform during early diastole, producing a dicrotic notch. In normal vessels, reflected waves return during diastole and cause the pulse pressure to be higher in the peripheral rather than central arteries. As arteries become stiffer with age, pulse wave velocity increases and the reflected wave reaches the central pulse in systole, boosting the SBP and left ventricular load.

To evaluate the appropriateness of central SBP as OV of BP, the literature deriving from database #15 was critically evaluated (see Table 1).

Increased arterial stiffness is a major factor contributing to the rise in SBP with ageing. This is due to both increased amplitude of the pressure wave and to increased wave velocity. Measurement of aortic pulse wave velocity is the best non-invasive measure of arterial stiffness and correlates with adverse cardiovascular outcomes [91–93]. Arterial stiffness has been suggested as a good tool for risk stratification and a target for intervention [94–96]. An increase in central arterial stiffness is accompanied by a parallelled increase in central BP [97]. In conclusion, the measurement of central SBP appears to be appropriate for the substantiation of health claims in the context of reduction/maintenance of BP.

3.1.7.4.1. Pulse wave analysis Direct central pressure assessment requires an invasive procedure which has the advantage of being a precise and reproducible technique. However, it is usually performed in clinical setting, only in patients scheduled for coronary angiography [98]. Therefore, several non-invasive methods and techniques for central BP estimation and pulse wave shape have been developed and validated [99]. High-fidelity sensors, tonometers and piezo-electric techniques rely on a mathematical transformation that allows the calculation of central SBP from radial tonometry data. Moreover, compared to

standard sphygmomanometry, pulse wave analysis can provide a better assessment of aortic pressure [91]. Applanation tonometry is the current gold standard for the measurement of aortic stiffness. Pulse wave velocity is calculated by measuring the transit time of the pulse waveform at two sites along the vasculature, providing a regional assessment of aortic stiffness. Different vasculature segments can be involved in the non-invasive measurement, including carotid-femoral (Class I, Level of Evidence A) and ankle-brachial. However, for the latter segment, longitudinal studies in USA and European countries are lacking. In order to enhance the uniformity of arterial stiffness studies, the sites of measurement should be clearly stated. Owing to the lack of evidence about the association with incident cardiovascular outcomes, single-point estimates of pulse wave velocity are not recommended. Moreover, for each subject, a duplicate of the measure of pulse wave velocity should be performed in the supine position after 10 min of rest (at least), controlling the environmental noise and temperature. If the two measurements show a difference higher than 0.5 m/s using the median value, a triplicate is needed. A limitation of the technique is represented by the requirement of trained expert personnel for obtaining reproducible results [98].

On the basis of current evidence, pulse wave analysis seems to be appropriate for measuring central SBP. Furthermore, pulse wave analysis appears an appropriate MM of arterial stiffness, assessed as augmentation index (AIx) and return time of reflected wave.

3.1.7.5. Point diastolic blood pressure As mentioned in the Section 3.1.7.1, BP varies between a maximal SBP (≤140 mmHg) corresponding to the contraction of the ventricle, and a minimal DBP (≤90 mmHg) corresponding to the relaxation of the ventricle. The arterial pressure wave has an initial rapid rising phase followed by an early systolic peak, followed by a second late systolic bulge. DBP is the minimum pressure at the end of the runoff period. With ageing, the increase in arterial stiffness leads to a rise in SBP. In the meanwhile, a reduction in aortic volume, which in turn causes a decline in DBP, can occur so leading to isolated systolic hypertension.

To evaluate the appropriateness of point DBP as OV of BP, the literature deriving from database #14 was critically evaluated (see Table 1).

SBP and DBP are usually highly correlated. However, with ageing SBP may increase as vessels become stiffer, whereas DBP may decline leading to an increase in differential BP. As for SBP, several factors, including emotion (white coat effect), exercise, and body temperature, may influence point DBP [75,76]. In addition, DBP displays a circadian rhythm characterized by an increase in the early morning hours, high values during the daytime, an afternoon fall and clear nocturnal dipping. For the above reasons, the isolated measurement of DBP may provide limited information compared to the extended measurements made by monitoring systems (i.e. 24 h ABPM) which may be useful when high BP variability is observed between repeated measurements during the same or different visits. Because a DBP

reduction may lead to an increased differential BP if not accompanied by a reduction of SBP, the measurement of point DBP as a biomarker of BP appears to be appropriate for the substantiation of health claims only when it is accompanied by the corresponding SBP.

3.1.7.5.1. Office blood pressure Please refer to Section 3.1.7.1.1.

3.1.7.5.2. Home blood pressure Please refer to Section 3.1.7.1.2.

3.1.7.6. 24 h diastolic blood pressure As mentioned in the Section 3.1.7.1, BP varies between a maximal SBP (≤140 mmHg) corresponding to the contraction of the ventricle, and a minimal DBP (≤90 mmHg) corresponding to the relaxation of the ventricle. For a detailed description see Sections 3.1.7.1 and 3.1.7.5.

To evaluate the appropriateness of 24 h DBP as OV of BP, the literature deriving from database #14 was critically evaluated (see Table 1).

24 h ABPM systems provide detailed and useful information about the circadian rhythm of DBP (for a detailed description, see Section 3.1.7.2). Nevertheless, as mentioned above, despite SBP and DBP are usually highly correlated, SBP may increase with ageing as vessels become stiffer, whereas DBP may decline leading to an increase in differential BP.

On the basis of these considerations, the measurement of mean 24-h DBP appears to be appropriate for the substantiation of health claims in the context of maintenance/reduction of BP only if accompanied by a reduction in SBP.

3.1.7.6.1. 24 h ambulatory blood pressure monitoring Please refer to Section 3.1.7.2.1.

3.1.8. Improvement of endothelial function

3.1.8.1. Endothelium dependent vasodilation The endothelium is the inner lining of blood vessels and plays a central role in vascular homeostasis. In healthy individuals, the endothelium exerts a protective effect on the vasculature by regulating vascular tone, blood fluidity and clotting. Endothelium-derived hyperpolarizing factor, nitric oxide (NO) and prostacyclin (PGI2) are endothelium-derived factors with vasodilatory and antiproliferative effects. On the other side, endothelin 1, angiotensin II and ROS are molecules exerting vasoconstrictor effects. Endothelial cells are also able to produce antithrombotic (NO and PGI2) and prothrombotic molecules, including von Willebrand factor and plasminogen activator inhibitor-1. The disruption of the fine-tuned relative concentration of these factors, mainly due to reduced NO bioavailability, may lead to impaired vasodilation (endothelial dysfunction) which is the earliest clinically identifiable event in the process of atherosclerosis. Endothelial (dys) function can be determined by assessing the degree of flow mediated dilation (FMD), which reflects the endothelium-dependent capacity of arterial relaxation, mediated by the release of NO, in response to a hyperaemic stimulus [100]. FMD can be, therefore, considered a reliable variable of arterial reactivity.

To evaluate the appropriateness of endothelium dependent vasodilation as OV of endothelial function, the

literature deriving from database #16 was critically evaluated (see Table 1).

Endothelial dysfunction is the earliest stage of the atherosclerotic process and is characterized mainly by a reduction in NO availability. No structural lesions have been reported at this early stage. Vascular reactivity tests have become the most common and reliable methods for assessment of endothelial function [101]. In order to rule out the contribution of vascular endothelium-independent reactivity to the vasodilation induced by an endothelium-dependent stimulus, exogenous NO donors (e.g. glycerol-trinitrate) can be administered. A reduction or the complete absence of endothelium-dependent vasodilatation is an index of endothelial dysfunction [102,103]. In conclusion, the measurement of endothelium dependent vasodilation appears to be appropriate for the substantiation of health claims in the context of improvement of endothelial function.

3.1.8.1.1. Flow mediated dilation FMD is the most common and reliable method for the assessment of endothelial (dys)function. FMD is a non-invasive method, introduced in 1992, based on the measurement of brachial artery diameter changes after an increase in shear stress induced by reactive hyperaemia, usually induced by circulatory arrest in the forearm (suprasystolic cuff occlusion) for a period of 4−5 min. In the setting of a healthy endothelium, the increased shear stress stimulates the release of NO, inducing a reactive local vasodilation in the brachial artery [104,105]. FMD assessment in the brachial artery may mirror endothelial health status in all vascular districts including the coronary arteries [102]. Impaired FMD is associated with almost every condition predisposing to atherosclerosis and evidence from prospective studies shows that it is associated with cardiovascular events [105,106]. FMD is assessed by Doppler ultrasound [101]. The assessment of FMD with this technique requires a highly skilled sonographer and a suitable ultrasound machine. In addition, strict standardization is needed: differences in methodological approaches may, in fact, deeply affect the response magnitude, leading to misinterpretation of data, and may limit between-study comparisons. A rigorous methodology is paramount to reduce measurement variability in both single-centre and multi-centre studies. The main factors influencing the outcome variable are: 1) a highly-trained operator working in blind conditions, 2) a defined experimental setting, 3) the use of an adjustable stereotactic probe-holding device, 4) an automated computer-assisted brachial artery measurement. In addition, it is worth considering that FMD is often measured in one arm only, with no possibilities of correction for potential measurement-induced changes in the systemic haemodynamics, such as those resulting from alterations in the autonomous nervous system tone.

The administration of low-dose sublingual nitroglycerine should be performed for the evaluation of endothelium-independent FMD response, to quantify the influence of altered smooth muscle cell contractility on the dilation response. On the basis of current evidence, FMD seems to be appropriate for measuring the endothelium-dependent and independent vasodilation function in humans.

3.1.8.2. Endothelium independent vasodilation The human body usually is endowed with an adequate supply of antioxidants, obtained from various food sources; yet, an excess of free radicals may reduce NO bioavailability. Endothelial (dys)function can be determined by assessing FMD (see Section 3.1.8.1.1). The vasodilatation induced by exogenous NO donors, such as nitroglycerine, is called "endothelium independent".

To evaluate the appropriateness of endothelium independent vasodilation as OV of endothelial function, the literature deriving from database #16 was critically evaluated (see Table 1).

Vascular responses are determined by the functional status of the vasculature and by the microvasculature structure. Moreover, to separate endothelium-dependent from endothelium-independent responses, exogenous NO donors (e.g., glycerol-trinitrate) can be used. Impaired signalling of the NO-guanylate cyclase-cGMP-PKG cascade as well as inactivation or destruction of NO prior to smooth muscle signalling may induce endothelial dysfunction. Endothelial-independent dysfunction is related to alterations in vascular structures and in smooth muscle cells rather than to changes in the endothelium [102,103]. In conclusion, the measurement of endothelium independent vasodilation (nitroglycerine-mediated) as biomarker of endothelial function does not appear to be appropriate for the substantiation of health claims in the context of improved endothelial function. However, it can be used as a supportive evidence in combination with the appropriate OV (i.e. endothelium-dependent vasodilation).

3.1.8.2.1. Flow mediated dilation   Please refer to Section 3.1.8.1.1.

3.1.8.3. Systemic arterial compliance Large arteries, such as the aorta and the carotids, are distensible so that they can buffer the pulsatile systolic output of the ventricle. This buffering function is usually described as systolic arterial compliance (SAC). SAC is an important determinant of the cardiac load. The total arterial compliance is defined as the change in the arterial blood volume associated with a change in the distending pressure (diameter-pressure relationship). Arterial compliance decreases with ageing and in various diseases such as systolic hypertension and coronary artery disease (CAD) [107]. When SAC is decreased, arteries are stiffer and with reduced ability to smooth the pulsatile blood flow; hence, they are exposed to higher pressure peaks which may be detrimental to vessel wall integrity.

To evaluate the appropriateness of SAC as OV of endothelial function, the literature deriving from database #17 was critically evaluated (see Table 1).

While arterial BP offers a summary measure of the haemodynamic load, SAC reflects the overall opposition of large arteries to the pulsatile ventricular ejection of blood. The evaluation of blood volume changes in the systemic arterial tree is difficult and, because arterial compliance is

strictly associated to BP fluctuations, there is no single reference value to normalize arterial compliance. In addition, SAC may differ when measured at different points of the non-linear pressure-volume curve and it is influenced by wall stiffness, arterial size and wall thickness [108]. SAC is estimated using simple decay time or area methods, which require the expression of aortic pressure as a function of time and cardiac output [109,110]. The two-area and pulse pressure methods are the most accurate and versatile methods and can be applied at all locations. Lumped or whole-body compliance, usually evaluated as the ratio of cardiac stroke volume to pulse pressure, is not useful because it is based on the assumption that the arterial tree is a single compartment and because it is affected by peripheral changes [111]. In conclusion, the measurement of SAC as biomarker of endothelial function does not appear to be appropriate for the substantiation of health claims in the context of improved endothelial function.

3.1.8.4. Nitrite/nitroso-species Nitrite/nitroso-species (RXNO), i.e. S-nitrosothiols, N-nitrosamines and iron-nitrosyl species, are produced by the interaction of NO and ROS and are key components of the redox regulation/signalling network [112]. A depletion of circulating RXNO has been associated with endothelial dysfunction [113]. Intravenous administration of S-nitrosothiols has been shown to induce vasodilation. More recently, N-nitroso proteins and iron-nitrosyl complexes have been suggested to serve as stores of plasma NO. Although the human body has an adequate supply of antioxidants (self-produced or obtained from foods) to neutralize free radicals, ROS accumulation may disrupt NO balance and make the endothelium overly permeable with the entry of different substances into body tissues.

To evaluate the appropriateness of nitrite/nitroso species as OV of endothelial function, the literature deriving from database #18 was critically evaluated (see Table 1).

RXNOs may be a suitable diagnostic marker for long-term changes in nitric oxide synthase 3 activity, due to their long half-life. RXNOs, but not nitrite, are associated with endothelial dysfunction because of their peculiar biochemical features, first of all half-life. Further studies are needed to test whether the reduction in plasmatic RXNO reflects impaired NO formation, accelerated decomposition, and/or consumption of RXNOs and whether these processes play a causal role in the pathogenesis of atherosclerosis. However, plasma RXNO levels may represent a marker of NO bioavailability and can be useful in identifying a potential mechanism of endothelial (dys)function. In conclusion, the measurement of RXNO alone does not appear appropriate to be used alone for the substantiation of health claims in context of endothelial function. However, it can be considered supportive of a mechanism underlying the claimed effect.

3.1.8.4.1. Reductive gas-phase luminescence Chemiluminescence can detect only free NO in gaseous state. NO metabolites have thus to be converted into free NO prior to quantification. The concentration of nitrosylated and nitrosate species is determined after reductive cleavage with subsequent assessment of the NO released into the gas phase by its chemiluminescent reaction with ozone (O_3) [114]. O3 is combined with NO to form NO2 in its activated state; a proportion of the latter arises in an electronically excited state (NO2*), which, on decay to its ground state, emits light in the near-infrared region (above 600 nm) and can be quantified by a photomultiplier. Because the intensity of the emitted light is proportional to the concentration of NO in the reaction chamber, the amount of NO in the original sample can be evaluated using proper calibration curves. It is possible to detect up to 1 pM of NO gas in the reaction chamber. However, the sensitivity of the method should be determined using calibration against a known amount of NO gas. This method has been validated for use in different biological tissues (plasma, whole blood, and red blood cells) and has some advantages: a) high specificity for NO; b) good reproducibility; c) possibility of using turbid or coloured samples; d) low cost [113]. On the other hand, this method has the disadvantage of requiring gaseous NO with ensuing low sample throughput. On the basis of the current evidence, reductive gas-phase chemiluminescence seems to be appropriate for measuring the RXNO.

3.1.9. Reduction of platelet aggregation

3.1.9.1. Percent of inhibition in platelet aggregation Platelets are involved in primary haemostasis. They first adhere to the sub-endothelium of a damaged vessel and then become activated, providing a surface triggering coagulation and favouring the formation of a stable coagulum that prevents bleeding and facilitates vessel repair. The haemostatic process results from a fine balance between the fluidity of blood and the tendency to plug formation after vessel injury. Thrombosis occurs because this delicate balance is altered. Arterial thrombosis occurs mainly after injury of vessel walls due to atherosclerosis, while venous thrombosis occurs in areas of stasis [115].

To evaluate the appropriateness of the percent of inhibition in platelet aggregation as OV of platelet aggregation, the literature deriving from database #19 was critically evaluated (see Table 1).

Hyperactive platelets are involved in atherothrombosis, inflammation and angiogenesis and are associated with acute coronary syndromes, cerebral infarction, and peripheral vascular disease [116]. Tests of platelet aggregation are affected by platelet count, making them unsuitable in severe thrombocytopenia. In clinical practice, many laboratories use agonists (ADP, collagen, ristocetin, adrenaline, thrombin receptor activating peptide, thromboxane A2, or arachidonic acid) and different dilutions depending upon the results of initial tests and the suspected abnormality [117]. Moreover, the interpretation of platelet aggregation traces can be difficult. In conclusion, the maintenance of normal platelet aggregation appears to be appropriate for the substantiation of health claims in the context of

reduction of platelet aggregation, provided that measurements are performed using accepted protocols.

3.1.9.1.1. Light transmission aggregometry The available tests of platelet aggregation assess the ability of various agonists to induce *in vitro* activation of platelets and platelet-to-platelet activation. Aggregometry uses platelet rich plasma but whole blood aggregometry can also be used. Commonly used agonists are ADP, collagen, ristocetin, adrenaline, thrombin receptor activating peptide, thromboxane A2 (TXA2) or arachidonic acid. Light transmission aggregometry (LTA) was invented in the 1960's and revolutionized the diagnosis of primary haemostatic defects. Conventional LTA, with a full panel of agonists, requires large blood volumes and a great expertise for evaluating test performance and interpreting the results. When an agonist is added, the platelets aggregate and absorb less light, the transmission increases and this is detected photometrically. Among functional tests, *in vitro* LTA is still regarded as the gold standard for measuring platelet function. By adding a panel of agonists to stirred platelets, it is possible to obtain more information about different aspects of platelet function [117−119]. However, an international standardization of this technique is still lacking. This test, which is coupled with the evaluation of stored and releasable platelet nucleotide content, is still applied in many laboratories with the aim of diagnosing platelet defects. LTA findings cannot however be easily extrapolated to the (patho)physiological setting because separated platelets undergo stirring under low shear conditions and form aggregates only after addition of agonists, conditions which do not mimic the aggregation triggered by vessel wall damage. Importantly, most recent aggregometers are entirely automated and easy to use. In conclusion, LTA is appropriate for measuring percent of inhibition in platelet aggregation.

3.1.9.2. Thromboxane A2 generation Thrombin induces the production and release of TXA2 from activated platelets. TXA2 is synthesized from the 20-carbon polyunsaturated arachidonic acid, which is cleaved from phospholipids and released during platelet activation. As other soluble platelet agonists, e.g. ADP and serotonin, TXA2 is released from stimulated platelets to amplify platelet activation and to recruit additional circulating platelets. TXA2 is a potent inducer of platelet aggregation, vasoconstriction and bronchoconstriction, and has been involved in a series of major diseases [120,121]. Because of its very short half-life (about 30 s), TXA2 functions as an autocrine or paracrine mediator in tissues surrounding the site of production.

To evaluate the appropriateness of TX A2 generation as OV of platelet aggregation, the literature deriving from database #20 was critically evaluated (see Table 1).

TXA2 participates to the cascade of events preceding actual cell activation. Described as "the most significant parameter related to platelet activation", TXA2 is inhibited by acetylsalicylic acid, which may limit the usefulness of TXA2 levels as *in vivo* marker of platelet activity in individuals taking this drug [122]. TXA2 generation occurs only after, and as a consequence of, the beginning of platelet aggregation, providing information about its extent [120]. For this reason, and not representing a direct measure of the claimed effect, TXA2 does not appear to be appropriate for the substantiation of health claims in the context of reduced platelet aggregation.

3.1.9.3. P-selectin P-selectin (P-sel) is a glycoprotein constitutively expressed in the α−granules of platelets and in the Weibel-Palade bodies of endothelial cells or released in soluble form. In response to physiological platelet activation, soluble P-sel translocates to platelet and endothelial cell surface promoting formation of platelet-leukocyte aggregate and bridges between leukocytes and endothelium. Soluble P-sel is the heaviest of the known selectins, with a molecular weight of 140 kDa. The detection of soluble P-sel has recently become one of the mostly used strategies to test platelet activation [123].

To evaluate the appropriateness of P-sel as OV of platelet aggregation, the literature deriving from database #21 was critically evaluated (see Table 1).

There is a growing body of evidence supporting the idea that soluble P-sel is a measure of platelet activation. Increased levels of soluble P-sel may be indicative of an increased risk of adverse cardiovascular events in patients with existing ischaemic heart disease [124]. Soluble P-sel may also be a marker of platelet activation during haemodialysis [125]. Contrarily to beta-thromboglobulin, increased levels of soluble P-sel are not caused by *ex vivo* platelet activation and are not associated with platelet count. However, it cannot be excluded that raised soluble P-sel levels depend upon other coagulation factors or are associated with post-thrombotic phenomena [126]. Indeed, raised soluble P-sel may be the result of platelet activation by thrombin, its fractions, or other factors leading to hypercoagulability. In addition, according to some researchers, a quote of soluble P-sel may arise from the endothelium. Thus, the specificity of soluble P-sel as marker of platelet activation is debated [123,127]. In conclusion, the measurement of plasma soluble P-sel is not appropriate for the substantiation of health claims in the context of reduced platelet aggregation.

3.1.10. Maintenance of normal homocysteine

3.1.10.1. Homocysteine Homocysteine, a sulfhydryl-containing amino acid, is an intermediate product of the synthesis of the amino acids methionine and cysteine. Normal levels of plasma homocysteine range from 5 to 15 μmol/L.

To evaluate the appropriateness of homocysteine as outcome variable, the literature deriving from database #22 was critically evaluated (see Table 1).

Plasma homocysteine is a potentiator of blood platelet response to agonists. While homocysteine functions as a pro-aggregatory agent, its acetylated form has the opposite effect.

However, normal homocysteine concentrations reflect a condition of normal metabolism and thus, in the framework of the function claim, its direct measure appears to be appropriate for the substantiation of health claims linked to maintenance of normal homocysteine.

3.1.10.1.1. LC-MS/MS LC-MS/MS is a relatively new method and has been reported to be precise and accurate

for measuring plasma total homocysteine concentrations, using small volumes of plasma and being suitable for routine use [128]. A satisfactory LC-MS/MS method for the measurement of total homocysteine in dried blood spots is also available; this method might be useful in routine screening for raised plasma concentrations of total homocysteine [129]. There are some advantages that suggest to use this method for large-scale population-based studies, including: a) high throughput; b) low cost; c) simple sample pre-treatment procedures. On the basis of current evidence, LC-MS/MS seems to be appropriate for measuring homocysteine.

3.1.11. Maintenance of normal venous blood flow

3.1.11.1. Venous reflux Healthy veins contain bicuspid valves assisting unidirectional flow from the lower limb toward the heart. The valves of the venous system may become incompetent and blood can flow backwards (venous reflux). Venous reflux is a pathological condition characterized by impaired return of blood and increased venous pressure that may lead to venous stasis and eventually microangiopathy. There are several predisposing conditions to venous reflux such as ageing, male sex, heredity, obesity, physical activity, multiparity and prolonged standing.

To evaluate the appropriateness of venous reflux as OV of normal venous blood flow, the literature deriving from database #23 was critically evaluated (see Table 1).

Venous reflux is a pathologic condition affecting mostly body extremities. When valve leaflets close improperly, vein pressure increases greatly leading to vein dilatation and valve incompetence. Venous stasis secondary to venous reflux may cause fluid leakage into the interstitial space with consequent oedema. The use of duplex scanning to evaluate reflux in the deep and superficial veins should be performed with the patient in the upright position, with the leg rotated outward, heel on the ground, and weight on the opposite limb [130–133]. There is presently no clear threshold to identify pathological refluxes. In the literature, both 500 ms and 1000 ms have been used as cut-points to identify a pathological reflux. The Committee of the Society for Vascular Surgery and the American Venous Forum recommends 500 ms as the cut-off value for saphenous, tibial, deep femoral, and perforating vein incompetence, and 1000 ms for femoral and popliteal vein incompetence [134]. In conclusion, the measurement of venous reflux appears to be appropriate for the substantiation of health claims related to the maintenance of normal venous blood flow.

3.1.11.1.1. Duplex doppler The veins are usually visualized by means of Duplex ultrasound, which combines Doppler flow with imaging information. This method allows the assessment not only of blood flow and velocity, but also the estimation of the vein diameter and of the degree of obstruction. Duplex Doppler is the main clinical tool used to evaluate the venous system for the diagnosis of chronic venous disease, especially in the lower limbs [132,133,135]. The major limitation of this method is that reliable information can only be achieved by trained and experienced operators. On the basis of the current evidence, duplex ultrasound seems to be appropriate for the evaluation of chronic venous disease, especially the phenomenon of venous reflux and the great saphenous diameter and the popliteal vein diameter (see Sections 3.1.11.2 and 3.1.11.3).

3.1.11.2. Great saphenous diameter Venous anatomy is highly variable in some parts and less variable in other parts of the lower limbs. Typically, the Great saphenous vein has a diameter ranging from 3 to 5 mm. This diameter can increase from 1 to 3 mm in response to hyperthermia, prolonged standing, the Valsalva manoeuvre, menstruation and pregnancy. The great saphenous vein is the vein most commonly responsible for varicose vein disease. Signs and symptoms include swelling, and heaviness up to frank pain. Prolonged volume overload and venous hypertension may result in venous dilatation and stasis, which represent predisposing conditions to thrombophlebitis and phlebothrombosis.

To evaluate the appropriateness of the great saphenous diameter as OV of normal venous blood flow, the literature deriving from database #24 was critically evaluated (see Table 1).

Studies using the great saphenous vein diameter as a marker for venous haemodynamic impairment have led to contradictory results. The diameter of the great saphenous vein can vary with patient positioning, Valsalva manoeuvres, and body mass index. Standardization of great saphenous vein assessment is difficult due to the variable anatomical configuration of the great saphenous vein. When measured distally to the terminal valve, the great saphenous vein diameter will usually be larger than when it is measured at the saphenous-femoral junction. Several procedures to measure the saphenous diameter are available but standardization in this region is difficult. The more reproducible method is to measure great saphenous vein diameter at the proximal thigh, although reflux is relatively unrelated to diameter in this region [136]. In conclusion, the measurement of great saphenous diameter as a marker of venous blood flow does not appear to be sufficient for the substantiation of health claims related to the maintenance of normal venous blood flow.

3.1.11.2.1. Duplex doppler Please refer to Section 3.1.11.1.1.

3.1.11.3. Popliteal vein diameter The popliteal vein is formed by the junction of the anterior and posterior tibial veins at the lower border of the popliteus muscle; it ascends through the popliteal fossa to the aperture in the Adductor magnus muscle, where it becomes the femoral vein. In the lower part of its course, it is placed medially to the artery; between the heads of the Gastrocnemius muscle, it is superficial to that vessel, while it is close to its lateral side above the knee-joint. It receives tributaries corresponding to the branches of the popliteal artery, and it also receives the small saphenous vein. The valves in the popliteal vein are usually four. The popliteal vein carries blood from the lower leg to the heart. The mean diameter

of the popliteal vein is 0.62 ± 0.06 cm in the recumbent position and 0.84 ± 0.1 cm in the erect position.

To evaluate the appropriateness of the popliteal vein diameter as OV of normal venous blood flow, the literature deriving from database #25 was critically evaluated (see Table 1).

An increase in popliteal vein diameter is associated with an increased risk of venous reflux [137]. Several procedures are available to measure popliteal diameter but standardization in this region is difficult due to the physiological variation of the vein anatomy [138]. The measurement procedure should be reported, especially if multiple operators are involved. Because leg extension produces compression of the vein, the ultrasonographic assessment should be performed with the limb slightly flexed [139]. In conclusion, the measurement of popliteal vein diameter does not appear to be appropriate for the substantiation of health claims related to the maintenance of normal venous blood flow.

3.1.11.3.1. Duplex doppler Please refer to Section 3.1.11.1.1.

3.2. Risk reduction claims Art. 14.1 (a)

3.2.1. Coronary heart disease
CHD is the direct effect of CAD on the heart. CAD develops when plaques grow within the walls (atherosclerosis) of the coronary arteries that become narrow and rigid, reducing blood flow to the heart. The heart becomes starved of oxygen and the vital nutrients it needs to work properly. CHD may be chronic, consisting in a progressive narrowing of the coronary arteries or acute, resulting from a sudden plaque complication (rupture and thrombus formation). This may lead to complete vessel obstruction and acute myocardial ischaemia. Because CAD often develops over decades, it can go unnoticed until the first heart attack. The traditional risk factors for CAD are age, smoking, high BP, diabetes, high cholesterol, and family history. In detail, the ageing process increases the risk of narrowing the coronary arteries and men are at greater risk of CAD than women. Women have an in increase in CAD risk after the menopause. High levels of cholesterol in blood contribute to the risk plaque formation and atherosclerosis.

3.2.1.1. Systolic blood pressure To evaluate the appropriateness of SBP as risk factor for CHD, the literature deriving from database #14 was critically evaluated (see Table 1).

Despite SBP and DBP are strictly correlated, SBP is more robustly associated with CVD than DBP [69,71,74]. Day-time and night-time BP values and changes obtained with treatment are related to each other, but the prognostic value of night-time BP in predicting cardiovascular events has been found to be superior to that of day-time BP (dipping phenomenon), which is undetectable with punctual BP determination. BP may increase with the ageing process as blood vessels become stiffer. Furthermore, several factors, including emotion (white coat effect), exercise, temperature, race and circadian fluctuations may influence punctual BP levels [75,76]. Therefore, isolated measurement of BP could provide limited information with respect to monitoring (e.g. 24 h ABPM) which may be useful in unveiling

hypertension when a high BP variability is registered between measurements during the same or different visits. Importantly, patients in which the nocturnal decline in BP is reduced or abolished (non-dipper), are at increased risk for cardiovascular complications than are individuals with a dipper circadian rhythm [79,84].

In conclusion, SBP can be considered as a risk factor to be used for the substantiations of health claims in the context of reduced risk of CHD.

3.2.1.1.1. Office, home and 24 h ambulatory BP Please refer to Sections 3.1.7.1.1, 3.1.7.1.2 and 3.1.7.2.1.

3.2.1.2. LDL-C To evaluate the appropriateness of LDL-C as risk factor for CHD, the literature deriving from database #12 was critically evaluated (see Table 1).

Multiple clinical trials have established beyond any reasonable doubt that the reduction of TC or LDL-C is associated with a clinically relevant reduction in cardiovascular mortality. LDL-C usually makes up 60–70% of serum TC. There are some lifestyle behaviours that decrease blood LDL-C, such as eating vegetables, eating less fat and doing daily exercise. Statins are the drugs usually employed for lowering cholesterol concentration, but, more recently, other LDL-lowering drugs, such as inhibitors of Nieman Pick C1 like 1 protein and inhibitors of proprotein convertase subtilisin/kexin type 9, have been shown to be effective in reducing cardiovascular risk [140]. Thus, it can be safely stated that lowering the LDL-C concentration lowers the cardiovascular risk [45,46,48–51]. The greater the LDL-C reduction, the more benefit there is to be gained. Some studies show that the number of LDL particles may be a better predictor of risk than LDL-C. LDL particle size may also be important when assessing risk but its evaluation is not currently endorsed by clinical guidelines [53].

In conclusion, LDL-C can be considered a modifiable risk factor to be used for the substantiation of health claims in the context of reduced risk of CHD.

3.2.1.2.1. Enzymatic assays Please refer to Section 3.1.6.1.1.

3.2.1.3. HDL-C As mentioned above, HDL has been extensively investigated because it extracts cholesterol from tissues and delivers it to the liver, where it may be converted into bile acids and excreted. Low HDL-C is an independent risk factor for CVD [43–46,48,49,58–61].

To evaluate the appropriateness of HDL-C as risk factor for CHD, the literature deriving from database #12 was critically evaluated (see Table 1).

HDL are believed to exert many cardioprotective activities, including the promotion of the reverse cholesterol transport, a process through which excess cholesterol is delivered from peripheral tissues back to the liver. It has been widely reported that low HDL-C levels (below 40 mg/dL in men and 50 mg/dL in women) are strongly associated with high cardiovascular risk, independently of LDL or TC levels. It is therefore understandable that most clinical studies have used HDL-C as the metric for quantifying HDL cardioprotective effects. Recent studies have evaluated HDL function, together with HDL levels, to fully define the cardioprotective potential of this class of lipoproteins

[43−46,49,57−61]. In particular, HDL capacity to promote cell cholesterol efflux has been proposed as novel target to reduce risk of CHD [47,57]. However, a causal relationship between HDL-C levels and CVD in humans currently is disputed by a number of key findings, the most noteworthy of them being: 1) Neither cholesteryl ester transfer protein -inhibitors nor niacin, both of which increase HDL-C, reduce cardiovascular mortality; 2) A number of mendelian randomization studies (a research design which tests the hypothesis of a causal relationship between genetically determined HDL-C levels and clinical events) have consistently found no evidence of a causal link between HDL-C levels and CHD risk [141]. Thus, HDL-C presently should be regarded as a strong independent indicator of a fraction of cardiovascular risk, which cannot be corrected via HDL-C raising treatments.

In conclusion, HDL-C cannot be used alone for the substantiation of health claims in the context of reduced risk of CHD.

3.2.1.3.1. Enzymatic assays Please refer to Section 3.1.6.2.1.

3.2.1.4. Total cholesterol Cholesterol, a steroid with a secondary hydroxyl group in the C3 position, is an important component of mammalian cell membranes where it contributes to intracellular transport, cell signalling, and maintenance of membrane fluidity. Cholesterol can be found in blood, bile, and in all tissues insofar as it is a key component of cell membranes. Brain and adrenal glands are organs with high cholesterol content. The measurement of serum TC is important for the diagnosis and classification of hyperlipoproteinemias. Maintenance of normal blood TC is important because elevated cholesterol levels are associated with the development of atherosclerosis and CVD [47,51]. Elevated cholesterol levels may occur also with hypothyroidism, nephrotic syndrome, diabetes, and biliary cirrhosis. There is a strong association between elevated serum TC and the incidence of CVD and recent studies suggest that altered cholesterol homeostasis is associated with the development of a chronic inflammatory state [43,45,51].

To evaluate the appropriateness of TC as risk factor for CHD, the literature deriving from database #12 was critically evaluated (see Table 1).

Cholesterol levels are influenced by diet, age, sex and hormonal status. TC has received most attention because several studies showed that CVD can be prevented by reducing TC [46]. A number of cardiovascular risk calculators use TC to quantify cardiovascular risk [49,51,142]. However, it should be recognized that the relationship between TC and CVD is mostly rooted in the non-HDL-C fraction (mostly LDL-C), usually representing the bulk of TC. TC is recommended to be used for the estimation of total cardiovascular risk by means of the SCORE system (as currently suggested by the European Society of Cardiology) [43,44,48]. However, in specific cases, TC may be misleading; this is especially true in women who have high HDL-C levels and in subjects with diabetes or the metabolic syndrome, who often have low HDL-C levels. For an adequate risk analysis, at least HDL-C and LDL-C should be analysed.

In conclusion, TC is not appropriate to be used alone for the substantiation of health claims in the context of reduced risk of CHD.

3.2.1.4.1. Enzymatic assays The cholesterol concentration is determined enzymatically with an assay that quantifies both cholesterol esters and free cholesterol [143,144]. Most available assays are of high quality, but the method used should be evaluated against the available reference methods and controlled in international quality programs. Cholesterol esters are enzymatically hydrolysed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase to yield the corresponding ketone and hydrogen peroxide coproducts. H_2O_2 is then detected using 10-acetyl-3,7-dihydroxyphenoxazine, a highly sensitive and stable probe for hydrogen peroxide. There are two types of probes for the enzymatic determination of H_2O_2: chromogenic and fluorogenic. In general, chromogenic probes are less sensitive than fluorogenic probes. On the basis of current evidence, the enzymatic assay is appropriate for measuring serum/plasma TC concentration.

3.2.1.5. Fasting triglycerides Cross-sectional and cohort studies have shown that atherogenic dyslipidemia is a risk factor for CVD [57,145–148]. This explains why high TG and low HDL are two of the components of the so-called Metabolic Syndrome.

To evaluate the appropriateness of fasting TG as risk factor for CHD, the literature deriving from database #13 was critically evaluated (see Table 1).

The biological intra-individual variability of TG concentration is related mostly to lifestyle, disease and use of medications. Although the traditional method to assess TG levels is following an overnight fasting, a non-fasting measurement may be more informative. Some studies have shown that non-fasting TG levels are better predictors of CVD compared to fasting TG levels which, are usually displaced by HDL-C in multivariable models. However, in a number of intervention RCTs fibric acid derivatives were effective in reducing CHD risk at least in the subgroup of patients with overt "atherogenic dyslipidemia". In this subgroup a minimal increase in HDL-C and a robust reduction in TG were evident [48]. This observation, coupled with the failure of pure HDL-C modifiers (see 3.2.1.3) to reduce cardiovascular risk, strongly suggests that TG reduction may be causally linked to the reduction in CHD risk, albeit only in patients with "atherogenic dyslipidemia". Furthermore, mendelian randomization studies consistently demonstrated, in sharp contrast to HDL-C that genetically determined TG levels are causally linked to CHD risk. However, although TG have been proposed as a modifiable CHD risk factor in individuals with "atherogenic dyslipidemia", the case is much less strong than for LDL-C, [149]. It remains to be determined

whether, in the clinical arena, non-fasting TG, small dense LDL or TRL are better predictor or modifiers of CHD risk than fasting TG. A small number of individuals display isolated fasting hypertriglyceridemia, which quite often exceeds 1000 mg/dL. In these individuals, the immediate risk is not of a cardiovascular event, but of acute pancreatitis.

In conclusion, fasting TG may not be appropriate to estimate changes in CHD risk, because their relationship to CHD disease is fraught with too many confounders. Therefore, fasting TG are not appropriate to be used alone for the substantiation of health claims in the context of reduced CHD risk.

3.2.1.5.1. Enzymatic assays Please refer to Section 3.1.6.3.1.

3.2.1.6. Homocysteine High levels of homocysteine may negatively affect cardiovascular function through several mechanisms: i) increased formation of ROS inducing a reduction in NO availability; ii) increased thickening of the arterial wall; iii) increased metalloproteinase activity; iv) impaired platelet function leading to increased platelet adhesion and aggregation. This latter mechanism links elevated blood plasma homocysteine levels with the occurrence of a more thrombogenic vascular wall profile and with the propensity of more "aggressive" circulating platelets, with increased risk of forming firmer fibrin clots and thrombus formation [150]. For this reason, the maintenance of normal homocysteine levels is beneficial.

To evaluate the appropriateness of homocysteine as risk factor for CHD, the literature deriving from database #22 was critically evaluated (see Table 1).

Plasma homocysteine is a specific sensitizer of blood platelet response to agonists. The mechanisms through which elevated homocysteine levels induce vascular injury and promote thrombosis remain unclear. Vascular lesion caused by hyperhomocysteinemia includes endothelial cell lesion, vascular smooth muscle growth, increased platelet adhesiveness, increased LDL-C oxidation with deposition on the vascular wall and direct activation of the coagulation cascade [151]. It is still debated in the literature whether homocysteine may directly influence platelet function or whether the effect is secondary to its action on the endothelium, blood cells, or other factors. High plasma homocysteine levels, due to genetic defects, causes CHD. It was therefore hypothesized that higher levels might appreciably increase CHD risk [152]. While an increase in homocysteine concentration is associated with an increased risk of CHD, a reduction in homocysteine concentration has not generally been shown to reduce this risk [153,154]. Retrospective studies originally suggested a strong relationship, but subsequent prospective observational studies suggested weaker associations. Some epidemiologic studies, but not others, indicated that the elevated homocysteine represents a risk factor for total death, CHD death, or cardiovascular mortality in the general population [150]. So, there is no clear consensus that decreasing homocysteine concentration decreases CHD

risk, and therefore it cannot used alone for the substantiation of health claims in the context of reduced risk of CHD.

3.2.1.6.1. LC-MS/MS Please refer to Section 3.1.10.1.1.

3.2.2. Stroke

A stroke occurs when an interruption or a severe reduction of the blood supply to a part of the brain makes the brain tissue lacking of oxygen and nutrients. There are two types of stroke:

1) Ischaemic stroke (most common): it is usually caused by a lack of blood flow as a result of an obstruction within a blood vessel supplying blood to the brain. The underlying condition is the development of atherosclerosis (fatty deposits lining the vessel walls) and is the same process that causes CHD. A clot may form in an artery in the brain itself (cerebral thrombosis), or in another location in the circulatory system, usually the heart and large arteries of the chest or neck (cerebral embolism) enters the bloodstream and travels through the brain's blood vessels until it reaches vessels too small where it becomes lodged.

2) Haemorrhagic stroke (least common): happens as a consequence when a weakened blood vessel in the brain (aneurysms or arteriovenous malformations) leaks or bursts (ruptures). The blood generally accumulates so compressing the surrounding brain tissues. There are two types of haemorrhagic stroke called intracerebral (inside the brain tissue) and subarachnoid (near the surface of the brain). High BP and/or diseases are usually the causes of this type of stroke.

The main risk factors for a stroke include age, cigarette smoking, high BP, high blood cholesterol, diabetes, alcohol and illegal drug use, previous stroke or transient ischaemic attack, existing heart and blood vessel diseases, including disorders of heart rhythm (such as atrial fibrillation), CHD and peripheral arterial disease.

The risk of stroke begins to increase at BP readings higher than 120/80 mm of mercury (mmHg). At younger ages, men have a higher risk of stroke than women, but women are more likely to die of strokes than men. Women who take birth control pills also are at slightly higher risk of stroke. African-Americans, Alaska Native and American-Indian have a higher risk of stroke than do people of other races.

3.2.2.1. Systolic blood pressure To evaluate the appropriateness of SBP as risk factor for stroke, the literature deriving from database #14 was critically evaluated (see Table 1).

Day-time and night-time BP values and changes obtained with treatment are related to each other. BP may increase with the ageing process as blood vessels become stiffer. Furthermore, several factors, including

emotion (white coat effect), exercise, temperature, and circadian fluctuations may influence punctual BP levels [75,76]. Therefore, as previously mentioned, isolated measurement of BP could provide limited information with respect to monitoring (i.e. 24 h ABPM) which may be useful in unveiling hypertension when a high BP variability is registered between measurements during the same or different visits. Importantly, patients in which the nocturnal decline in BP is reduced or abolished (non-dipper), are at increased risk for cerebrovascular complications than are individuals with a dipper circadian rhythm [79,84]. . Within-individual systolic BP variability is a risk factor for stroke and cardiovascular events, independent of mean absolute BP level. It is well known that lowering SBP by 10 mmHg or diastolic BP by 5 mmHg using any of the main classes of BP lowering drugs reduces the risk of stroke by approximately a third [155].

In conclusion, SBP can be considered as a risk factor to be used for the substantiations of health claims in the context of reduced risk of stroke.

3.2.2.1.1. Office, home and 24 h ambulatory blood pressure Please refer to Sections 3.1.7.1.1, 3.1.7.1.2 and 3.1.7.2.1.

3.2.3. Hypertension

As a term used to define a condition of high or raised BP, hypertension is characterized by the long-term force of the blood against the artery wall which contributes to heart disease, stroke, kidney failure, as well as premature death. Even if it rarely causes symptoms in the early stages, damage to blood vessels and heart may occur. An early detection, in conjunction to an adequate treatment and a good control of this pathology, can provide important heath and economic benefits preventing the complications of hypertension, which may lead to expensive invasive interventions, including carotid or cardiac bypass surgery. Values lower than 140 and 90 mmHg have been long considered as normal for adults. However, in 2004, the Seventh Report of Joint National Committee on Prevention, Detection, Evaluation, and Treatment of high blood pressure proposed a reclassification of BP levels. This revision was determined by the new data on lifetime risk of hypertension and the increase in the risk of cardiovascular complications associated with values of BP around the cutoff of "normal" BP. It has been introduced the "prehypertension" category which ranges from 120 to 139 mmHg systolic and 80−89 mmHg diastolic BP. Subjects within these intervals have high risk of developing hypertension but can retard the progression of BP to hypertensive levels with age or prevent hypertension entirely adopting healthy lifestyles. At the present, the normal values of BP are considered those corresponding to <120/80 mmHg. It has been established that hypertension starts from ≥140/90 mmHg and can be classified as "stage 1" (140−159/90−99 mmHg) and "stage 2" (≥160/100 mmHg).

In 2000, worldwide prevalence of hypertension was estimated to be 26.4%, expected to increase involving until 1.56 billion in 2025. This phenomenon can be attributed to population growth, ageing and behavioural risk factors, such as unhealthy diet, excess intake of alcohol, lack of physical activity, excess body weight and exposure to persistent stress. People with hypertension often suffer from other detrimental health conditions, such as obesity and/or diabetes mellitus that, in conjunction with high BP, play an important role increasing the risk of CVD and kidney failure. However, estimates on mortality prevalence attribute to hypertension approximately 9 million deaths every year, with a disproportion in low- and middle-income countries due to the weakness of healthcare systems.

3.2.3.1. Arterial stiffness Arterial stiffness can be explained as a condition in which the ability of expansion and contraction of an artery in response to pressure modifications is limited. This vascular impairment takes origin from a complex interaction between endogenous (structural, cellular, hormonal) and exogenous (dietary) factors. Adverse structural and functional alterations within the vessel wall cause a reduction of arterial compliance. Raised central arterial stiffening represents a manifestation of ageing process as well as a consequence of several cardiometabolic diseases, including diabetes, atherosclerosis and chronic renal disturbs. Owing to the availability of several techniques and calculations used to evaluate arterial stiffness, its assessment is challenging [98]. Among the various measures to assess arterial stiffness, pulse pressure is one of the most used [94].

To evaluate the appropriateness of arterial stiffness as a risk factor for hypertension, the literature deriving from database #26 was critically evaluated (see Table 1).

Several measures can be used to assess arterial stiffness. Among these, pulse pressure is frequently chosen. However, cardiac function plays a role on the determination of pulse pressure which can be affected by heart rate, stroke volume and the pattern of ventricular ejection [156]. Therefore, as the gold standard measure of arterial stiffness, aortic pulse wave velocity is preferred, owing to its less sensitivity to cardiac function. Measures of wave reflection can be considered as potential surrogates of arterial stiffness [157]. Among these, AIx has been proposed. It represents the percentage of central pulse pressure influenced by a late systolic pressure rise due to overlap between the forward and reflected pressure waves [92]. The reason why AIx can be considered a measure of aortic stiffness depends on the fact that in presence of an increment of aortic pulse wave velocity, reflected waves should return to the heart earlier, and, as a result, overlap between the forward and reflected waves progressively increases and pressure augmentation becomes greater [96]. Thus, AIx and aortic stiffness appears strongly related in young adults, whereas this relation fails after about 60 years of age when augmentation tends to decrease and aortic pulse wave velocity, pulse pressure and CVD risk increase dramatically. On the basis of these observations, wave reflection contributes minimally to the rise of pulse pressure in the elderly [158]. A major contribution is due to an increase of aortic impedance. As a result, forward wave amplitude rises, while the blood flow remains

constant or decreases. Pressure wave reflection in the arterial system helps the blood to return to the aorta during diastole and enhances the diastolic perfusion of coronary beds [159]. To obtain a surrogate marker of arterial stiffness, it has been assumed that the inflection point marks the time of return of the reflected wave. However, several reports have shown that the time-related changes of the inflection point are associated poorly with the change of arterial system [156]. This lack of association together with the simplistic assumption of a single-tube model with distal reflection impedes an accurate estimate of pulse wave velocity by means of the return time. Likewise, a return time estimated from the foot of the reflected wave does not give information on stiffness. In other words, pulse wave velocity is an inappropriate surrogate measure of aortic stiffness [99].

However, according to the AHA statement, pulse wave velocity is a reasonable clinical measure of arterial stiffness (class IIa, Level of Evidence A) [98].

Despite arterial stiffness can be considered as associated with increased blood pressure (see 3.1.7.4), there is no sufficient evidence demonstrating that a reduction in arterial stiffness univocally leads to a reduction in hypertension. For this reason, arterial stiffness, neither evaluated through the calculation of AIx, nor through measurement of the return time of reflected wave, could be considered supportive but not sufficient for the substantiation of hypertension risk reduction claims. Other outcome variables need to be evaluated for this purpose, such as SBP and DBP.

3.2.3.1.1. Pulse wave analysis Please refer to Section 3.1.7.4.1.

3.3. Claims referring to Children's development Art. 14.1 (b)

3.3.1. Protection of DNA, protein and lipid oxidative damage

The protection of DNA, proteins and lipids from oxidative damage has been considered a beneficial effect in the context of child development (in detail, referred to infants and young children from birth to three years of age) [160]. The claim refers to two claims previously assessed with a favourable outcome on protection of DNA, proteins and lipids from oxidative damage [161,162].

For the three types of macromolecules (DNA, proteins and lipids), several OVs and related MMs have been proposed.

For the description and the critical evaluation of the OVs and MMs, please see:

- Protection of DNA: see Section 3.1.3
- Protection of lipids: see Section 3.1.4
- Protection of proteins: see Section 3.1.5

4. Conclusions

To date a high number of requests for authorization of health claims pursuant to Article 13(5) and 14 of Regulation (EC) No 1924/2006 has received a negative opinion

from EFSA. One of the most critical limitations is the design of human intervention studies aiming to proof efficacy, including the proper choice of OVs and of their MMs. The present paper provides information concerning the collection, collation and critical analysis of claimed effects, OVs and MMs that have been proposed so far in the context of protection against oxidative damage and cardiovascular health compliant with the European Regulation. The information provided in this work could serve as basis for EFSA to develop further guidance to applicants in the preparation of applications for authorization of health claims in the context of protection against oxidative damage and cardiovascular health. In addition, it could be useful for applicants during the design or selection of human intervention studies aimed to substantiate such health claims. Finally, the results of this critical review work might have an impact on general research and will be useful for the design of human studies, independently from health claims substantiation. In this scenario, further research is needed to validate emerging OVs and the related MMs that were not included in this work (due to the project search strategy applied), but could be used in the near future for the substantiation of health claims.

In addition, it is worth repeating that many other issues, such as a proper sample size and an adequate statistical analysis, are required for receiving a positive opinion from EFSA.

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