

**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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28 **Keywords:** health claims, claimed effect, outcome variable, method of measurement, oxidative damage,  
29 cardiovascular health

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31 **Acronyms:**

32 8-Iso-PGF2 $\alpha$ : 8-iso-15(S)-Prostaglandin F2 $\alpha$

33 8-OHdG: 8-hydroxy-2-deoxy-guanosine

34 8-oxo-dG: 8-oxo-2-deoxyguanosine

35 ABPM: Ambulatory blood pressure monitoring

36 ADHP: 10-acetyl-3,7-dihydroxyphenoxazine

37 ADP: Area under the diastolic decay portion of the pulse pressure curve

38 ADP: Adenosine diphosphate

39 AIx: Augmentation Index

40 ARE: Antioxidant response element

41 ATP: Adenosine Triphosphate

42 AUC: Area under the curve

43 BP: Blood pressure

44 BPV: Blood pressure variability

45 CAD: Coronary artery disease

46 CAT: Catalase

47 CETP: Cholesteryl ester transfer protein

48 CHD: Coronary heart disease

49 CL-HPLC: Chemiluminescence-based high pressure liquid chromatography

50 CM: Chylomicrons

51 COX: Cyclooxygenase

52 CRP: C-reactive protein

53 CV: Cardiovascular

54 CVD: Cardiovascular disease

55 DAP: Dihydroxyacetone phosphate

56 DBP: Diastolic blood pressure

57 DNA: Deoxyribonucleic acid

58 DNP: Dinitrophenyl

59 DNPH: Dinitrophenylhydrazine

60 ECD: Electrochemical detection

61 EDHF: Endothelium-derived hyperpolarization factor

62 EDTA: Ethylenediaminetetraacetic acid

63 ELISA: Enzyme-linked immunosorbent assay

64 Endo III: Endonuclease III

65 eNOS: endothelial NO synthase

66 ET-1: Endothelin-1

67 F2-IsoPs: F2-isoprostanes

68 FL: Fluorescence

69 FMD: Flow-mediated dilation

70 FPG: Formamidopyrimidine DNA glycosilase

71 G1P: Glycerol-1-phosphate

72 GC: Gas chromatography

73 GCL:  $\gamma$ -glutamylcysteine ligase

74 GPO: Glycerol phosphate oxidase

75 GSH: Glutathione

76 GSH-Px: Glutathione peroxidase

77 GSSG: Glutathione disulfide

78 HDL: High-density lipoprotein

79 HDL-C: High-density lipoprotein cholesterol

80 HO: Heme oxygenase

81 HPLC: High performance liquid chromatography

82 IDL: Intermediate-density lipoprotein

83 IsoPs: Isoprostanes

84 LC-MS/MS: Liquid chromatography tandem mass spectrometry

85 LDL: Low-density lipoprotein

86 LDL-C: Low-density lipoprotein cholesterol

87 LTA: Light transmission aggregometry

88 MDA: Malondialdehyde

89 MS: Mass spectrometry

90 NEFA: Non-esterified fatty acids

91 Non-HDL-C: Non-high density lipoprotein cholesterol

92 Ox-LDL: Oxidized LDL

93	PAI-1: Plasminogen activator inhibitor-1
94	PC: Phosphatidylcholine
95	PCOOH: Phosphatidylcholine hydroperoxide
96	PCSK9: proprotein convertase subtilisin/kexin type 9
97	PDP: End-diastolic aortic blood pressure
98	PGI2: prostacyclin 2
99	P-sel: P-selectin
100	PSP: End-systolic aortic blood pressure
101	R: Total peripheral resistance (mean arterial BP divided by the mean blood volume flow)
102	RCT: randomized controlled trial
103	ROS: Reactive oxygen species
104	RXNO: Nitroso/nitrosyl species
105	SAC: Systemic arterial compliance
106	SBP: Systolic blood pressure
107	SBs: Strand breaks
108	SOD: Superoxide dismutase
109	SV: stroke volume
110	TBA: Thiobarbituric acid
111	TBARS: thiobarbituric acid reactive substances
112	TC: Total cholesterol
113	TG: Triglycerides
114	TRAP: Thrombin receptor activating peptide agonist
115	TRL: Triglycerides rich lipoproteins
116	TXA2: Thromboxane A2
117	TXB2: Thromboxane B2
118	US: ultrasound
119	UV: Ultraviolet detection
120	VLDL: Very-low-density lipoprotein
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## **1 INTRODUCTION**

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## **5 ACKNOWLEDGMENTS**



127    **ABSTRACT**

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

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

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

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

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## 155 1 INTRODUCTION

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

168 Nevertheless, to date a huge number of unacceptable requests of authorisation to apply health claims on food  
169 has been proposed from stakeholders to the European Food Safety Authority (EFSA). The main concerns  
170 include the insufficient characterization of food/food constituent(s), the lack of beneficial physiological  
171 effect of the proposed claimed effect and, above all, the quality of the studies provided for the scientific  
172 substantiation of the claims. The most critical points involve the design and the strength of the studies  
173 provided in the application, including the proper choice of outcome measures and their methods of  
174 measurement.

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

182 The present manuscript gathers information in the context of protection against oxidative damage and  
183 cardiovascular health, reporting the results obtained from the collection, collation and critical analysis of the  
184 information in relation to claimed effects, outcome variables (OVs), and methods of measurement (MMs).

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## 186 **2 STRATEGY**

187 The manuscript refers to outcome variables and methods of measurement collected from the relative  
188 Guidance document (EFSA 2011), from the applications for authorization of health claims under Articles  
189 13.5 and 14 of Regulation 1924/2006 related to oxidative damage and cardiovascular health  
190 (ec.europa.eu/nuhclaims/), as well as from comments received during public consultations. The OVs and  
191 their MMs were considered only if the food/food constituent(s) was sufficiently characterized and the  
192 claimed effect, suitably defined, provided a beneficial physiological effect. Following this decision tree, 11  
193 claimed effects with 32 outcome variables were evaluated under Article 13.5, whereas 4 disease risk  
194 reduction claims and 1 claimed effect referred to children development were selected under the Article 14.  
195 For each OV, all the MMs proposed in the scientific opinions and/or in the Guidance documents were  
196 included in the evaluation. If no methods were proposed or any proposed method was considered  
197 inappropriate, also the best or the most widely used method was included. Subsequently, different databases  
198 of references were created on PubMed based on the keywords defined from each OV, in order to permit a  
199 specific critical analysis of the OVs and the MMs. The critical evaluation for each outcome variable and  
200 method of measurement was performed following a review of the literature deriving from the so obtained  
201 databases.

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## 203 **3 CRITICAL EVALUATION OF OUTCOME VARIABLES AND METHODS OF MEASUREMENT**

### 204 ***3.1 CLAIMS FALLING UNDER ART. 13(5)***

#### 205 **3.1.1 Protection of cells against oxidative damage**

##### 206 ***3.1.1.1 Antioxidant enzymes***

207 The harmful effects of free radicals and peroxides are controlled *in vivo* by a wide spectrum of anti-oxidative  
208 defence mechanisms, including vitamins (e.g. E and C), carotenoids, other metabolites (e.g. glutathione) and  
209 antioxidant enzymes. These compounds represent a first line of defence against oxidative stress, acting  
210 through the removal of key reactive oxygen species, and play an important role in the human antioxidant

211 network protecting cells and biomolecules from excessive oxidative damage. The main enzymes in this line  
212 of defence include superoxide dismutase (SOD, catalyzing dismutation of the superoxide anion into H<sub>2</sub>O<sub>2</sub>),  
213 catalase (CAT, detoxifying H<sub>2</sub>O<sub>2</sub>) and glutathione peroxidase (GSH-Px, removing H<sub>2</sub>O<sub>2</sub> and converting lipid  
214 peroxy radicals to nontoxic alcohols) [9]. Low levels of active antioxidant enzymes promote oxidative  
215 stress, with increased endogenous formation of malondialdehyde (MDA) and isoprostanes (IsoPs) and trigger  
216 inflammatory processes, leading to endothelial dysfunction.

217 To evaluate the appropriateness of antioxidant enzymes as outcome variable of antioxidant status and  
218 antioxidant defence, database #1 was generated (See Tab.1).

219 Antioxidant enzymes have been measured as biological indicators of many pathological conditions,  
220 including hyperlipidemia, atherosclerosis and diabetes. However, the antioxidant defence system is a  
221 complex network, with interactions and synergistic mechanisms that must be taken into account for a critical  
222 evaluation. In detail, when a single measure of antioxidant status is considered in isolation, it is often  
223 difficult to critically evaluate whether the food/food component up-regulates the antioxidant enzyme (to  
224 increase body's antioxidant protection), or it acts as a pro-oxidant agent (causing oxidative stress itself and  
225 forcing the body to up-regulate the antioxidant enzyme to protect itself against the pro-oxidative component).  
226 In addition, antioxidant enzymes exhibit a wide inter-individual variability, which should be considered  
227 when they are measured to substantiate a possible health benefit linked to the intake of a specific food or  
228 food component.

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

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#### 235 3.1.1.1.1 Enzymatic assays

236 The most important parameter determining the biological impact of the antioxidant enzymes is their activity.  
237 The measurement of the activity of the antioxidant enzymes is traditionally performed by enzymatic assays  
238 and native gels [10]. The activity assay requires 10-fold more protein than the gel assays but has the

239 advantage of giving a quantitative result; on the contrary, the native gel requires a lower amount of protein,  
240 but has the drawback of providing only qualitative results.

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

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

248 Besides enzymatic assays, standardized immunohistochemical and immunofluorescence techniques are  
249 commonly used to determine endogenous antioxidants in most laboratories. The antibodies used in these  
250 applications are commercially available and can be used on both fresh or fixed tissues and cells.

251 Immunohistochemical analysis is another method for determining cell-specific antioxidant expression levels.  
252 However, it does not measure the activity of the enzyme and, because the protein can be expressed while  
253 remaining inactive (especially in disease conditions), immunohistochemical analysis is not the ideal method  
254 for measuring the antioxidant defence status.

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

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#### 258 3.1.1.2 Heme oxygenase

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

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

264 To evaluate the appropriateness of HO as outcome variable of antioxidant status and antioxidant defence, the  
265 literature deriving from database #2 was critically evaluated. HO is the enzyme responsible for physiologic  
266 heme degradation into equimolar amounts of CO and biliverdin, and releases free iron. The degradation of

267 heme is considered a critical step in cellular defence, because of the pro-oxidant heme removal and the  
268 increased production of bilirubin and CO. It has been suggested that the inducible form HO-1 may represent  
269 a non-specific response to oxidative stress conferring protection against oxidative stress[11]. Moreover, the  
270 increase of HO-1 activity is correlated with the increase of CuZn SOD activity, suggesting a synergistic  
271 antioxidant action of the two enzymes.

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

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#### 277 3.1.1.2.1 Chromatographic techniques

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

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

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

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

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

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

297

### 298 3.1.1.3 Glutathione

299 Glutathione (GSH,  $\gamma$ -L-glutamyl-L-cysteinyl-glycine) is a tri-peptide present in relatively high levels in  
300 mammalian cells (1–10 mM). Micromolar concentrations are typically detected in plasma, while very high  
301 concentrations are present in erythrocytes.

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

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

317 To evaluate the appropriateness of GSH as outcome variable of antioxidant status and antioxidant defence,  
318 the literature obtained from database #3 was critically evaluated (see Tab.1).

319 GSH levels reported in the literature may exhibit a 10-fold variation and errors may arise during GSH  
320 determination (e.g. GSH autooxidation or haemolysis causing overestimation in view of the very high GSH  
321 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 bio-molecules 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.

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#### 3.1.1.3.1 Chromatographic techniques

Several analytical methods for the measurement of GSH are available. The measurement of glutathione 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-UV, with electrochemical detection (ECD) and fluorescence (FL) 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



350 haemolysis may cause overestimation in plasma samples. HPLC coupled with ECD has some advantages,  
351 allowing the simultaneous determination of GSH and GSSG without prior treatment or derivatization. More  
352 recently, mass spectrometric (MS) detection has been proposed for the quantification of blood GSH and  
353 GSSG contents, once again without derivatization steps, owing to the sensitivity and selectivity of the  
354 method[14]. The comparison between methods is often difficult, especially because of the high variations of  
355 GSH levels reported in the literature (partially due to errors arising from different phases of GSH  
356 determination) and because GSH and GSSG can be expressed relative to different parameters (e.g. tissue  
357 weight, cell number).  
358 However, on the basis of current evidence, HPLC methods employing electrochemical or mass spectrometric  
359 detectors can be properly used for the measurement of GSSG and GSH in biological fluids.

360

### 361 **3.1.2 Protection against non-oxidative DNA damage**

#### 362 *3.1.2.1 Spontaneous DNA SBs*

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

368 To evaluate the appropriateness of spontaneous DNA SBs as outcome variable of generic DNA damage, the  
369 literature deriving from database #5 was critically evaluated (see Tab.1).

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

372 It is widely recognised that DNA damage, mainly in the event of double SBs, plays a major role in  
373 mutagenesis, carcinogenesis (e.g. in chromosomal rearrangements and deletions, and in mitotic  
374 recombination in somatic cells), and ageing. SBs may produced by several exogenous agents, such as  
375 ionizing radiation, but also occurs spontaneously during cellular processes. Oxidative DNA damage caused  
376 by reactive oxygen species (ROS) generated during metabolic endogenous processes makes a significant

377 contribution to SB formation. To repair this potentially lethal damage, eukaryotic cells have evolved a  
378 variety of repair mechanisms, collectively known as non-homologous DNA end joining.  
379 As DNA SBs may be induced by several factors, not only by oxidative processes, this outcome variable  
380 cannot be considered appropriate for the substantiation of health claims in the context of protection against  
381 oxidative damage to DNA. However, as spontaneous DNA SBs are associated with an altered cell function  
382 spontaneous DNA SBs are appropriate for the substantiation of health claims in the context of protection  
383 against generic DNA damage.

384

#### 385 3.1.2.1.1 Comet assay

386 The Comet Assay is a very simple, fast and cheap technique for the detection of oxidative and non-oxidative  
387 DNA damage in single eukaryotic cells. In its traditional version, the alkaline comet assay detects DNA  
388 migration caused by strand breaks, alkaline labile sites, as well as repair sites. Individual cells are embedded  
389 in a thin agarose gel on a microscope slide. A lysis step removes all cellular proteins from the cells. DNA is  
390 then allowed to unwind under alkaline/neutral conditions, and subjected to electrophoresis during which  
391 damaged DNA migrates away from the nucleus. The DNA is then stained with a DNA-specific fluorescent  
392 dye, and the gel is read for the amount of fluorescence in head and tail. The extent of DNA liberated from the  
393 head of the comet is directly proportional to the amount of DNA damage. The term "comet" refers to the  
394 image of DNA migration obtained through the electrophoresis gel, which often resembles a comet. The  
395 traditional comet assay can be appropriately applied to measure spontaneous DNA strand breaks (SBs).  
396 However, owing to the lack of specificity for oxidative damage, it is not appropriate for evaluating *in vivo*  
397 oxidative damage to DNA. The same consideration can be made for other variants of the Comet Assay,  
398 which determine resistance against oxidative modification using *ex vivo* pro-oxidant challenges (e.g. H<sub>2</sub>O<sub>2</sub>).  
399 On the other hand, specific oxidized bases can be readily detected using the Comet Assay by incorporating in  
400 the assay (after cell lysis) a step in which DNA is incubated with a lesion-specific enzyme that converts the  
401 altered bases (purines or pyrimidines) to strand breaks. The most common enzymes employed for this  
402 purpose are formamidopyrimidine DNA glycosylase (FPG), which recognizes and removes oxidatively  
403 damaged purines (e.g., 8-oxo-7,8-dihydroguanine) and endonuclease III (Endo III) for pyrimidines. This  
404 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 outcome variable of oxidized damage to DNA, the literature deriving from database #4 was critically evaluated (see Tab.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 it is not appropriate for the substantiation of health claims in the context of protection against oxidative damage to DNA.

438

#### 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, GC-MS, and 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.

455

#### 3.1.3.1.2 *Comet assay*

Please refer to 3.1.2.1.1 “Comet assay”

458

### 3.1.4 **Protection against oxidative damage to lipids**

#### 3.1.4.1 *F2-isoprostanes*

461 F2-isoprostanes (F2-IsoPs) are a relatively newly discovered class of molecules, originating from oxidation  
462 of polyunsaturated fatty acids. They include a series of prostaglandin F2a-like compounds produced *in vivo*  
463 by non-enzymatic peroxidation of arachidonic acid, esterified in phospholipids and subsequently hydrolysed  
464 to a free acid form by platelet-activating factor acetylhydrolase[18]. F2-IsoPs are released from the cell  
465 membrane into circulation by phospholipases and can be quantified in all human tissues and biological  
466 fluids, including plasma and urine. A high level of F2-IsoPs has been observed in a number of conditions  
467 associated with increased oxidative stress, including diabetes and neurodegenerative and pulmonary disease.  
468 Compared to other markers of oxidative damage, F2-IsoPs are very specific and chemically stable end-  
469 products of polyunsaturated fatty acid peroxidation, and, for this reason, are the most well studied markers of  
470 oxidative stress[19]. Among the F2-isoprostane isomers, 8-Iso-PGF2 $\alpha$  has very high concentration in  
471 biological fluids and is the most commonly evaluated.

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

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

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

484

#### 485 3.1.4.1.1 *Chromatographic techniques*

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

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

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

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

499

#### 500 *3.1.4.1.2 Immunological techniques*

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

508 Studies comparing immunological methods to GC/MS have shown at least 30-fold higher levels with  
509 immunoassays, with no clear correlation with GC/MS[21]. Part of this difference may be because  
510 immunoassay methods, while being able to discriminate against most isomers, are unable to prevent cross-  
511 reactivity with all or some of their metabolites.

512 On the basis of these considerations, more studies are required comparing immunoassay methods to mass  
513 spectrometry or more specific and accurate ELISA kits need to be developed. Until then, the use of  
514 immunological techniques in measuring F2-IsoPs remains questionable.

515

#### 516 3.1.4.2 Oxidized LDL

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

527 To evaluate the appropriateness of oxidized LDL as outcome variable of oxidized damage to lipids, the  
528 literature deriving from database #7 was critically evaluated (see Tab.1).

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

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

536 Human studies evaluating the association of Ox-LDL with atherosclerosis or cardiovascular events have  
537 been highly conflicting, and many clinical trials failed to prove the oxidative theory of atherosclerosis.  
538 Despite the present absence of a convincing link between the levels of Ox-LDL and atherosclerosis onset or  
539 development, in the framework of the functional claim, Ox-LDL appears to be an appropriate outcome  
540 variable for the substantiation of health claims related to the reduction of oxidative damage to lipids.

541

#### 542 3.1.4.2.1 *Immunological techniques*

543 The assessment of human circulating Ox-LDL has become common after the introduction of several ELISA  
544 procedures, based on sandwich and competitive assays, partly because of the availability of commercial kits.  
545 Extensively oxidized LDL may have a very short half-life in human plasma, because they are rapidly cleared  
546 from the circulation via scavenger receptors. However, small but significant amounts of oxidized LDL are  
547 detectable in normal plasma using specific monoclonal antibodies. However, some studies reported that  
548 slight variations in the procedure were associated with substantial differences in the results[25].  
549 On the basis of the current evidence, immunological methods can be applied for measuring Ox-LDL, but  
550 great care should be used when evaluating the characteristics of a given assay, considering that even small  
551 variations in sensitivity and specificity may have profound effects on the obtained results.

552

#### 553 3.1.4.3 Phosphatidylcholine hydroperoxides

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

559 To evaluate the appropriateness of PCOOH as outcome variable of oxidized damage to lipids, the literature  
560 deriving from database #8 was critically evaluated (see Tab.1).

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

565 When evaluating lipid damage, the effect of the antioxidant systems in plasma should also be taken into  
566 account, considering that it has been reported that some glutathione peroxidases can directly reduce the  
567 concentrations of phospholipid hydroperoxide [26]. On the basis of the current evidence, PCOOH in blood or  
568 tissue is a sensitive and specific index of lipid peroxidation *in vivo* and an appropriate marker of lipid  
569 peroxidation to be used for the substantiation of health claims in the context of protection against oxidative



570 damage to lipids. However, the combined analysis of PCOOH and F2-isoprostanes (markers of PUFA  
571 oxidation) is preferable.

572

#### 573 3.1.4.3.1 *High pressure liquid chromatography*

574 Increased interest in lipid peroxidation has accelerated the development of techniques for the quantification  
575 of lipid peroxides in biological fluids. To this aim, many methods have been developed, mainly using liquid  
576 chromatography, which separates molecules in solution using their different affinity between a liquid mobile  
577 phase and a solid stationary phase. One of the most precise and accurate methods for the detection of lipid  
578 hydroperoxides is a chemiluminescence-based high-performance liquid chromatography assay (CL-HPLC).  
579 CL-HPLC systems were developed and applied for the hydroperoxide-specific determination of PCOOH in  
580 biological tissues, such as human blood plasma. This approach involves the separation of  
581 phosphatidylcholines from total plasma lipids with normal-phase silica gel HPLC and post-column detection  
582 of the hydroperoxide-dependent chemiluminescence of PCOOH. The method in use appear to be highly  
583 sensitive and specific for lipid hydroperoxides[27].

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

586

#### 587 3.1.4.4 Malondialdehyde

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

594 To evaluate the appropriateness of MDA as outcome variable of oxidized damage to lipids, the literature  
595 deriving from database #9 was critically evaluated (see Tab.1).

596 For many years, MDA has been used as a biomarker of lipid oxidation. However, its validity has been widely  
597 criticized for problems with post sampling formation and for the lack of specificity, both from biological and

598 methodological perspectives [28, 29]. As the biological perspective is concerned, MDA is not just a marker  
599 of lipid peroxidation.

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

608

#### 609 3.1.4.4.1 Chromatographic techniques

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

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

618 On the other hand, the use of HPLC or gas chromatography (GC) in repeated-measure studies appears to be  
619 appropriate for the detection and quantification of MDA derivatives, due to high specificity and sensitivity.

620 A number of HPLC methods have been developed based either on direct measurement of MDA or MDA-  
621 adducts or detection from UV/Vis and fluorescence instruments. Several methods have been proposed for  
622 quantifying both free MDA (unbound, detected without any hydrolytic sample treatment) or total MDA  
623 (bound to matrix molecules, requiring an hydrolytic step). The chromatographic approach is more advisable,  
624 as it allows a significant improvement of the specificity, including a step where the MDA-TBA adduct is  
625 quantified without confounding[30].

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

628

### 629 **3.1.5 Protection against oxidative damage to proteins**

#### 630 *3.1.5.1 Oxidative changes of amino acids in proteins*

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

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

644 To evaluate the appropriateness of oxidative changes of amino acids in proteins as outcome variable of  
645 oxidized damage to proteins, the literature deriving from database #10 was critically evaluated (see Tab.1).  
646 The ability to quantify distinct amino acid oxidation products in plasma or serum is a valuable tool for  
647 exploring the roles of different oxidation pathways in the pathogenesis of atherosclerosis and other  
648 diseases[31, 33].

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

653 appropriate for the substantiation of health claims in the context of protection against oxidative damage to  
654 proteins.

655

#### 656 *3.1.5.1.1 Chromatographic techniques*

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

667 On the basis of current evidence, the use of chromatographic techniques with mass spectrometry detection is  
668 appropriate for the in vivo measurement of oxidative damage to proteins[31].

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

672

#### 673 *3.1.5.2 Protein oxidation by-products (e.g. protein carbonyls)*

674 Oxidative damages to proteins, lipids, or DNA are all deleterious and may occur concomitantly[35].

675 However, proteins are possibly the most immediate vehicle for inflicting oxidative damage to cells, because  
676 they are more often catalysts rather than stoichiometric mediators. CO groups (aldehydes and ketones) are  
677 produced on protein side chains when they are oxidized. Protein carbonyls are therefore the biomarker that is  
678 generally used to estimate protein oxidation. These moieties are chemically stable, a useful feature for both  
679 sample storage and analytical detection[36]. Protein carbonyl derivatives can also be generated through  
680 oxidative cleavage of proteins by either the  $\alpha$ -amidation pathway or by oxidation of glutamyl side chains,

681 leading to formation of peptides in which the N-terminal amino acid is blocked by an  $\alpha$ -ketoacyl  
682 derivative[35]. Lysine, arginine, proline, and histidine are the amino acid residues most prone to form  
683 carbonyl derivatives.

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

686 Protein carbonyls are the most commonly measured products of protein oxidation in biological samples.  
687 However, they can be better regarded as a measure of the balance between oxidative and anti-oxidative  
688 processes involving proteins [35, 36]. Their use as a measure of oxidative damage may help identifying  
689 individuals at risk of developing diseases associated with oxidative damage to proteins[37]. However, there  
690 is a considerable variability in the basal levels of protein carbonyls in some tissues, also depending on how  
691 the carbonyl assay is performed, and the specificity is related to the tissue/sample used in the analysis[35,  
692 36]. Even if this bias would not be relevant in the framework of an RCT, many interfering factors, changing  
693 irrespectively of oxidative stress, could invalidate the use of protein carbonyls. Therefore, based on current  
694 data, the measurement of protein oxidation by-products, such as protein carbonyls, should be used only in  
695 combination with direct markers of oxidative damage to proteins *in vivo* for the substantiation of health  
696 claims in the context of improved protection against oxidative.

697

#### 698 3.1.5.2.1 Colorimetric procedures

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

708 However, this essay does not provide any information on the extent of oxidative damage to a particular  
709 protein in a complex mixture. In addition, this method requires a relatively high amount of proteins, is time-  
710 consuming and has high variability owing to many washing steps. Moreover, the quantitative derivatization  
711 requires a large amount of reagents.

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

716 The spectrophotometric DNPH assay coupled to protein fractionation by HPLC is far more sensitive than the  
717 spectrophotometric method alone, implying smaller quantities of the sample for the quantification of  
718 carbonyl content [38, 39].

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

722

#### 723 3.1.5.2.2 *ELISA methods*

724 In the late 90s, an ELISA method using an anti- DNP antibody was developed for measuring total protein  
725 CO groups. In its original version, the protein sample reacts with DNPH and is then adsorbed to the wells of  
726 an ELISA plate before reacting with anti-DNPH antibody[40].

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

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

736

### 737 **3.1.6 Improvement of blood lipid profile**

#### 738 *3.1.6.1 LDL-C*

739 As other fat-like substances, cholesterol needs specific proteins (lipoproteins) to be carried in the blood  
740 stream. Lipoproteins are usually classified on the basis of their density. In detail, from the lowest to the  
741 highest density, lipoproteins are classified as chylomicrons (CM), very low density lipoprotein (VLDL),  
742 intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL).  
743 The lipoproteins have different affinities for and behave differently with the transported cholesterol. A LDL-  
744 C particle is a microscopic micelle whose outer rim is made of lipoprotein and whose centre is made of  
745 cholesterol. Elevated LDL-C levels in the blood are associated with an increased risk of atherosclerosis and  
746 cardiovascular diseases (CVD) mostly because: 1) LDL-C tend to deposit into the walls of arteries; 2) The  
747 white blood cells try to digest LDL-C inside the arterial wall and in doing so they oxidize it and make it more  
748 toxic; 3) LDL-C attract other inflammatory cells inside arterial wall, creating persistent inflammation [41-  
749 50].

750 To evaluate the appropriateness of LDL-C as outcome variable of blood lipid profile, the literature deriving  
751 from database #12 was critically evaluated (see Tab.1).

752 LDL-C usually represents up to 60-70% of total serum cholesterol. The main protein found in LDL is  
753 apolipoprotein-B (apo-B) which contains a binding site that causes LDL to be deposited in the extracellular  
754 matrix of many tissues. LDL-C infiltration, accumulation and oxidation in the intima of endothelial vessels is  
755 one of the early and main features in the atherosclerotic process [45, 46, 48-52]. LDL oxidation is a  
756 prerequisite for macrophage uptake via unregulated macrophage scavenger receptors to form foam cells  
757 which accumulate in the plaque lipid core [53]. LDL particle size may also be important when assessing risk  
758 but its evaluation is not currently endorsed by clinical guidelines [54]. In conclusion, the measurement of  
759 blood LDL-C concentration appears to be appropriate for the substantiation of health claims in the context of  
760 improved lipid profile.

761

#### 762 *3.1.6.1.1 Enzymatic assays*

763 A number of methods are available for the determination of LDL-C [55]. The enzymatic assay involves the  
764 dissolution of non-LDL lipoprotein particles (HDL, VLDL and CM) but leaves LDL particles intact. The  
765 degradation of the dissolved cholesterol fraction is then carried out using two enzymes (cholesterol esterase  
766 and cholesterol oxidase). The remaining LDL particles are then solubilized and the soluble cholesterol and  
767 cholesterol esters are oxidized by cholesterol oxidase to produce hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). There are two  
768 types of probes for the enzymatic determination of H<sub>2</sub>O<sub>2</sub>: chromogenic and fluorogenic. In general,  
769 chromogenic probes are less sensitive than fluorogenic probes. In some studies, LDL-C has been calculated  
770 using Friedewald formula ( $\text{LDL-C} = \text{TC} - \text{HDL-C} - \text{TG}/2.2$  if concentrations are given in mmol/L, or  $\text{LDL-C}$   
771  $= \text{TC} - \text{HDL-C} - \text{TG}/5$ ) if given in mg/dL [56]. The use of this formula is associated with some problems: 1)  
772 methodological errors may accumulate because the formula requires three separate analyses of TC, TG, and  
773 HDL-C; 2) a constant cholesterol/TG ratio in VLDL is assumed, which is not always true 3) blood must be  
774 obtained under fasting conditions, which is sometimes impractical. For these reasons, many guidelines  
775 discourage the use of Friedewald formula [57, 58]. On the basis of current evidence, the enzymatic assay  
776 seems to be appropriate for measuring blood LDL-C and can be used for the substantiation of health claims  
777 in the context of improved blood lipid profile and of reduced risk of coronary heart diseases (see Section  
778 3.2.1.1).

779

#### 780 3.1.6.2 HDL-C

781 HDL contain cholesterol, phospholipids and triglycerides. HDL has been extensively investigated because it  
782 extracts cholesterol from tissues and delivers it to the liver, where it may be converted into bile acids and  
783 excreted (reverse cholesterol transport).

784 To evaluate the appropriateness of HDL-C as outcome variable of blood lipid profile, the literature deriving  
785 from database #12 was critically evaluated (see Tab.1).

786 HDL-C exerts cardioprotective effects, mainly by promoting reverse cholesterol transport, a process by  
787 which excess cholesterol is delivered from peripheral tissues back to the liver. Specifically, cholesterol efflux  
788 from macrophages to HDL-C plays an important role in protecting from the development and progression of  
789 atherosclerosis [47, 59]. HDL composition can be modified by oxidative mechanisms, which reduce the  
790 protein's ability to promote reverse cholesterol transport. In addition, recent studies have suggested to



791 include the evaluation of HDL function, together with HDL levels, to fully define the cardioprotective  
792 potential of this class of lipoproteins[43-46, 49, 59-63]. In conclusions, in the framework of functional  
793 claims, HDL-C can be considered an appropriate outcome variable for the substantiation of health claims in  
794 the context of improved lipid profile.

795

#### 796 *3.1.6.2.1 Enzymatic assays*

797 The concentration of HDL-C may be determined enzymatically with an assay that quantifies both cholesterol  
798 esters and free cholesterol [64]. Most of the available assays are of high quality, but the method used should  
799 be validated against the available reference methods. During the procedure, serum samples react with  
800 polyethylene glycol and all the VLDL and LDL are precipitated, while HDL remains in the supernatant.  
801 Cholesterol esters may be hydrolysed via cholesterol esterase into fatty acids and free cholesterol, making it  
802 possible to detect separately the two types of molecules in the presence and absence of cholesterol esterase.  
803 Cholesterol is then oxidized by cholesterol oxidase into the ketone cholest-4-ene-3-one, producing hydrogen  
804 peroxide. In the presence of peroxidase, the generated hydrogen peroxide reacts to form a photometrically  
805 measurable product. There are two types of probes for the enzymatic determination of H<sub>2</sub>O<sub>2</sub>: chromogenic  
806 and fluorogenic. The intensity of absorbance or fluorescence is proportional to cholesterol concentration and  
807 is easily measured. In general, chromogenic probes are less sensitive than fluorogenic ones. On the basis of  
808 the current evidence, enzymatic assays are appropriate to measure serum/plasma HDL-C concentration to be  
809 used for the substantiation of health claims in the context of Improved blood lipid profile and of Reduced  
810 risk of coronary heart disease (see Section 3.2.1.2).

811

#### 812 *3.1.6.3 Fasting triglycerides*

813 In the human body, triglycerides (TG) are primarily stored in adipocytes. TG are mobilized from adipocytes  
814 into the bloodstream, usually as NEFA and glycerol, under enzymatic, hormonal and neural control. TG are  
815 hydrophobic and travel in the bloodstream primarily by means of lipoproteins (triglyceride-rich lipoproteins,  
816 TRL), which are secreted by the liver (VLDL) and by the gut (CM). The American Heart Association  
817 presently suggests serum TG levels  $\leq 100$  mg/dL as “optimal” and  $\leq 150$  mg/dl as “acceptable”[65]. Low TG

818 levels are often associated with low HDL-C and high levels of small dense LDL and TRL remnants, a  
819 condition usually referred to as “atherogenic dyslipidaemia”.  
820 To evaluate the appropriateness of fasting TG as outcome variable of blood lipid profile, the literature  
821 deriving from database #13 was critically evaluated (see Tab.1).  
822 Overnight fasting represents the traditional and most widely used method for the assessment of TG levels for  
823 two main reasons: 1) the lower variability of TG measurements in the fasting state vs. the postprandial state;  
824 2) the almost exclusive use of the Friedewald equation to estimate LDL-C ( $LDL = TC - HDL - TG/5.0$   
825  $mg/dL$ ), which requires fasting TG concentration. Post-prandial TG assessment is generally dependent on the  
826 consumed fat amount. The ingestion of a high-fat meal results in a rise of circulating TG levels and in the  
827 production of TRL (CM and, secondarily, VLDL). During this physiological process these lipoproteins and  
828 their subclasses undergo variations in concentration and composition and are metabolized to atherogenic  
829 remnants. “Postprandial hyperlipidaemia” is defined as the quantitative/qualitative alteration of this normal  
830 process. However, the correlation between fasting and non-fasting triglyceride concentrations is usually high.  
831 In conclusions, in the framework of functional claims, the reduction of fasting TG levels is considered  
832 beneficial and blood TG measurement can be considered an appropriate outcome variable for the  
833 substantiation of health claims in the context of improved blood lipid profile.

834

#### 835 *3.1.6.3.1 Enzymatic assays*

836 TG are generally measured using accurate and cheap enzymatic techniques[66]. Such techniques are based  
837 on a lipase-induced enzymatic hydrolysis of the triglycerides to glycerol and free fatty acids. The glycerol  
838 released by this chemical reaction is measured by a coupled enzymatic reaction system. In the first analytical  
839 step, TG are hydrolysed to glycerol and free fatty acids by lipoprotein lipase. Glycerol is then  
840 phosphorylated by ATP forming glycerol-1-phosphate (G1P) and ADP. G1P is then oxidized by glycerol  
841 phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and  $H_2O_2$ .  $H_2O_2$  is then coupled with 4-  
842 aminoantipyrine and sodium N-ethyl-N-(3-sulfopropyl)m-anisidine by means of peroxidase to produce a  
843 quinoneimine dye that shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is  
844 directly proportional to the triglyceride content of the sample. There are two types of probes for the  
845 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 coronary heart disease (See Section 3.2.1.4).

#### 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 minutes after the consumption of a meal containing fat, and by a subsequent peak that occurs 3-4 hours 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[67].

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

Measurement of fasting TG is the traditional method to assess plasma TG levels. However, compared to fasting, a non-fasting measurement may be more informative even if there is no consensus about the normal range for non-fasting TG that can make results difficult to interpret. The ingestion of a high-fat meal results indeed in a rise in circulating TG and in the production of TRL (CM and, secondarily, VLDL). During this physiological process, these plasma lipoproteins and their subclasses undergo variations in concentration and composition and are metabolized to atherogenic remnants that appear to promote early atherogenesis with a possible negative impact on endothelial function. In addition, 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 (CRP). Standardized post-prandial testing should be used for post-

874 prandial plasma TG assessment [68-70]. In conclusion, the measurement of post-prandial blood TG  
875 concentration seems to be an appropriate outcome variable to use in combination with other biomarkers (e.g.  
876 HDL-C and LDL-C) for the substantiation of health claims in the context of post-prandial lipid profile.

877

#### 878 3.1.6.4.1 *Enzymatic assays*

879 Please refer to 3.1.6.3.1 “Enzymatic assay”.

880

### 881 3.1.7 Maintenance/reduction of blood pressure

#### 882 3.1.7.1 *Point systolic blood pressure*

883 The heart is a pump of the “two-stroke” variety, with a filling and an emptying phase. Because both the left  
884 and right hearts perform their work in a cyclic fashion, flow is pulsatile. Blood pressure (BP) is measured in  
885 millimetres of mercury (mm Hg). The mean BP in the large systemic arteries is approximately 95 mm Hg.  
886 This is a single time-averaged value but BP varies between from a systolic maximum (SBP,  $\leq 140$  mm Hg)  
887 corresponding to the contraction of the ventricle to a diastolic minimum, (DBP,  $\leq 90$  mm Hg) corresponding  
888 to the relaxation of the ventricle. Pulse pressure is the difference between SBP and DBP. The arterial  
889 pressure wave has an initial rapid rising phase followed by an early systolic peak, followed by a second late  
890 systolic bulge. The maximum SBP may occur either during the early or late wave, DBP is the minimum  
891 pressure at the end of the runoff period. Arterial pressure is one of the most important and widely assessed  
892 cardiovascular risk factors [71-75].

893 To evaluate the appropriateness of SBP as outcome variable of BP, the literature deriving from database #14  
894 was critically evaluated (see Tab.1).

895 BP homeostasis is complex and several factors can influence BP levels [71, 73, 76]. BP increases with  
896 ageing as blood vessels become stiffer. Emotions (as in the case of white coat effects, also called doctor’s  
897 office effect), exercise and increased body temperature are known factors increasing point BP[77, 78]. BP  
898 levels increase during the day and decrease during the night, as a result of circadian fluctuations. For the  
899 above reasons, the single measurement of point BP may provide limited information compared to the  
900 extended measurements made by (i.e. 24 h ABPM). ABPM is useful when high BP variability is observed  
901 during the same or different visits ABPM has also the ability to discriminate “dippers”, from “non-dippers”

902 i.e. the subjects in whom the physiological nocturnal decline of blood pressure is reduced or abolished [79-  
903 81]. In conclusion, SBP appears to be appropriate for the substantiation of health claims in the context of  
904 maintenance/reduction of BP, even though studies must be designed to account for intra-individual  
905 variability and SBP must be measured according to standardized protocols.

906

#### 907 *3.1.7.1.1 Office blood pressure*

908 Office BP should be assessed using a manual sphygmomanometer in both arms as a 10% difference in right  
909 and left arm readings exists. BP measurement requires that: 1) the arm is supported, with upper arm at heart  
910 level and feet on the floor (back supported, legs uncrossed); 2) BP is measured after at least 5 minutes  
911 resting; 3) BP should not be assessed when under stress, within 30 minutes of caffeine or tobacco  
912 consumption, or following exercise. For study purposes, due to the “white coat effect” and physiological  
913 fluctuations, BP should be measured every 2 minutes by the same investigator until two measurements, both  
914 systolic and diastolic, differ no more than 5 mmHg. The average of three readings (systolic and diastolic)  
915 should be calculated. To date, concerns about the accuracy of automated Office BP readings have been  
916 raised, particularly for their use in clinical studies[71, 80-84].

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

920

#### 921 *3.1.7.1.2 Home BP*

922 Affordable and validated automated BP devices, are commercially available for home BP self-measurement.  
923 These devices should be checked for accuracy at least every 1 to 2 years. Although home BP monitors may  
924 be inaccurate, depending on the accuracy threshold used, home BP measurement data usually correlate well  
925 with daytime BP ambulatory values[78, 81-83, 85, 86]. It has been argued that the ease of use of the  
926 electronic devices (aneroid sphygmomanometers) and their relative insensitivity to observer bias, can  
927 balance any inherent inaccuracy of the sphygmomanometer method[87]. Standardization of home BP  
928 measurements are currently lacking. On the basis of the current evidence, home BP does not appear to be  
929 appropriate for measuring point BP to be used in the substantiation of health claims.

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### *3.1.7.2 Point diastolic blood pressure*

As mentioned in the Section “Point systolic BP”, BP varies between a maximal SBP ( $\leq 140$  mm Hg) corresponding to the contraction of the ventricle, and a minimal DBP ( $\leq 90$  mm Hg) 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 DBP as outcome variable of BP, the literature deriving from database #14 was critically evaluated (see Tab.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 BP, several factors, including emotion (white coat effect), exercise, and body temperature, may influence point DBP[77, 78]. 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. ABPM systems have also the advantage to identify “non-dippers” i.e. subjects in whom the physiological nocturnal decline in BP is reduced or abolished[79-81]. In conclusion, 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.

#### *3.1.7.2.1 Office blood pressure*

Please refer to Section 3.1.7.1.1.

#### *3.1.7.2.2 Home blood pressure*

Please refer to Section 3.1.7.1.2

### 958 3.1.7.3 24 h Systolic blood pressure

959 As mentioned above, arterial pressure wave has an initial rapid rising phase followed by an early systolic  
960 peak, followed by a second late systolic bulge. The mean 24-h systolic BP gives a better approximation of  
961 the “true” BP[88].

962 To evaluate the appropriateness of 24h SBP as outcome variable of BP, the literature deriving from database  
963 #14 was critically evaluated (see Tab.1).

964 Repeated 24 h ABPM systems provide detailed and useful information about the circadian rhythm of BP:  
965 day-time and night-time BP profiles, day-night BP differences, morning BP surge and nocturnal dipping.  
966 Specifically, the ABPM is the sole tool to identify the absence of a nocturnal fall in BP, known as “non-  
967 dipping” status [79-81]. In addition, ABPM enables the identification of the white-coat phenomenon or of  
968 masked hypertension [77, 78, 89].

969 Finally, as several other factors, including emotions, exercise, and body temperature may influence point BP  
970 levels, the isolated measurement of BP could provide limited information with respect to monitoring  
971 systems, which may also be used when high BP variability is registered between measurements during the  
972 same or different visits[90]. In conclusion, the measurement of 24-h SBP appears to be appropriate to  
973 measure BP for the substantiation of health claims in the context of maintenance/reduction of BP.

974

### 975 3.1.7.3.1 24 h ambulatory blood pressure

976 ABPM is a non-invasive method that measure BP over a 24-hour period, thus recording BP variability not  
977 recovered by single measurements [84, 88]. ABPM devices consist of a BP cuff that is worn on the non-  
978 dominant arm and which is attached to a recorder worn on a belt. The ABPM device registers BP  
979 periodically (usually at 15- or 30-min intervals) during 24 hours, including sleep, with a total number of  
980 readings between 50 and 100. BP data are stored in the device and subsequently downloaded into device-  
981 specific computer software. ABPM allows the recording of average daytime, night-time, and 24-hour blood  
982 pressures (calculated as the arithmetic mean of all measurements and/or by the mean of hourly averages in  
983 the respective periods) which are the most commonly used variables[91]. ABPM devices are accurate  
984 enough for routine clinical use in most individuals. Factors such as age, weight, sex, and severity of  
985 hypertension may be associated with influence the measurement error but the differences are small enough to

986 be unlikely to affect clinical practice. On the basis of current evidence, 24-h ambulatory BP seems to be  
987 appropriate for measuring mean 24-h BP to be used for the substantiation of health claims in the context of  
988 maintenance/reduction of BP.

989

#### 990 *3.1.7.4 24 h diastolic blood pressure*

991 As mentioned in the Section “Point diastolic BP”, BP varies between a maximal SBP ( $\leq 140$  mm Hg)  
992 corresponding to the contraction of the ventricle, and a minimal DBP ( $\leq 90$  mm Hg) corresponding to the  
993 relaxation of the ventricle. For a detailed description see Section 3.1.7.2

994 To evaluate the appropriateness of 24h DBP as outcome variable of BP, the literature deriving from database  
995 #14 was critically evaluated (see Tab.1).

996 Repeated 24 h ABPM systems provide detailed and useful information about the circadian rhythm of DBP  
997 (for a detailed description, see Section 3.1.7.3). Nevertheless, as mentioned in Section 3.1.7.2, despite SBP  
998 and DBP are usually highly correlated, SBP may increase with ageing as vessels become stiffer, whereas  
999 DBP may decline leading to an increase in differential BP.

1000 On the basis of these considerations, the measurement of mean 24-h DBP appears to be appropriate for the  
1001 substantiation of health claims in the context of maintenance/reduction of BP only if accompanied by a  
1002 reduction in SBP.

1003

#### 1004 *3.1.7.4.1 24 h ambulatory blood pressure*

1005 Please refer to Section 3.1.7.3.1

1006

#### 1007 *3.1.7.5 Area under blood pressure curve*

1008 Arterial pressure is one of the most important and widely assessed cardiovascular risk factors[71-75, 92-94].

1009 The area under blood pressure curve (AUC BP) takes into account both the magnitude and the temporal  
1010 persistence of BP variations[95]. Specifically, repeated measures of SBP or DBP are taken during a time  
1011 period and graphically plotted. A software calculates the integral of the curve that is called AUC. The AUC  
1012 of both SBP and DBP can be calculated.



1013 To evaluate the appropriateness of AUC BP as outcome variable of BP, the literature deriving from database  
1014 #14 was critically evaluated (see Tab.1).

1015 AUC BP represents a time-weighted measure of BP and can accommodate the short-term fluctuations of an  
1016 individual's change pattern, thus correcting for short-term measurement variability [96]. The area under the  
1017 blood pressure curve can play a useful and complementary role in the evaluation of 24-h ABPM. One  
1018 limitation of the AUC measure is that it is strictly dependent on the baseline threshold considered [91]. In  
1019 conclusion, the measurement of AUC BP as a biomarker of BP appears to be appropriate for the  
1020 substantiation of health claims in the context of maintenance/reduction of BP, but studies need to be well  
1021 designed.

1022

#### 1023 *3.1.7.5.1 Home blood pressure*

1024 Please refer to Section 3.1.7.1.2

1025

#### 1026 *3.1.7.5.2 Office blood pressure*

1027 Please refer to Section 3.1.7.1.1

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#### 1029 *3.1.7.6 Central systolic blood pressure*

1030 Central BP is the pressure in the aorta, which not only serves as a conduit during systole but also acts as a  
1031 reservoir for blood during diastole. The pressure generated by left ventricle ejection causes a pressure pulse  
1032 wave through the arterial tree, which is reflected mainly in distal arteries, and interacts with the incident  
1033 waveform during early diastole, producing a dicrotic notch. In normal vessels, reflected waves return during  
1034 diastole and cause the pulse pressure to be higher in the peripheral rather than central arteries. As arteries  
1035 become stiffer with age, pulse wave velocity increases and the reflected wave reaches the central pulse in  
1036 systole, boosting the SBP and left ventricular load.

1037 To evaluate the appropriateness of central SBP as outcome variable of BP, the literature deriving from  
1038 database #15 was critically evaluated (see Tab.1).

1039 Increased arterial stiffness is a major factor contributing to the rise in SBP with ageing. This is both due to  
1040 increased amplitude of the pressure wave and to increased wave velocity. Measurement of aortic pulse wave

1041 velocity is the best non-invasive measure of arterial stiffness and correlates with adverse cardiovascular  
1042 outcomes[97-99]. Arterial stiffness has been suggested as a good tool for risk stratification and a target for  
1043 intervention[100-102]. An increase in central arterial stiffness is accompanied by a paralleled increase in BP.  
1044 In conclusion[103], the measurement of central SBP appears to be appropriate for the substantiation of health  
1045 claims in the context of reduction/maintenance of BP.

1046

#### 1047 *3.1.7.6.1 Pulse wave analysis*

1048 Direct central pressure assessment requires an invasive procedure which has the advantage of being a precise  
1049 and reproducible technique. However, it is usually performed in clinical setting, only in patients scheduled  
1050 for coronary angiography[104]. Therefore, several non-invasive methods and techniques for central BP  
1051 estimation and pulse wave shape have been developed and validated[105]. High-fidelity sensors, tonometers  
1052 and piezo-electric techniques rely on a mathematical transformation that allows the calculation of central  
1053 SBP from radial tonometry data. Moreover, compared to standard sphygmomanometry, pulse wave analysis  
1054 can provide a better assessment of aortic pressure[97]. Applanation tonometry is the current gold standard  
1055 for the measurement of aortic stiffness. Pulse wave velocity is calculated by measuring the transit time of the  
1056 pulse waveform at two sites along the vasculature, providing a regional assessment of aortic stiffness.  
1057 Different vasculature segments can be involved in the non-invasive measurement, including carotid-femoral  
1058 (Class I, Level of Evidence A) and ankle-brachial. However, for the latter segment, longitudinal studies in  
1059 USA and European countries are lacking. In order to enhance the uniformity of arterial stiffness studies, the  
1060 sites of measurement should be clearly stated. Owing to the lack of evidence about the association with  
1061 incident cardiovascular outcomes, single-point estimates of pulse wave velocity are not recommended.  
1062 Moreover, for each subject, a duplicate of the measure of pulse wave velocity should be performed in the  
1063 supine position after 10 minutes of rest (at least), controlling the environmental noise and temperature. If the  
1064 two measurements show a difference higher than 0.5 m/s using the median value, a triplicate is needed. A  
1065 limitation of the technique is represented by the requirement of trained expert personnel for obtaining  
1066 reproducible results[104].

1067 On the basis of current evidence, pulse wave analysis seems to be appropriate for measuring central SBP.  
1068 Furthermore, pulse wave analysis appears an appropriate method of measurement of arterial stiffness,  
1069 assessed as augmentation index (AIx) and return time of reflected wave.

1070

### 1071 **3.1.8 Improvement of endothelial function**

#### 1072 *3.1.8.1 Endothelium dependent vasodilation*

1073 The endothelium is the inner lining of blood vessels and plays a central role in vascular homeostasis. In  
1074 healthy individuals, the endothelium exerts a protective effect on the vasculature by regulating vascular tone,  
1075 blood fluidity and clotting. Endothelium-derived hyperpolarizing factor (EDHF), nitric oxide (NO) and  
1076 prostacyclin (PGI<sub>2</sub>) are endothelium-derived factors with vasodilatory and antiproliferative effects. On the  
1077 other side, endothelin 1 (ET-1), angiotensin II and ROS are molecules exerting vasoconstrictor effects.  
1078 Endothelial cells are also able to produce antithrombotic (NO and PGI<sub>2</sub>) and prothrombotic molecules,  
1079 including von Willebrand factor and plasminogen activator inhibitor-1 (PAI-1). The disruption of the fine-  
1080 tuned relative concentration of these factors, mainly due to reduced NO bioavailability, may lead to impaired  
1081 vasodilation (endothelial dysfunction) which is the earliest clinically identifiable event in the process of  
1082 atherosclerosis. Endothelial (dys)function can be determined by assessing the degree of Flow mediated  
1083 dilation (FMD), which reflects the endothelium-dependent capacity of arterial relaxation, mediated by the  
1084 release of NO, in response to a hyperemic stimulus[106]. FMD can be, therefore, considered a reliable  
1085 variable of arterial reactivity.

1086 To evaluate the appropriateness of endothelium dependent vasodilation as outcome variable of endothelial  
1087 function, the literature deriving from database #16 was critically evaluated (see Tab.1).

1088 Endothelial dysfunction is the earliest stage of the atherosclerotic process and is characterized mainly by a  
1089 reduction in NO availability. No structural lesions have been reported at this early stage. Vascular reactivity  
1090 tests have become the most common and reliable methods for assessment of endothelial function[107]. In  
1091 order to rule out the contribution of vascular endothelium-independent reactivity to the vasodilation induced  
1092 by an endothelium-dependent stimulus, exogenous NO donors (e.g. glycerol-trinitrate) can be administered.  
1093 A reduction or the complete absence of endothelium-dependent vasodilatation is an index of endothelial

dysfunction[108-111]. 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.

1096

#### 1097 *3.1.8.1.1 Flow mediated dilation*

1098 FMD is the most common and reliable method for the assessment of endothelial (dys)function. FMD is a  
1099 non-invasive method, introduced in 1992, based on the measurement of brachial artery diameter changes  
1100 after an increase in shear stress induced by reactive hyperaemia, usually induced by circulatory arrest in the  
1101 forearm (suprasystolic cuff occlusion) for a period of 4 to 5 minutes. In the setting of a healthy endothelium,  
1102 the increased shear stress stimulates the release of NO, inducing a reactive local vasodilation in the brachial  
1103 artery[112, 113]. FMD assessment in the brachial artery may mirror endothelial health status in all vascular  
1104 districts including the coronary arteries[109, 114]. Impaired FMD is associated with almost every condition  
1105 predisposing to atherosclerosis and evidence from prospective studies shows that it is associated with  
1106 cardiovascular events[113, 115]. FMD is assessed by Doppler ultrasound[107]. The assessment of FMD with  
1107 this technique requires a highly skilled sonographer and a suitable ultrasound machine. In addition, strict  
1108 standardization is needed: differences in methodological approaches may, in fact, deeply affect the response  
1109 magnitude, leading to misinterpretation of data, and may limit between-study comparisons. A rigorous  
1110 methodology is paramount to reduce measurement variability in both single-centre and multi-centre studies.  
1111 The main factors influencing the outcome variable are: 1) a certified operator, 2) a defined experimental  
1112 setting, 3) the use of an adjustable stereotactic probe-holding device, 4) an automated computer-assisted  
1113 brachial artery measurement. The administration of low-dose sublingual nitroglycerine should be performed  
1114 for the evaluation of endothelium-independent FMD response, to quantify the influence of altered smooth  
1115 muscle cell contractility on the dilation response. On the basis of current evidence, FMD seems to be  
1116 appropriate for measuring the endothelium-dependent and independent vasodilation function in humans.

1117

#### 1118 *3.1.8.2 Endothelium independent vasodilation*

1119 The human body usually is endowed with an adequate supply of antioxidants, obtained from various food  
1120 sources; yet, an excess of free radicals may reduce NO bioavailability. Endothelial (dys)function can be  
1121 determined by assessing FMD, which reflects the endothelium-dependent relaxation of the artery, mediated

1122 by the release of NO, in response to a hyperaemic stimulus, and is considered a reliable variable of vascular  
1123 reactivity of the arterial vasculature[106]. The vasodilatation produced by NO donors, such as nitroglycerine,  
1124 is called "endothelium independent".

1125 To evaluate the appropriateness of endothelium independent vasodilation as outcome variable of endothelial  
1126 function, the literature deriving from database #16 was critically evaluated (see Tab.1).

1127 Vascular responses are determined by the functional status of the vasculature and by the microvasculature  
1128 structure. Moreover, to separate endothelium-dependent from endothelium-independent responses, direct  
1129 non-NO donors or exogenous NO donors (e.g., glycerol-trinitrate) can be used. Impaired signalling of the  
1130 NO-guanylate cyclase-cGMP-PKG cascade as well as inactivation or destruction of NO prior to smooth  
1131 muscle signalling may induce endothelial dysfunction. Endothelial-independent dysfunction is related to  
1132 alterations in vascular structures and in smooth muscle cells rather than to changes in the endothelium[108-  
1133 111]. In conclusion, the measurement of endothelium independent vasodilation (nitroglycerine-mediated) as  
1134 biomarker of endothelial function appears to be appropriate for the substantiation of health claims in the  
1135 context of improved endothelial function.

1136

#### 1137 *3.1.8.2.1 Flow mediated dilation*

1138 Please refer to Section 3.1.8.1.1

1139

#### 1140 *3.1.8.3 Systemic arterial compliance*

1141 Large arteries, such as the aorta and the carotids, are distensible so that they can buffer the pulsatile systolic  
1142 output of the ventricle. This buffering function is usually described as systolic arterial compliance (SAC).  
1143 SAC is an important determinant of the cardiac load. The total arterial compliance is defined as the change in  
1144 the arterial blood volume associated with a change in the distending pressure (diameter-pressure  
1145 relationship). Arterial compliance decreases with ageing and in various diseases such as systolic  
1146 hypertension and coronary artery disease [116]. When SAC is decreased, arteries are stiffer and with reduced  
1147 ability to smooth the pulsatile blood flow; hence, they are exposed to higher pressure peaks which may be  
1148 detrimental to vessel wall integrity.

1149 To evaluate the appropriateness of SAC as outcome variable of endothelial function, the literature deriving  
1150 from database #17 was critically evaluated (see Tab.1).  
1151 While arterial BP offers a summary measure of the hemodynamic load, SAC reflects the overall opposition  
1152 of large arteries to the pulsatile ventricular ejection of blood. The evaluation of blood volume changes in the  
1153 systemic arterial tree is difficult and, because arterial compliance is strictly associated to BP fluctuations,  
1154 there is no single reference value to normalize arterial compliance. In addition, SAC may differ when  
1155 measured at different points of the non-linear pressure-volume curve and it is influenced by wall stiffness,  
1156 arterial size and wall thickness[117, 118]. SAC is estimated using simple decay time or area methods, which  
1157 require the expression of aortic pressure as a function of time and cardiac output[119, 120]. The two-area and  
1158 pulse pressure methods are the most accurate and versatile methods and can be applied at all locations.  
1159 Lumped or whole-body compliance, usually evaluated as the ratio of cardiac stroke volume to pulse pressure,  
1160 is not useful because it is based on the assumption that the arterial tree is a single compartment and because  
1161 it is affected by peripheral changes[121]. In conclusion, the measurement of SAC as biomarker of  
1162 endothelial function does not appear to be appropriate for the substantiation of health claims in the context of  
1163 improved endothelial function.

1164

#### 1165 3.1.8.3.1 *Ultrasound technique*

1166 Systemic arterial compliance can be defined as the change in arterial blood volume associated with a given  
1167 change of arterial BP and is generally measured as pressure (carotid artery)-volume (aortic outflow)  
1168 relationship. SAC is usually evaluated with an ultrasound technique that measures the bidimensional changes  
1169 in the area of the brachial artery associated with pressure changes over a wide range of pressures[122]. SAC  
1170 can be simply evaluated as the  $SV/\Delta P$  ratio, where SV is the stroke volume and  $\Delta P$  is the pulse pressure.  
1171 However, one of the most popular methods to measure SAC, the so-called “area method”, employs a  
1172 different relationship:  $SAC = ADP / [R * (PSP - PDP)]$ . Here, ADP is the area under the diastolic decay portion  
1173 of the pulse pressure curve (from end systole to end diastole), R is the total peripheral resistance calculated  
1174 as the mean arterial BP divided by the mean blood volume flow, PSP is the end-systolic aortic BP, and PDP  
1175 is the end-diastolic aortic BP. The pressure waveform representing the aortic root driving pressure is  
1176 obtained from the carotid artery waveform with applanation tonometry of the proximal right carotid artery.

1177 Central SBP is obtained by calibration of the pressure obtained by tonometry against the simultaneously  
1178 measured brachial BP[120]. The main problem associated with the use of US scanners to measure SAC is  
1179 their low resolution, which makes it difficult to detect small changes in vessel diameter. For this reason, their  
1180 use is typically limited to larger and more accessible arteries, such as carotid arteries and the abdominal  
1181 aorta. In addition, most of the measures necessary for SAC evaluation are local and their extrapolation in  
1182 terms of systemic compliance may not be appropriate. Lastly, the measurement technique is quite expensive  
1183 and highly operator-dependent. Therefore, an experienced operator is central to obtain reproducible  
1184 measurements. On the other hand, ultrasound has the advantage of being non-invasive[123]. In conclusion,  
1185 the use of SAC measured by duplex US scanners is insufficient for the substantiation of a health claim  
1186 concerning the improvement of endothelial function.

1187

#### 1188 *3.1.8.4 Nitrite/nitroso-species*

1189 Nitrite/nitroso-species (RXNO), i.e. S-nitrosothiols, N-nitrosamines and iron-nitrosyl species, are produced  
1190 by the interaction of NO and ROS and are key components of the redox regulation/signalling network[124].  
1191 A depletion of circulating RXNO has been associated with endothelial dysfunction [125]. Intravenous  
1192 administration of S-nitrosothiols has been shown to induce vasodilation. More recently, N-nitroso proteins  
1193 and iron-nitrosyl complexes have been suggested to serve as stores of plasma NO. Although the human body  
1194 has an adequate supply of antioxidants (self-produced or obtained from foods) to neutralize free radicals,  
1195 ROS accumulation may disrupt NO balance and make the endothelium overly permeable with the entry of  
1196 different substances into body tissues.

1197 To evaluate the appropriateness of nitrite/nitroso species as outcome variable of endothelial function, the  
1198 literature deriving from database #18 was critically evaluated (see Tab.1).

1199 RXNOs may be a suitable diagnostic marker for long-term changes in nitric oxide synthase 3 (eNOS)  
1200 activity, due to their long half-life. RXNOs, but not nitrite, are associated with endothelial dysfunction  
1201 because of their peculiar biochemical features, first of all half-life. Further studies are needed to test whether  
1202 the reduction in plasmatic RXNO reflects impaired NO formation, accelerated decomposition, and/or  
1203 consumption of RXNOs and whether these processes play a causal role in the pathogenesis of  
1204 atherosclerosis. However, plasma RXNO levels may represent a marker of NO bioavailability and can be

1205 useful in identifying a potential mechanism of endothelial (dys)function[126-128]. In conclusion, the  
1206 measurement of RXNO alone does not appear appropriate to be used alone for the substantiation of health  
1207 claims in the context of endothelial function. However, it can be considered supportive of a mechanism  
1208 underlying the claimed effect.

1209

#### 1210 *3.1.8.4.1 Reductive gas-phase luminescence*

1211 Chemiluminescence can detect only free NO in gaseous state. NO metabolites have thus to be converted into  
1212 free NO prior to quantification. The concentration of nitrosylated and nitrosate species is determined after  
1213 reductive cleavage with subsequent assessment of the NO released into the gas phase by its  
1214 chemiluminescent reaction with ozone (O<sub>3</sub>) [129, 130]. O<sub>3</sub> is combined with NO to form NO<sub>2</sub> in its  
1215 activated state; a proportion of the latter arises in an electronically excited state (NO<sub>2</sub>\*), which, on decay to  
1216 its ground state, emits light in the near-infrared region (above 600 nm) and can be quantified by a  
1217 photomultiplier. Because the intensity of the emitted light is proportional to the concentration of NO in the  
1218 reaction chamber, the amount of NO in the original sample can be evaluated using proper calibration curves.  
1219 It is possible to detect up to 1 pM of NO gas in the reaction chamber. However, the sensitivity of the method  
1220 should be determined using calibration against a known amount of NO gas. This method has been validated  
1221 for use in different biological tissues (plasma, whole blood, and red blood cells) and has some advantages: a)  
1222 high specificity for NO; b) good reproducibility; c) possibility of using turbid or colored samples; d) low  
1223 cost[125]. On the other hand, this method has the disadvantage of requiring gaseous NO with ensuing low  
1224 sample throughput. On the basis of the current evidence, reductive gas-phase chemiluminescence seems to  
1225 be appropriate for measuring the RXNO.

1226

#### 1227 **3.1.9 Reduction of platelet aggregation**

##### 1228 *3.1.9.1 Percent of inhibition in platelet aggregation*

1229 Platelets are involved in primary haemostasis. They first adhere to the sub-endothelium of a damaged vessel  
1230 and then become activated, providing a surface triggering coagulation and favouring the formation of a  
1231 stable coagulum that prevents bleeding and facilitates vessel repair. The haemostatic process results from a  
1232 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[131]. To evaluate the appropriateness of the percent of inhibition in platelet aggregation as outcome variable of platelet aggregation, the literature deriving from database #19 was critically evaluated (see Tab.1). Hyperactive platelets are involved in atherothrombosis, inflammation and angiogenesis and are associated with acute coronary syndromes, cerebral infarction, and peripheral vascular disease[132, 133]. 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[134, 135]. 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.

1246

#### 1247 *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 (TRAP), 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[134, 136-138]. 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

1261 extrapolated to the (patho)physiological setting because separated platelets undergo stirring under low shear  
1262 conditions and form aggregates only after addition of agonists, conditions which do not mimic the  
1263 aggregation triggered by vessel wall damage. Importantly, most recent aggregometers are entirely automated  
1264 and easy to use. In conclusion, LTA is appropriate for measuring percent of inhibition in platelet  
1265 aggregation.

1266

### 1267 *3.1.9.2 Thromboxane A2 generation*

1268 Thrombin induces the production and release of TXA2 from activated platelets. TXA2 is synthesized from  
1269 the 20-carbon polyunsaturated arachidonic acid, which is cleaved from phospholipids and released during  
1270 platelet activation. As other soluble platelet agonists, e.g. ADP and serotonin, TXA2 is released from  
1271 stimulated platelets to amplify platelet activation and to recruit additional circulating platelets. TXA2 is a  
1272 potent inducer of platelet aggregation, vasoconstriction and bronchoconstriction, and has been involved in a  
1273 series of major diseases [139-141]. Because of its very short half-life (about 30 seconds), TXA2 functions as  
1274 an autocrine or paracrine mediator in tissues surrounding the site of production.

1275 To evaluate the appropriateness of thromboxane A2 generation as outcome variable of platelet aggregation,  
1276 the literature deriving from database #20 was critically evaluated (see Tab.1).

1277 TXA2 participates to the cascade of events preceding actual cell activation. Described as “the most  
1278 significant parameter related to platelet activation”, TXA2 is inhibited by aspirin, a drug widely used. This  
1279 may limit the usefulness of TXA2 levels as in vivo marker of platelet activity[140, 142]. TXA2 is not a true  
1280 marker of platelet activation in its native behaviour (blood vessels) as its production occurs after the  
1281 beginning of platelet aggregation, providing information about what has already happened[139]. In  
1282 conclusion, TXA2 does not appear to be appropriate for the substantiation of health claims in the context of  
1283 reduced platelet aggregation.

1284

### 1285 *3.1.9.2.1 Enzyme immunoassays*

1286 TXA2 is one of the COX metabolites of arachidonic acid. TXA2 is rapidly converted to thromboxane  
1287 (TXB2), which is a biologically inactive metabolite with a long-life in the circulation because of its chemical  
1288 stability. For this reason, TXA2 levels are frequently monitored by measuring TXB2 in serum or urine. In

1289 enzyme immunoassays for TXB2 evaluation, the hapten molecule is labelled with  $\beta$ -galactosidase. The  
1290 formed immunoprecipitate is subjected to a fluorometric assay of  $\beta$ -galactosidase, and TXB2 is detectable in  
1291 the range of pmol. Known amounts of TXB2 are added to plasma to evaluate the correlation between the  
1292 added and measured thromboxane TXB2. The signal obtained with the enzymatic immunoassay is inversely  
1293 proportional to the amount of TXB2 in the sample. It is difficult to accurately measure circulating levels of  
1294 TXB2 in animals as plasma and urinary levels of the molecule are primarily due to ex vivo platelet activation  
1295 and intra-renal production, respectively [143]. The main limitation of TXB2 is that platelets are not directly  
1296 assayed. In addition, it may be not completely platelet-specific[144]. On the basis of current evidence, the  
1297 enzyme immunoassay seems to be appropriate for measuring TXA2 generation.

1298

#### 1299 3.1.9.3 *P-Selectin*

1300 P-selectin (P-sel) is a glycoprotein constitutively expressed in the  $\alpha$ -granules of platelets and in the Weibel-  
1301 Palade bodies of endothelial cells or released in soluble form. In response to physiological platelet activation,  
1302 soluble P-sel translocates to platelet and endothelial cell surface promoting formation of platelet-leukocyte  
1303 aggregate and bridges between leukocytes and endothelium. Soluble P-sel is the heaviest of the known  
1304 selectins, with a molecular weight of 140 kDa. The detection of soluble P-sel has recently become one of the  
1305 most important methods to test platelet activation [145, 146].

1306 To evaluate the appropriateness of P-sel as outcome variable of platelet aggregation, the literature deriving  
1307 from database #21 was critically evaluated (see Tab.1).

1308 There is a growing body of evidence supporting the idea that soluble P-sel is a measure of platelet activation.  
1309 Increased levels of soluble P-sel may be indicative of an increased risk of adverse cardiovascular events in  
1310 patients with existing ischemic heart disease[147, 148]. Soluble P-sel may also be a marker of platelet  
1311 activation during haemodialysis[149]. Contrarily to beta-thromboglobulin, increased levels of soluble P-sel  
1312 are not caused by ex vivo platelet activation and are not associated with platelet count. However, it cannot be  
1313 excluded that raised soluble P-sel levels depend upon other coagulation factors or are associated with post-  
1314 thrombotic phenomena[150]. Indeed, raised soluble P-sel may be the result of platelet activation by  
1315 thrombin, its fractions, or other factors leading to hypercoagulability. In addition, according to some  
1316 researchers, a quote of soluble P-sel may arise from the endothelium. Thus, the specificity of soluble P-sel as

1317 marker of platelet activation is debated[146, 151, 152]. In conclusion, the measurement of plasma soluble P-  
1318 sel is not sufficient alone for the substantiation of health claims in the context of reduced platelet  
1319 aggregation.

1320

#### 1321 3.1.9.3.1 *ELISA methods*

1322 In the late 90s, an ELISA method using an anti- DNP antibody for measuring total protein CO groups has  
1323 been developed. In its original version, the protein sample reacts with DNPH and is then adsorbed to the  
1324 wells of an ELISA plate before reacting with anti-DNPH antibody. Soluble P-sel was usually determined  
1325 using ELISA kit in EDTA plasma. The limitations the ELISA test include the need of expensive and  
1326 specialized equipment; moreover, as for colorimetric procedure, ELISA methods are usually susceptible to  
1327 interferences by molecules other than proteins. The ELISA test has however some important advantages,  
1328 such as the requirement of only microgram amounts (about 60 µg) of protein, which is similar to the  
1329 requirement for the HPLC method, compared with the 10 mg required for optimal results with clinical  
1330 samples using the colorimetric assay. This expanded their use when only limited amounts of protein are  
1331 viable for analysis[148]. In addition, the ELISA test is easier to use, less labour-intensive, and handles more  
1332 samples per day than the colorimetric assay.[153] On the basis of current evidence, ELISA seems to be  
1333 appropriate for measuring plasma soluble P-sel.

1334

### 1335 3.1.10 Maintenance of normal homocysteine

#### 1336 3.1.10.1 *Homocysteine*

1337 Homocysteine, a sulfhydryl-containing amino acid, is an intermediate product of the synthesis of the amino  
1338 acids methionine and cysteine. Normal levels of plasma homocysteine range from 5 to 15 µmol/L.

1339 To evaluate the appropriateness of homocysteine as outcome variable, the literature deriving from database  
1340 #22 was critically evaluated (see Tab.1).

1341 Plasma homocysteine is a potentiator of blood platelet response to agonists. Interestingly, while  
1342 homocysteine functions as a pro-aggregatory agent, its acetylated form has the opposite effect. The  
1343 mechanisms by which elevated levels of homocysteine induce vascular injury and promote thrombosis  
1344 remain unclear. Most findings have been obtained in *in vitro* studies employing very high concentrations of

1345 homocysteine. One of the questions in the literature is whether homocysteine directly influences platelets or  
1346 whether its effect is secondary to the action on the endothelium, blood cells, or other factors.  
1347 However, homocysteine reflects a condition of normal metabolism so, in the framework of the functional  
1348 claim, and on the basis of current data present in literature, its direct measure appears to be appropriate for  
1349 the substantiation of health claims linked to maintenance of normal homocysteine.

1350

#### 1351 *3.1.10.1.1 LC-MS/MS*

1352 LC-MS/MS is a relatively new method and has been reported to be precise and accurate for measuring  
1353 plasma total homocysteine concentrations, using small volumes of plasma and being suitable for routine  
1354 use[154]. A satisfactory LC-MS/MS method for the measurement of total homocysteine in dried blood spots  
1355 is also available; this method might be useful in routine screening for raised plasma concentrations of total  
1356 homocysteine[155, 156]. There are some advantages that suggest to use this method for large-scale  
1357 population-based studies, including: a) high throughput; b) low cost; c) simple sample pre-treatment  
1358 procedures. On the basis of current evidence, LC-MS/MS seems to be appropriate for measuring  
1359 homocysteine.

1360

### 1361 **3.1.11 Maintenance of normal venous blood flow**

#### 1362 *3.1.11.1 Great saphenous diameter*

1363 Venous anatomy is highly variable in some parts and less variable in other parts of the lower limbs.  
1364 Typically, the Great saphenous vein has a diameter ranging from 3 to 5 mm. This diameter can increase from  
1365 1 to 3 mm in response to hyperthermia, prolonged standing, the Valsalva maneuver, menstruation and  
1366 pregnancy. The great saphenous vein is the vein most commonly responsible for varicose vein disease. Signs  
1367 and symptoms include swelling, and heaviness up to frank pain. Prolonged volume overload and venous  
1368 hypertension may result in venous dilatation and stasis which represent predisposing conditions to  
1369 thrombophlebitis and phlebothrombosis.  
1370 To evaluate the appropriateness of the great saphenous diameter as outcome variable of normal venous blood  
1371 flow, the literature deriving from database #23 was critically evaluated (see Tab.1).

1372 Studies using the great saphenous vein diameter as a marker for venous hemodynamic impairment have led  
1373 to contradictory results. The diameter of the great saphenous vein can vary with patient positioning,  
1374 Valsalva manoeuvres, and body mass index. Standardization of great saphenous vein assessment is difficult  
1375 due to the variable anatomical configuration of the great saphenous vein. When measured distally to the  
1376 terminal valve, the great saphenous vein diameter will usually be larger than when it is measured at the  
1377 saphenous-femoral junction. Several procedures to measure the popliteal diameter are available but  
1378 standardization in this region is difficult. The more reproducible method is to measure great saphenous vein  
1379 diameter at the proximal thigh, although reflux is relatively unrelated to diameter in this region [157]. The  
1380 great saphenous vein diameter, as measured by duplex scanning, can have substantial inter-observer  
1381 variability and can also vary depending on time of day, patient positioning, and room temperature so that the  
1382 measurement technique must be reported in detail[158, 159]. In conclusion, the measurement of great  
1383 saphenous diameter as a marker of venous blood flow does not appear to be sufficient for the substantiation  
1384 of health claims related to the maintenance of normal venous blood flow.

1385

#### 1386 *3.1.11.1.1 Duplex doppler*

1387 The veins are usually visualized by means of Duplex ultrasound, which combines Doppler flow with  
1388 imaging information. This method allows the assessment not only of blood flow and velocity, but also the  
1389 estimation of the vein diameter and of the degree of obstruction. Duplex Doppler is the main clinical tool  
1390 used to evaluate the venous system for the diagnosis of chronic venous disease, especially in the lower limbs  
1391 [158-161]. The major limitation of this method is that reliable information can only be achieved by trained  
1392 and experienced operators. On the basis of the current evidence, duplex ultrasound seems to be appropriate  
1393 for the evaluation of chronic venous disease, especially the great saphenous diameter and the popliteal vein  
1394 diameter (see Section 3.1.11.2) and the phenomenon of venous reflux (see Section 3.1.11.3).

1395

#### 1396 *3.1.11.2 Popliteal vein diameter*

1397 The popliteal vein is formed by the junction of the anterior and posterior tibial veins at the lower border of  
1398 the popliteus muscle; it ascends through the popliteal fossa to the aperture in the Adductor magnus muscle,  
1399 where it becomes the femoral vein. In the lower part of its course, it is placed medially to the artery; between

1400 the heads of the Gastrocnemius muscle it is superficial to that vessel, while it is close to its lateral side above  
1401 the knee-joint. It receives tributaries corresponding to the branches of the popliteal artery, and it also receives  
1402 the small saphenous vein. The valves in the popliteal vein are usually four. The popliteal vein carries blood  
1403 from the lower leg to the heart. The mean diameter of the popliteal vein is  $0.62 \pm 0.06$  cm in the recumbent  
1404 position and  $0.84 \pm 0.1$  cm in the erect position.

1405 To evaluate the appropriateness of the popliteal vein diameter as outcome variable of normal venous blood  
1406 flow, the literature deriving from database #24 was critically evaluated (see Tab.1).

1407 An increase in popliteal vein diameter is associated with an increased risk of venous reflux[162, 163].

1408 Several procedures are available to measure popliteal diameter but standardization in this region is difficult  
1409 due to the physiological variation of the vein anatomy[164]. The measurement procedure should be reported,  
1410 especially if multiple operators are involved. Because leg extension produces compression of the vein, the  
1411 ultrasonographic assessment should be performed with the limb slightly flexed [165]. In conclusion, the  
1412 measurement of popliteal vein diameter does not appear to be appropriate for the substantiation of health  
1413 claims related to the maintenance of normal venous blood flow.

1414

#### 1415 *3.1.11.2.1 Duplex doppler*

1416 Please refer to Section 3.1.11.1.1

1417

#### 1418 *3.1.11.3 Venous reflux*

1419 Healthy veins contain bicuspid valves assisting unidirectional flow from the lower limb toward the heart.

1420 The valves of the venous system may become incompetent and blood can flow backwards (venous reflux).

1421 Venous reflux is a pathological condition characterized by impaired return of blood and increased venous  
1422 pressure that may lead to venous stasis and eventually microangiopathy. There are several predisposing  
1423 conditions to venous reflux such as aging, male sex, heredity, obesity, physical activity, multiparity and  
1424 prolonged standing.

1425 To evaluate the appropriateness of venous reflux as outcome variable of normal venous blood flow, the  
1426 literature deriving from database #25 was critically evaluated (see Tab.1).

1427 Venous reflux is a pathologic condition affecting mostly body extremities. When valve leaflets close  
1428 improperly, vein pressure increases greatly leading to vein dilatation and valve incompetence. Venous stasis  
1429 secondary to venous reflux may cause fluid leakage into the interstitial space with consequent oedema. The  
1430 use of duplex scanning to evaluate reflux in the deep and superficial veins should be performed with the  
1431 patient in the upright position, with the leg rotated outward, heel on the ground, and weight on the opposite  
1432 limb[158, 159, 166-169]. There is presently no clear threshold to identify pathological refluxes. In the  
1433 literature, both 500 ms and 1000 ms have been used as cut-points to identify a pathological reflux. The  
1434 Committee of the Society for Vascular Surgery and the American Venous Forum recommends 500 ms as the  
1435 cut-off value for saphenous, tibial, deep femoral, and perforating vein incompetence, and 1000 ms for  
1436 femoral and popliteal vein incompetence[170]. In conclusion, the measurement of venous reflux appears to  
1437 be appropriate for the substantiation of health claims related to the maintenance of normal venous blood  
1438 flow.

1439

#### 1440 *3.1.11.3.1 Duplex doppler*

1441 Please refer to Section 3.1.11.1.1

1442

### 1443 **3.2 REDUCTION OF DISEASE RISK CLAIMS ART. 14.1 (a)**

#### 1444 **3.2.1 Coronary heart disease**

1445 Coronary heart disease (CHD) is the direct effect of coronary artery diseases (CAD) on the heart. CAD  
1446 develops when plaques grow within the walls (atherosclerosis) of the coronary arteries that become narrow  
1447 and rigid, reducing blood flow to the heart. The heart becomes starved of oxygen and the vital nutrients it  
1448 needs to work properly. CHD may be chronic, consisting in a progressive narrowing of the coronary arteries  
1449 or acute, resulting from a sudden plaque complication (rupture and thrombus formation). This may lead to  
1450 complete vessel obstruction and acute myocardial ischemia. Because coronary artery disease often develops  
1451 over decades, it can go unnoticed until the first heart attack. The traditional risk factors for coronary artery  
1452 disease are age, smoking, high BP, diabetes, high cholesterol, and family history. In detail, the ageing  
1453 process increases the risk of narrowing the coronary arteries and men are at greater risk of CAD than women.



1454 Women have an increase in CAD risk after the menopause. High levels of cholesterol in blood contribute  
1455 to the risk plaque formation and atherosclerosis.

1456

#### 1457 3.2.1.1. LDL-C

1458 To evaluate the appropriateness of LDL-C as risk factor for CHD, the literature deriving from database #12  
1459 was critically evaluated (see Tab.1).

1460 Multiple clinical trials have established beyond any reasonable doubt that the reduction of TC or LDL-C is  
1461 associated with a clinically relevant reduction in cardiovascular mortality. LDL-C usually makes up 60-70%  
1462 of serum TC. There are some lifestyle behaviours that decrease blood LDL-C, such as eating vegetables,  
1463 eating less fat and doing daily exercise. Statins are the drugs usually employed for lowering cholesterol  
1464 concentration, but, more recently, other LDL-lowering drugs, such as inhibitors of Nieman Pick C1 like 1  
1465 protein and inhibitors of proprotein convertase subtilisin/kexin type 9 (PCSK9), have been shown to be  
1466 effective in reducing cardiovascular risk[171]. Thus, it can be safely stated that lowering the LDL-C  
1467 concentration lowers the cardiovascular risk[45, 46, 48-52]. The greater the LDL-C reduction, the more  
1468 benefit there is to be gained. Some studies show that the number of LDL particles may be a better predictor  
1469 of risk than LDL-C. LDL particle size may also be important when assessing risk but its evaluation is not  
1470 currently endorsed by clinical guidelines [54].

1471 In conclusion, LDL-C can be considered a modifiable risk factor to be used for the substantiation of health  
1472 claims in the context of reduced risk of CHD.

1473

#### 1474 3.2.1.1.1 Enzymatic assays

1475 Please refer to Section 3.1.6.1.1

1476

#### 1477 3.2.1.2 HDL-C

1478 As mentioned above, HDL has been extensively investigated because it extracts cholesterol from tissues and  
1479 delivers it to the liver, where it may be converted into bile acids and excreted. Low HDL-C is an independent  
1480 risk factor for CVD[43-46, 48, 49, 60-63].

1481 To evaluate the appropriateness of HDL-C as risk factor for CHD, the literature deriving from database #12  
1482 was critically evaluated (see Tab.1).

1483 HDL are believed to exert many cardioprotective activities, including the promotion of the reverse  
1484 cholesterol transport, a process through which excess cholesterol is delivered from peripheral tissues back to  
1485 the liver. It has been widely reported that low HDL-C levels (below 40 mg/dL in men and 50 mg/dL in  
1486 women) are strongly associated with high cardiovascular risk, independently of LDL or TC levels. It is  
1487 therefore understandable that most clinical studies have used HDL-C as the metric for quantifying HDL  
1488 cardioprotective effects and why this parameter could be proposed for the substantiation of health claims. In  
1489 addition, it is important to notice that recent studies suggested to include the evaluation of HDL function,  
1490 together with HDL levels, to fully define the cardioprotective potential of this class of lipoproteins[43-46,  
1491 49, 59-63]. In particular, HDL capacity to promote cell cholesterol efflux has been proposed as novel target  
1492 whose modulation may help to reduce risk of coronary heart disease [47, 59]. However, a causal relationship  
1493 between HDL-C levels and cardiovascular disease in humans currently is disclaimed by a number of key  
1494 findings, the most noteworthy of them being: 1) Neither CETP-inhibitors nor niacin, both of which increase  
1495 HDL-C, reduce cardiovascular mortality; 2) A number of mendelian randomization studies (a research  
1496 design which tests the hypothesis of a causal relationship between genetically determined HDL-C levels and  
1497 clinical events) have consistently found no evidence of a causal link between HDL-C levels and  
1498 cardiovascular risk [172]. Thus, HDL-C presently should be regarded as a strong independent indicator of a  
1499 fraction of cardiovascular risk, which cannot be corrected via HDL-C raising treatments.

1500 In conclusion, HDL-C cannot be used alone for the substantiation of health claims in the context of reduced  
1501 risk of CHD.

1502

1503 *3.2.1.2.1 Enzymatic assays*

1504 Please refer to Section 3.1.6.2.1

1505

1506 *3.2.1.3 Total cholesterol*

1507 Cholesterol, a steroid with a secondary hydroxyl group in the C3 position, is an important component of  
1508 mammalian cell membranes where it contributes to intracellular transport, cell signalling, and maintenance

1509 of membrane fluidity. Cholesterol can be found in blood, bile, and in all tissues insofar as it is a key  
1510 component of cell membranes. Brain and adrenal glands are organs with high cholesterol content. The  
1511 measurement of serum TC is important for the diagnosis and classification of hyperlipoproteinemias.  
1512 Maintenance of normal blood TC is important because elevated cholesterol levels are associated with the  
1513 development of atherosclerosis and cardiovascular disease[47, 51]. Elevated cholesterol levels may occur  
1514 also with hypothyroidism, nephrotic syndrome, diabetes, and biliary cirrhosis. There is a strong association  
1515 between elevated serum TC and the incidence of cardiovascular disease and recent studies suggest that  
1516 altered cholesterol homeostasis is associated with the development of a chronic inflammatory state [43, 45,  
1517 51].

1518 To evaluate the appropriateness of total cholesterol as risk factor for CHD, the literature deriving from  
1519 database #12 was critically evaluated (see Tab.1).

1520 Cholesterol levels are influenced by diet, age, sex and hormonal status. TC has received most attention  
1521 because several studies showed that CVD can be prevented by reducing TC[46]. A number of cardiovascular  
1522 risk calculators use TC to quantify cardiovascular risk[49, 51, 52, 173]. However, it should be recognized  
1523 that the relationship between TC and cardiovascular disease is mostly rooted in the non-HDL-C fraction  
1524 (mostly LDL-C), usually representing the bulk of TC. TC is recommended to be used for the estimation of  
1525 total cardiovascular risk by means of the SCORE system (as currently suggested by the European Society of  
1526 Cardiology)[43, 44, 48]. However, in specific cases, TC may be misleading; this is especially true in women  
1527 who have high HDL-C levels and in subjects with diabetes or the metabolic syndrome, who often have low  
1528 HDL-C levels. For an adequate risk analysis, at least HDL-C and LDL-C should be analysed.

1529 In conclusion, TC cannot be used alone for the substantiation of health claims in the context of reduced risk  
1530 of CHD, because the simultaneous measurement of HDL-C and LDL-C is preferred.

1531

#### 1532 3.2.1.3.1 Enzymatic assays

1533 The cholesterol concentration is determined enzymatically with an assay that quantifies both cholesterol  
1534 esters and free cholesterol [174, 175]. Most available assays are of high quality, but the method used should  
1535 be evaluated against the available reference methods and controlled in international quality programs.

1536 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<sub>2</sub>O<sub>2</sub> is then detected using ADHP, a highly sensitive and stable probe for hydrogen peroxide. There are two types of probes for the enzymatic determination of H<sub>2</sub>O<sub>2</sub>: 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.

1543

#### 1544 *3.2.1.4 Fasting triglycerides*

Cross-sectional and cohort studies have shown that atherogenic dyslipidemia is a risk factor for CVD[59, 176-183]. 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 Tab.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 cardiovascular disease 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 cardiovascular 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 are evident[48]. This observation, coupled with the failure of pure HDL-C modifiers (see “HDL-”) to reduce cardiovascular risk, strongly suggests that TG reduction may be causally linked to the reduction in CV 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 CV risk. However, although TG have been proposed as a modifiable CV risk factor in individuals with “atherogenic dyslipidemia”, the case is much less strong than for LDL-C, [184]. It remains to be determined whether, in the clinical arena, non-fasting TG, small dense LDL or TRL are better than TG a predicting and modifying cardiovascular outcomes. 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, TG may not be appropriate to estimate changes in cardiovascular risk, because their relationship to cardiovascular disease is fraught with too many confounders. Therefore, TG are 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*

Please refer to Section 3.1.6.3.1

#### *3.2.1.5 Homocysteine*

High levels of homocysteine may negatively affect cardiovascular function through several mechanisms: i) increased formation of reactive oxygen species 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[185]. 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 Tab.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[186]. 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[187]. While an increase in homocysteine concentration is associated with an increased risk of coronary heart disease, a reduction in

1593 homocysteine concentration has not generally been shown to reduce this risk [188, 189]. Retrospective  
1594 studies originally suggested a strong relationship, but subsequent prospective observational studies suggested  
1595 weaker associations. Some epidemiologic studies, but not others, indicated that the elevated homocysteine  
1596 represents a risk factor for total death, CHD death, or cardiovascular mortality in the general population  
1597 [185]. So, there is no clear consensus that decreasing homocysteine concentration decreases global  
1598 cardiovascular risk, therefore it cannot be used alone for the substantiation of health claims in the context of  
1599 reduced risk of CHD.

1600

#### 1601 3.2.1.5.1. *LC-MS/MS*

1602 Please refer to Section 3.1.10.1.1

1603

#### 1604 3.2.1.6 Systolic blood pressure

1605 To evaluate the appropriateness of SBP as risk factor for CHD, the literature deriving from database #15 was  
1606 critically evaluated (see Tab.1).

1607 Despite SBP and DBP are strictly correlated, SBP is more robustly associated with CVD than DBP[71, 73,  
1608 76]. Day-time and night-time BP values and changes obtained with treatment are related to each other, but  
1609 the prognostic value of night-time BP in predicting cardiovascular events has been found to be superior to  
1610 that of day-time BP (dipping phenomenon), which is undetectable with punctual BP determination. BP may  
1611 increase with the ageing process as blood vessels become stiffer. Furthermore, several factors, including  
1612 emotion (white coat effect), exercise, temperature, race and circadian fluctuations may influence punctual BP  
1613 levels[77, 78]. Therefore, isolated measurement of BP could provide limited information with respect to  
1614 monitoring (e.g. 24 h ABPM) which may be useful in unveiling hypertension when a high BP variability is  
1615 registered between measurements during the same or different visits. Importantly, patients in which the  
1616 nocturnal decline in BP is reduced or abolished (non-dipper), are at increased risk for cardiovascular  
1617 complications than are individuals with a dipper circadian rhythm [79-81].

1618 In conclusion, SBP can be considered as a risk factor to be used for the substantiations of health claims in the  
1619 context of reduced risk of CHD.

1620

1621 3.2.1.6.1 *Office, home and 24 h ambulatory BP*

1622 Please refer to Sections 3.1.7.1.1, 3.1.7.1.2 and 3.1.7.3.1

1623

## 1624 3.2.2 **Stroke**

1625 A stroke occurs when an interruption or a severe reduction of the blood supply to a part of the brain makes  
1626 the brain tissue lacking of oxygen and nutrients. There are two types of stroke:

1627 1) Ischemic stroke (most common): it is usually caused by a lack of blood flow as a result of an  
1628 obstruction within a blood vessel supplying blood to the brain. The primary condition is the development of  
1629 atherosclerosis (fatty deposits on the inner lining of the vessel walls) and is the same process that causes  
1630 CHD. A clot may form in an artery in the brain itself (cerebral thrombosis) or in another site in the  
1631 circulatory system like the heart and large arteries of the chest or neck (cerebral embolism). In this case, it  
1632 enters the bloodstream and moves through the brain's blood vessels up to reach vessels too small where it  
1633 becomes lodged.

1634 2) Haemorrhagic stroke (least common): happens as a consequence of a weakened blood vessel in the  
1635 brain (aneurysms or arteriovenous malformations) that leaks or bursts (ruptures). The blood generally  
1636 accumulates so compressing the near brain tissues. There are two types of haemorrhagic stroke called  
1637 intracerebral (inside the brain tissue) and subarachnoid (near the surface of the brain). High BP and/or  
1638 diseases are usually the causes of this type of stroke.

1639 The main risk factors for a stroke include high BP, smoking, increasing age, high blood cholesterol, diabetes,  
1640 alcohol and illegal drug use, personal or family history of stroke or transient ischemic attack, existing heart  
1641 disease (coronary heart disease, cardiomyopathy, heart failure, and atrial fibrillation).

1642 The risk of stroke begins to increase at BP readings higher than 120/80 millimeters of mercury (mm Hg). At  
1643 younger ages, men have a higher risk of stroke than women, but women are more likely to die of strokes than  
1644 are men. Women who take birth control pills also are at slightly higher risk of stroke. African-Americans,  
1645 Alaska Native and American-Indian have a higher risk of stroke than do people of other races.

1646

1647 3.2.2.1 SBP

1648 To evaluate the appropriateness of SBP as risk factor for stroke, the literature deriving from database #14  
1649 was critically evaluated (see Tab.1).

1650 Day-time and night-time BP values and changes obtained with treatment are related to each other. BP may  
1651 increase with the ageing process as blood vessels become stiffer. Furthermore, several factors, including  
1652 emotion (white coat effect), exercise, temperature, and circadian fluctuations may influence punctual BP  
1653 levels [77, 78]. Therefore, as previously mentioned, isolated measurement of BP could provide limited  
1654 information with respect to monitoring (i.e. 24 h ABPM) which may be useful in unveiling hypertension  
1655 when a high BP variability is registered between measurements during the same or different visits.

1656 Importantly, patients in which the nocturnal decline in BP is reduced or abolished (non-dipper), are at  
1657 increased risk for cerebrovascular complications than are individuals with a dipper circadian rhythm [79-81].

1658 Elevated BP is an established prognostic factor after acute stroke, although evidence as to the effect of BP  
1659 lowering on outcome is partly conflicting, with some large studies reporting near positive effects on  
1660 functional outcome but others, reporting neutral or near negative results[190, 191]. Blood pressure variability  
1661 (BPV) may be important in the acute stroke period. Within-individual systolic BPV is a risk factor for stroke  
1662 and cardiovascular events, independent of mean absolute BP level. It is well known that lowering SBP by  
1663 10mmHg or diastolic BP by 5 mmHg using any of the main classes of BP lowering drugs reduces the risk of  
1664 stroke by approximately a third[192].

1665 In conclusion, SBP can be considered as a risk factor to be used for the substantiations of health claims in the  
1666 context of reduced risk of stroke.

1667

#### 1668 *3.2.2.1.1 Office, home and 24 h ambulatory BP*

1669 Please refer to Sections 3.1.7.1.1, 3.1.7.1.2 and 3.1.7.3.1

1670

### 1671 **3.2.3 Hypertension**

1672 As a term used to define a condition of high or raised BP, hypertension is characterized by the long-term  
1673 force of the blood against the artery wall which contributes to heart disease, stroke, kidney failure, as well as  
1674 premature death. Even if it rarely causes symptoms in the early stages, damage to blood vessels and heart  
1675 may occur. An early detection, in conjunction to an adequate treatment and a good control of this pathology,



can provide important health and economic benefits preventing the complications of hypertension, which may lead to expensive invasive interventions, including carotid or cardiac bypass surgery. The American Heart Association has long considered <140/90 mmHg as normal values 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 cut-off of “normal” BP. It has been introduced the “prehypertension” category which ranges from 120-139 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  $\geq 140/90$  mmHg and can be classified as “stage 1” (140-159/90-99 mmHg) and “stage 2” ( $\geq 160/100$  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 cardiovascular diseases 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.

1696

#### 1697 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 aging process as well as a consequence of several cardio-metabolic diseases, including diabetes, atherosclerosis and chronic renal disturbs. Owing to the

1704 availability of several techniques and calculations used to evaluate arterial stiffness, its assessment is  
1705 challenging[104]. Among the various measures to assess arterial stiffness, pulse pressure is one of the most  
1706 used [100].

1707 To evaluate the appropriateness of arterial stiffness as risk factor for hypertension, the literature deriving  
1708 from database #26 was critically evaluated (see Tab.1).

1709 Several measures can be used to assess arterial stiffness. Among these, pulse pressure is frequently chosen.  
1710 However, cardiac function plays a role on the determination of pulse pressure which can be affected by heart  
1711 rate, stroke volume and the pattern of ventricular ejection [193]. Therefore, as the gold standard measure of  
1712 arterial stiffness, aortic pulse wave velocity is preferred, owing to its less sensitivity to cardiac function.  
1713 Measures of wave reflection can be considered as potential surrogates of arterial stiffness [194]. Among  
1714 these, AIx has been proposed. It represents the percentage of central pulse pressure influenced by a late  
1715 systolic pressure rise due to overlap between the forward and reflected pressure waves [98]. The reason why  
1716 AIx can be considered a measure of aortic stiffness depends on the fact that in presence of an increment of  
1717 aortic pulse wave velocity, reflected waves should return to the heart earlier, and, as a result, overlap  
1718 between the forward and reflected waves progressively increases and pressure augmentation becomes greater  
1719 [102]. Thus, AIx and aortic stiffness appears strongly related in young adults, whereas this relation fails after  
1720 about 60 years of age when augmentation tends to decrease and aortic pulse wave velocity, pulse pressure  
1721 and CVD risk increase dramatically. On the basis of these observations, wave reflection contributes  
1722 minimally to the rise of pulse pressure in the elderly[195]. A major contribution is due to an increase of  
1723 aortic impedance. As a result, forward wave amplitude rises, while the blood flow remains constant or  
1724 decreases. Pressure wave reflection in the arterial system helps the blood to return to the aorta during diastole  
1725 and enhances the diastolic perfusion of coronary beds [196]. To obtain a surrogate marker of arterial  
1726 stiffness, it has been assumed that the inflection point marks the time of return of the reflected wave.  
1727 However, several reports have shown that the time-related changes of the inflection point are associated  
1728 poorly with the change of arterial system[193]. This lack of association together with the simplistic  
1729 assumption of a single-tube model with distal reflection impedes an accurate estimate of pulse wave velocity  
1730 by means of the return time. Likewise, a return time estimated from the foot of the reflected wave does not

1731 give information on stiffness. In other words, pulse wave velocity is an inappropriate surrogate measure of  
1732 aortic stiffness[105].

1733 However, according to the AHA statement, pulse wave velocity is a reasonable clinical measure of arterial  
1734 stiffness (class IIa, Level of Evidence A)[104].

1735 In conclusions, neither the calculation of AIx, nor the measurement of the return time of reflected wave  
1736 appear appropriate haemodynamic parameters to be used alone for the assessment of the arterial stiffness  
1737 [193]. Moreover, the evidence of a reduction of arterial stiffness is supportive but not sufficient for the  
1738 substantiation of hypertension risk reduction claims. Other outcome variables need to be evaluated for this  
1739 purpose, such as SBP and DBP.

1740

1741 *3.2.3.1.1 Pulse wave analysis*

1742 Please refer to Section 3.1.7.6.1

1743

### 1744 **3.3 CLAIMS REFERRING TO CHILDREN'S DEVELOPMENT ART. 14.1 (b)**

#### 1745 **3.3.1 Protection of DNA, protein and lipid oxidative damage**

1746 The protection of DNA, proteins and lipids from oxidative damage has been considered a beneficial effect in  
1747 the context of child development (in detail, referred to infants and young children from birth to three years of  
1748 age)[197]. The claim refers to two claims previously assessed with a favourable outcome on protection of  
1749 DNA, proteins and lipids from oxidative damage [198, 199].

1750 For the three types of macromolecules (DNA, proteins and lipids), several outcome variables and related  
1751 methods of measurement have been proposed.

1752 For the description and the critical evaluation of the outcome variables and methods of measurement, please  
1753 see:

- 1754 - Protection of DNA: Sections 3.1.3
- 1755 - Protection of lipids: Sections 3.1.4
- 1756 - Protection of proteins: Sections 3.1.5

1757

1758

1759 **CONCLUSIONS**

1760 To date a high number of requests for authorization of health claims pursuant to Article 13(5) and 14 of  
1761 Regulation (EC) No 1924/2006 has received a negative opinion from the European Food Safety Authority.  
1762 One of the most critical limitations is the design of human intervention studies aiming to proof efficacy,  
1763 including the proper choice of outcome variables and of their methods of measurement. The present paper  
1764 provides information concerning the collection, collation and critical analysis of claimed effects, outcome  
1765 variables and methods of measurement in the context of protection against oxidative damage and  
1766 cardiovascular health compliant with the European Regulation. The information provided in this work could  
1767 serve as basis for EFSA to develop further guidance to applicants in the preparation of applications for  
1768 authorization of health claims in the context of protection against oxidative damage and cardiovascular  
1769 health. In addition, it could be useful for applicants during the design or selection of human intervention  
1770 studies aimed to substantiate such health claims.

1771

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1776 the views/any official position or scientific works of EFSA. To know about EFSA guidance documents and  
1777 other scientific outputs of EFSA, please consult its website at: <http://www.efsa.europa.eu>.

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