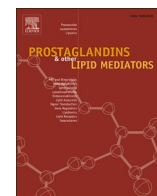


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Authors	Vejux, Anne;Ghzaiel, Imen;Mackrill, John J.;Dias, Irundika H.K.;Rezig, Leila;Ksila, Mohamed;Zarrouk, Amira;Nury, Thomas;Brahmi, Fatiha;El Midaoui, Adil;Meziane, Smail;Atanasov, Atanas G.;Hammami, Sonia;Latruffe, Norbert;Jouanny, Pierre;Lizard, Gérard
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Oxysterols, age-related-diseases and nutritherapy: Focus on 7-ketocholesterol and 7 β -hydroxycholesterol

Anne Vejux^{a,b,*}, Imen Ghzaïel^{b,c}, John J. Mackrill^d, Irundika H.K. Dias^e, Leila Rezig^{f,g}, Mohamed Ksila^b, Amira Zarrouk^{c,h}, Thomas Nury^b, Fatiha Brahmiⁱ, Adil El Midaoui^{j,k}, Smail Meziane^l, Atanas G. Atanasov^m, Sonia Hammami^c, Norbert Latruffe^{a,b}, Pierre Jouannyⁿ, Gérard Lizard^{b,**}

^a Centre des Sciences du Goût et de l'Alimentation, CNRS, INRAE, Institut Agro Dijon, Université de Bourgogne Europe, 21000 Dijon, France

^b Team 'Biochemistry of the Peroxisome, Inflammation and Lipid Metabolism' EA7270/Inserm, Université de Bourgogne Europe, 21000 Dijon, France

^c Lab-NAFS 'Nutrition-Functional Food & Vascular Health', Faculty of Medicine, University of Monastir, LR12ES05, Monastir 5000, Tunisia

^d Department of Physiology, University College Cork, Western Gateway Building, Western Road, Cork T12 XF62, Ireland

^e Aston Medical School, College of Health and Life Sciences, Aston University, Birmingham, UK

^f University of Carthage, National Institute of Applied Sciences and Technology, LR11ES26, LIP-MB 'Laboratory of Protein Engineering and Bioactive Molecules', Tunis 1080, Tunisia

^g University of Carthage, High Institute of Food Industries, 58 Alain Savary Street, El Khadra City, Tunis 1003, Tunisia

^h Faculty of Medicine of Sousse, avenue Mohamed Karaoui, 4002 Sousse, Tunisia

ⁱ Laboratory of Biomathematics, Biophysics, Biochemistry, and Scientometrics (L3BS), Faculty of Nature and Life Sciences, University of Bejaia, 06000 Bejaia, Algeria

^j Department of Biology, Faculty of Sciences and Techniques of Errachidia, Moulay Ismail University of Meknes, Meknes 50050, Morocco

^k Department of Pharmacology and Physiology, Faculty of Medicine, University of Montreal, Montreal, QC H3C 3J7, Canada

^l Institut Européen des Antioxydants, 1B Rue Victor de Lespinats, 54230 Neuves-Maisons, France

^m Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Magdalenka, Jastrzebiec, Poland

ⁿ Geriatric Internal Medicine Department (Champmaillot), University Hospital Center, Université de Bourgogne Europe, 21000 Dijon, France

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ABSTRACT

Age-related diseases are often associated with a disruption of RedOx balance that can lead to lipid peroxidation with the formation of oxysterols, especially those oxidized on carbon-7: 7-ketocholesterol (also known as 7-oxocholesterol) and 7 β -hydroxycholesterol. Like cholesterol, these oxysterols have 27 carbons, they are composed of a sterane nucleus and have a hydroxyl function in position 3. The oxysterols 7-ketocholesterol and 7 β -hydroxycholesterol are mainly formed by cholesterol autooxidation and are biomarkers of oxidative stress. These two oxysterols are frequently found at increased levels in the biological fluids (plasma, cerebrospinal fluid), tissues and/or organs (arterial wall, retina, brain) of patients with age-related diseases, especially cardiovascular diseases, neurodegenerative diseases (mainly Alzheimer's disease), ocular diseases (cataract, age-related macular degeneration), and sarcopenia. Depending on the cell type considered, 7-ketocholesterol and 7 β -hydroxycholesterol induce either caspase-dependent or -independent types of cell death associated with mitochondrial and peroxisomal dysfunctions, autophagy and oxidative stress. The caspase dependent type of cell death associated with oxidative stress and autophagy is defined as oxiapoptophagy. These two oxysterols are also inducers of inflammation. These biological features associated with the toxicity of 7-ketocholesterol, and 7 β -hydroxycholesterol are often observed in patients with age-related diseases, suggesting an involvement of these oxysterols in the pathophysiology of these disorders. The cytotoxic effects of 7-ketocholesterol and 7 β -hydroxycholesterol are counteracted on different cell models by representative nutrients of the Mediterranean diet: ω 3 and ω 9 fatty acids, polyphenols, and tocopherols. There are also evidences, mainly in cardiovascular diseases, of the benefits of α -tocopherol and phenolic compounds. These *in vitro* and *in vivo* observations on 7-ketocholesterol and 7 β -hydroxycholesterol, which are frequently increased in age-related diseases, reinforce

* Corresponding author at: Centre des Sciences du Goût et de l'Alimentation, CNRS, INRAE, Institut Agro Dijon, Université de Bourgogne Europe, 21000 Dijon, France.

** Correspondence to: PHYNOHA Consulting & Université Bourgogne Europe / Inserm, France.

E-mail addresses: anne.vejux@u-bourgogne.fr (A. Vejux), gerard.lizard@u-bourgogne.fr, gerardlizard21.1@gmail.com (G. Lizard).

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the interest of nutritherapeutic treatments to prevent and/or cure age-related diseases currently without effective therapies.

1. Cholesterol and cholesterol autoxidation products: formation and inactivation of 7-ketocholesterol and 7 β -hydroxycholesterol

Oxysterols are molecules obtained by the oxidation of cholesterol; they can be formed endogenously (by autoxidation, via specific enzymes or by both processes), and/or provided through the diet such as dairy, egg, and meat products [1]. Like cholesterol, oxysterols consist of a sterane nucleus, and an aliphatic side chain connected to the nucleus at carbon-17 (<https://lipidmaps.org/resources/lipidweb/index.php?page=lipids/simple/cholesterol/index.htm>). Oxidation of cholesterol can take place by the addition of oxygen to the sterane nucleus and/or the side chain either by autoxidation (type I or II autoxidation) [2,3], or by enzymes, most commonly Cytochrome P450 enzymes [1,4]. Type I autoxidation concerns oxidation reactions involving reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) (superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), nitric oxide (NO), and peroxynitrite (ONOO $^-$)). ROS and RNS can be generated by cellular metabolism, or by their decomposition into hydroxyl radicals by the dismutation of two superoxide anions into hydrogen, or by the Fenton reaction ($H_2O_2 + Me^{n+} \rightarrow HO^{\bullet} + OH^- + Me^{(n+1)+}$) where Me is a transition metal such as copper, iron, or aluminum; type II autoxidation concerns non-radical attacks by oxygen singlet ($^1\Delta_g O_2$ or 1O_2), hypochlorous acid (HOCl; corresponding oxyanion: hypochlorite (ClO $^-$) or ozone (O_3) [2,5]. Oxysterols resulting from oxidation on the sterane nucleus and/or on the side chain can be increased or decreased in several severe diseases: age-related diseases (cardiovascular, brain and ocular diseases, sarcopenia) [6], metabolic diseases (type 2 diabetes, metabolic syndrome) [7, 8], and genetic diseases (X-linked adrenoleukodystrophy (X-ALD), Niemann Pick disease) [9,10]. Several oxysterols, especially those oxidized on the side chain, interact with cellular receptors [11]. They can be ligands or activators of the following receptors: (i) nuclear receptors, such as liver X receptors (LXRs) α or β [12] and retinoic acid receptor-related orphan receptor α and γ (ROR α [NR1F1] and ROR γ [NR1F3]) [13], (ii) cytoplasmic receptors such as SREBP (sterol regulatory element binding transcription protein) [14], NPC1 (NPC intracellular cholesterol transporter 1 / Nieman-Pick type C1) [15], FXR (NR1H4, farnesoid X receptor alpha) [16], oxysterols binding proteins (OSBPs), OSBPs-related proteins (ORPs) [17,18] and cholesterol epoxide hydrolase (ChEH) (also named anti-estrogen binding site (AEBS); ChEH is a hetero-oligomeric complex comprising 3 β -hydroxysterol- Δ^8 - Δ^7 -isomerase (DBD7I) and 3 β -hydroxysterol- Δ^7 -reductase (DHC7R)) [19] as well as (iii) membrane receptors such as receptor tyrosine kinases [20] and the Epstein–Barr virus-induced gene 2 receptor (EBI2, also known as GPR183) [21–23]. Among the oxysterols involved in age-related diseases, there are, among others, those which are formed mainly by autoxidation on the carbon 7, such as 7-ketocholesterol (7KC) and 7 β -hydroxycholesterol (7 β -OHC). It is important to underline that 7KC can also be formed enzymatically from 7 β -OHC by the hydroxysteroid dehydrogenase type 2 (11 β -HSD2; HSD11B2 gene, OMIM 614232) which is mainly expressed in the kidney, colon, and placenta [24]. In addition, in patients with cerebrotendinous xanthomatosis or with Smith Lemli Opitz (SLO) syndrome, 7KC can be formed from 7-dehydrocholesterol (a direct precursor in cholesterol biosynthesis belonging to the Kandutsch–Russell pathway) [25] by the enzyme cholesterol-7 α -hydroxylase (CYP7A1) [26,27]. In addition, the enzyme hydroxysteroid dehydrogenase type 1 (11 β -HSD1; HSD11B1 gene, OMIM 600713) reduces 7KC to 7 β -OHC [28]. Noteworthy, during lipid peroxidation, which affects all lipids, cholesterol is less susceptible to free radical attacks than polyunsaturated fatty acids in body fluids, while the opposite occurs at the cellular level [29]. The preferential site of oxidation of cholesterol by highly reactive species is at C7 because a

relatively weak carbon–hydrogen bond. Therefore, increased levels of 7KC and 7 β -OHC in the body fluids, as well as tissues and organs affected by the diseases is a sign of strong oxidative stress; however, in contrast to plasma oxidation, cellular cholesterol is more susceptible to oxidation than cellular linoleates [29]. The detailed mechanisms leading to the formation of 7KC but also 7 β -OHC, and the associated metabolites, have been described by several authors [2,3,30,31]. The major oxysterol metabolite routes for 7KC and 7 β -OHC inactivation are esterification, sulfation, oxidation, and reduction. These different aspects of biogenesis and inactivation of 7KC and 7 β -OHC are summarized in Fig. 1. Metal ions (Me^{n+}) which are involved in the Fenton reaction, where Me is a transition metal (copper, iron, or aluminum) also contribute to the biogenesis of 7KC and 7 β -OHC via $HO^{\bullet}$ production, not only in the body but also during the processing of industrial meat [32].

Currently, several data support that 7KC and 7 β -OHC, as well as their metabolites and degradation products, can constitute suitable biomarkers for several pathologies especially age-related diseases and in the ageing process [8,33,34]. They could be also of interest as biomarkers in some “civilization” diseases (diabetes, metabolic syndrome) [35] and in food industry involved in the production of more or less highly processed foods to evaluate food quality: indeed, long term storage as well as the industrial methods used to prepare food can influence autoxidation and have detrimental consequences on food qualities [36].

2. Required precautions and available methods for the identification and quantification of 7-ketocholesterol and 7 β -hydroxycholesterol

To evaluate the part taken by 7KC and 7 β -OHC in cell and tissue samples, reliable and sensitive analytical methods are required. These methods need important expertise, and several cautions must be taken both for sample collection and storage, as well as during the pre-analytical and analytical steps to avoid artefactual results. The analysis of 7KC and 7 β -OHC encounters numerous challenges due most often to their low concentrations in biological fluids and tissues, typically in the nanomolar range, with cholesterol coexisting at concentrations 10^4 – 10^6 times higher in the same samples. Concentrations in the micromolar ranges have however been observed in advance stages of patients with the most aggressive forms of X-linked adrenoleukodystrophies (X-ALD) [9] as well as in patients with acid SMase-deficient Niemann-Pick disease [37]. High amounts of these oxysterols can also be observed in brain lesions of patients with Alzheimer’s disease [38] and in the biological fluids (cerebrospinal fluid, plasma, serum) [39,40]. In addition, it is important to underline that after fat-rich meals, very high levels of 7KC can be found in the plasma [41,42] suggesting that regular consumption of fat could progressively favor the accumulation of this oxysterol in various tissues and gradually trigger important dysfunctions, since 7KC has strong pro-oxidant and inflammatory activities [6,43]. 7KC concentration is also strongly increased in the plasma of patients with chronic artery disease (CAD), and it is reproducibly detected in more than 90 % of patients with CAD [44]. In addition, multiple regression analysis revealed that 7KC was an independent variable for CAD progression [45]. It is important to underline that presence of high concentrations of cholesterol not only disrupts chromatography and contaminates instruments, but also poses the risk of autoxidation during sample preparation, leading to the artifactual formation of oxysterols such as 7KC and 7 β -OHC. Therefore, care must be taken for accurate identification and quantification of 7KC and 7 β -OHC. Several chromatography-based analytical methods have been developed to avoid this problem [1–4]. New analytical methods

available on tissues samples including antibody - based methods [5], as well as mass spectrometry imaging of oxysterols and cholesterol have been applied for this purpose [46,47], with advantages and limitations.

Thorough understanding of the fundamental levels of 7KC and 7 β -OHC is closely tied to the experimental conditions employed throughout the analytical process. This encompasses various stages such as sample collection, extraction, fractionation, separation, detection, and quantification [6]. Notably, the strategies applied in sample preparation such as the type of sample collection tubes used, the number of freeze-thaw cycles, presence of antioxidant compounds and storage conditions significantly contribute to variability, impacting the stability of samples, as well as the overall recovery and profile of 7KC and 7 β -OHC.

Oxysterol analysis has been successfully performed using mass spectrometry (MS) methods coupled with gas or liquid chromatographic separation. Over the years, research has been advanced to tackle common analytical challenges of oxysterol analysis with the development of high-sensitive mass spectrometers. Different approaches such as chemical derivatisation to improve the ionisation characteristics of the 7KC and 7 β -OHC for subsequent MS detection have been successfully applied in literature [7]. Derivatisation is often employed to enhance the volatility of oxysterols for efficient separation. However, this method requires a multi-step sample preparation process, making it time-consuming and prone to potential errors.

Various liquid chromatography methods, including reverse-phase and normal-phase chromatography, have been developed for separating 7KC and 7 β -OHC [4]. Tandem mass spectrometry (MS/MS) enhances specificity by providing structural information through fragmentation patterns [8]. Another widely used method in MS is the multiple reaction monitoring (MRM) method that allows to selectively detect and quantify 7KC and 7 β -OHC based on the screening of specified precursor-to-fragment ion transitions [2]. Since this method uses targeted approach, it is not possible to screen for unknown oxysterols.

MS methods are superior to high-performance liquid chromatography (HPLC) coupled with UV or fluorescence detection. However, HPLC methods remains a valuable option, as this provides a fast, simple

technique to separate, identify, and quantify most of the oxysterols [9], particularly for laboratories without access to advanced mass spectrometers.

ELISA assays offer a high-throughput and cost-effective alternative for screening many molecules. However, its specificity can be a concern for oxysterols, as the antibodies used may cross-react with structurally similar sterol compounds. For localization studies, immunohistochemistry allows researchers to visualize the distribution of 7KC within tissues. This technique provides valuable insights into the spatial distribution of 7KC in cells and tissue [5]. However, the methods must be used with care and adapted to the types of cells and tissue samples studied. Currently, the potential of mass spectrometry imaging (MSI) to study cholesterol, its precursors, and its metabolites, including oxysterols, in human biopsies is a major challenge [47].

Despite the advancements in oxysterol analysis, challenges persist, such as the need for stable isotope-labelled internal standards for accurate quantification and the isomer interference during separation of 7 α -hydroxycholesterol and 7 β -OHC. Future research may focus on refining existing methods, exploring novel techniques, and developing standardized protocols to ensure reproducibility and comparability across studies [48,49]. The identification and quantification of 7KC and 7 β -OHC are pivotal for unravelling their roles in health and disease. Researchers must carefully select and validate analytical methods based on the specific requirements of their investigations, balancing sensitivity, specificity, and practical considerations. Continued advancements in analytical techniques will undoubtedly contribute to a deeper understanding of the complex roles played by these oxysterols in cellular processes and disease pathogenesis including age-related diseases and ageing process [49,50].

3. Identification of 7-ketocholesterol and 7 β -hydroxycholesterol in patients with age-related diseases

A more or less pronounced oxidative stress is a common feature of age-related diseases [51–53]. Consequently, several biomarkers of

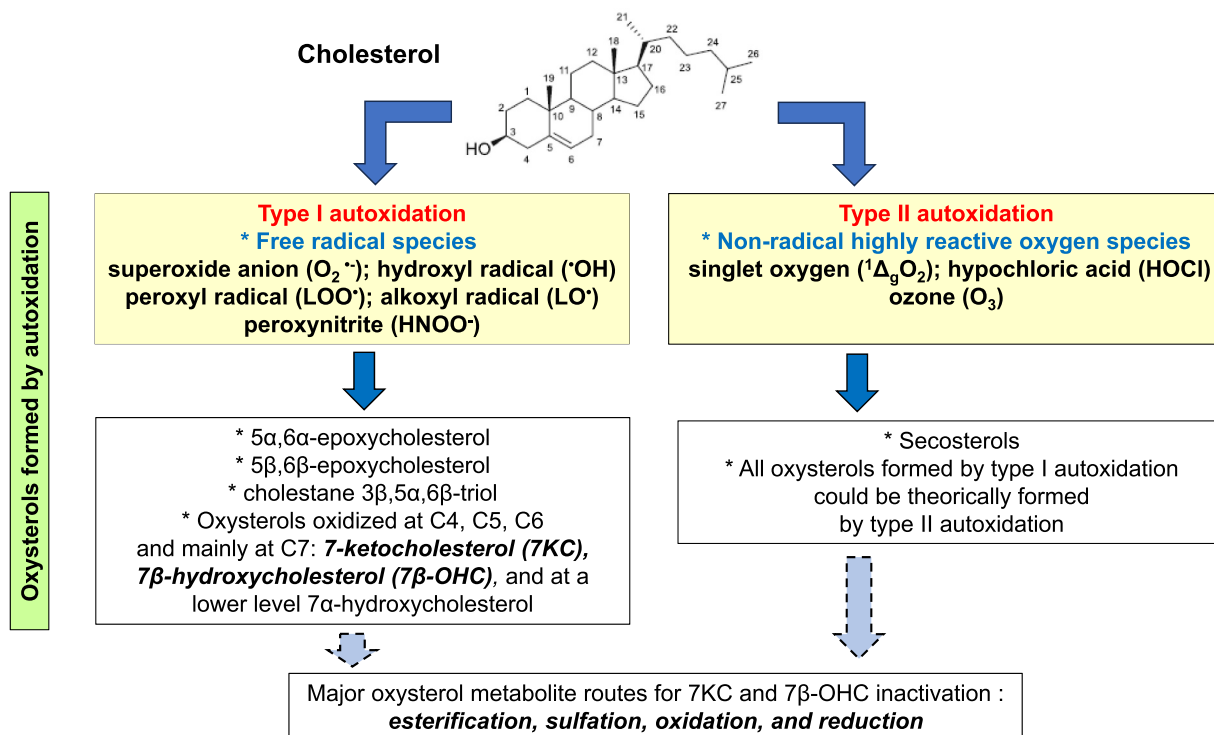


Fig. 1. Oxysterols formed by autoxidation. Details about the formation of 7-ketocholesterol (7KC) and 7 β -hydroxycholesterol (7 β -OHC) are given in the following references: Iuliano in 2011, and Zerbinati & Iuliano in 2017 [2,3]; Anderson *et al.* in 2020 [30] and Ghzaïel *et al.* in 2022 [31].

oxidative stress have been observed in these diseases, including lipid peroxidation biomarkers which reflect fatty acid oxidation (increased level of malondialdehyde (MDA) [54], 4-hydroxynonenal (4-HNE) [55], F(2)-isoprostanes [56], 9-hydroxy-10,12-octadecadienoic acid and 9-hydroxy-10,12-octadecadienoic acid (HODEs) [57], cholesterol autooxidation products (oxysterols: 7KC and 7 β -OHC), and enhanced levels of carbonylated proteins [58] and/or advanced glycation end products (AGEs) [59]. Overall, oxysterols, formed either by autooxidation and enzymatically, could be useful as biomarkers of aging to evaluate biological aging compared to chronological aging [33,34]. Whereas chronological age corresponds how old we are, biological age is how old our cells are depending on internal and external factors [60]. As a result, there is not necessarily a correlation between chronological and biological age, and thus we can appear younger or older than our age. Some biomarkers of oxidative stress, especially 7KC and 7 β -OHC, are most often described in biological fluids (cerebrospinal fluid, plasma) and in the cells and/or tissues of organs (atheroma plaques, heart, arteries and blood vessels, lens, retina, brain) affected by the disease [6,61]. In cardiovascular diseases, enhanced levels of 7KC and 7 β -OHC have been described both in the plasma and atheroma plaques where apoptotic cells can be simultaneously detected [8,62]. It has been clearly established that the cytotoxic components of oxidized low-density lipoproteins (LDL) were 7KC and 7 β -OHC [63]. The role of 7KC and 7 β -OHC is also widely suspected in vascular aging [64,65]. In age related macular degeneration and cataract enhanced levels of 7KC are found in drusen at the retinal level [66] and in the lens [67] respectively. In Alzheimer's disease enhanced levels of 7KC and 7 β -OHC have been observed in brain lesions [68], and in the cerebrospinal fluid and plasma [39,69]. In sarcopenia, significant increased levels of 7 β -OHC have been identified [70,71]. In breast cancer, which is the most common cancer in young and post-menopausal women, a significant increase in the level of 7KC was observed in the samples following tumor removal and the start of therapy compared to the sampling before [72]. *In vitro* data suggest that 7KC could modulate the efficiency of chemotherapeutic treatment (doxorubicin, tamoxifen) in breast cancer cells: 7-KC stimulates the efflux function of P-glycoprotein and reduced intracellular doxorubicin accumulation in MCF-7 human breast cancer cells (estrogen receptor (ER) positive cells); 7KC slightly decreases the efficacy of tamoxifen in MCF-7 cells, while an increased effect of tamoxifen and higher caspase 3/7 activity was observed in the human breast cancer BT-20 cell line (ER negative cells) [73,74]. Some authors suggested that 7KC and derivatives, due to its biological activities (stimulation of immune response, cell death induction) could be used as anti-tumoral drugs [75,76]. In severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection often observed in patients over 65 years old, enhanced levels of 7KC and 7 β -OHC were reported, and the contribution of these oxysterols in the pathophysiology of this infectious disease still remains not well understood [70,77]. However, accumulation of oxysterols, including 7KC, in the erythrocytes of COVID-19 patients has been proposed as a biomarker for case severity [78]. Altogether, data on the potential involvement of 7KC and 7 β -OHC in frequent age-related disorders is questionable since these oxysterols favor oxidative stress, inflammation and cell death which are hallmarks of several age-related diseases. The biological characteristics of these oxysterols also open new perspectives with therapeutic applications in several human diseases including age-related diseases, infectious and inflammatory diseases, and cancers [79,80].

3.1. Biological activities of 7-ketocholesterol and 7 β -hydroxycholesterol

The biological activities of 7KC and 7 β -OHC were initially described on cells of the vascular wall (endothelial cells and smooth muscle cells) and human monocytic U937 cells in the context of cardiovascular diseases [81–83] and cancer [84,85], respectively. Subsequently, the biological activities of 7KC and 7 β -OHC were studied on retinal epithelial cells, nerve cells, skeletal muscle cells and bone cells, since enhanced

levels of these oxysterols were found in patients with cardiovascular diseases, age-related macular degeneration, Alzheimer's disease, sarcopenia and osteoporosis [86]. Noteworthy, 7KC and 7 β -OHC are strong inducers of cell death, and the mode of cell death induced can be caspase-dependent or -independent, depending on the cell type considered. However, this cell death is always associated with an increased production of reactive oxygen species (ROS) and a rupture of RedOx homeostasis, as well as with organelle dysfunctions (mitochondria, peroxisome and/or lysosome) [86] (Fig. 2). In some cells, 7KC and 7 β -OHC can also induce inflammatory processes leading to the secretion of inflammatory cytokines [62] stimulation of the expression of adhesion molecules [87–89] and activation of matrix metalloproteinase [90,91]. In several cell types from different species, 7KC and 7 β -OHC induce a mode of cell death defined as oxiaoptophagy, described for the first time in human monocytic cells U937 in 2003 [92], which is considered as an hybrid cell death type [93], and could favor vascular diseases [94]. In U937 cells, oxiaoptophagy is associated with cytokine-dependent inflammation characterized by a secretion of MCP-1, MIP-1 β , TNF- α , and/or IL-8 secretion, the latter involving the MEK / ERK1/2 signaling pathway [62]. This type of cell death, which includes the activation of oxidative stress, apoptosis induction, and is associated with autophagic criteria (oxiaoptophagy: OXIidative stress + APOPTosis + autoPHAGY) [95] has been observed in the presence of 7KC and/or 7 β -OHC on murine nerve cells (oligodendrocytes, glial cells, neuronal cells) [96–99], in bone marrow mesenchymal stem cell from patients with acute myeloid leukemia [100] and in the murine osteoblastic MC3T3-E1 cell line [101]. Noteworthy, this type of cell death can also be induced by other oxysterols. Thus, oxiaoptophagy has been described on 158 N oligodendrocytes in the presence of 24S-hydroxycholesterol [102], in myeloma cell in the presence of 5,6-epoxycholesterol [103], with 25-hydroxycholesterol in murine L929 fibroblasts [104], and with 7 α , 25-dihydroxycholesterol in murine chondrocytes both *in vitro* and *in vivo* [105]. The detailed signaling pathways associated with 7KC- and 7 β -OHC-induced oxiaoptophagy are well described by Vejux *et al.* [86] and Nury *et al.* [5] which makes it possible i) to consider identifying pharmacological targets and ii) to search for natural or synthetic molecules as well as mixtures of molecules (such as edible oils) to oppose the toxicity of these compounds [5,106]. Currently, there is also preliminary data supporting that oxiaoptophagy can be induced by natural molecules, which are not sterols. In C6 rat glioma cells, 7 β -OHC induces a mode of cell death by autophagy (lethal autophagy) associated with oxidative stress and organelle dysfunctions [107]. In other cells, such as human fibroblasts, 7KC and 7 β -OHC induces a mode of cell death considered as necrosis [82] and in human retinal ARPE-19 cells, both caspase-dependent and -independent modes of cell death have been reported [108–111] with a simultaneous induction of IL-8 secretion [109]. In the immortalized mouse myoblast C2C12 cell line, 7KC and / or 7 β -OHC induce a caspase-independent mode of cell death associated with ROS overproduction, mitochondrial and peroxisomal changes [70,71] evocating ferroptosis [112,113] with a simultaneous secretion of IL-6, IL-8, and TNF- α [71]. Fig. 3 summarizes the main cytotoxic effects of 7 β -OHC (50 μ M; 24 h of treatment) [70,71] observed on C2C12 cells. The 7 β -OHC-induced cell death, was measured by flow cytometry after staining with fluoresceine diacetate (FDA); it was associated with loss of transmembrane mitochondrial potential ($\Delta\Psi$ m), measured using 3, 3'-dihexyloxycarbocyanine iodide (DiOC6(3)); overproduction of reactive oxygen species (ROS) was evaluated on whole cells and at the mitochondrial level after staining with dihydroethidine (DHE) and MitoSOX red, respectively, and peroxisomal changes was evaluated by transmission electron microscopy. Whereas few peroxisomes were observed in untreated C2C12 cells, several peroxisomes localized in vacuolar structures, evocating pexophagy [114,115], were detected in 7 β -OHC-treated cells. Under treatment with 7KC and 7 β -OHC, whatever the type of cells used and the type of cell death induced, an overproduction of ROS is always observed, associated with mitochondrial and peroxisomal dysfunctions.

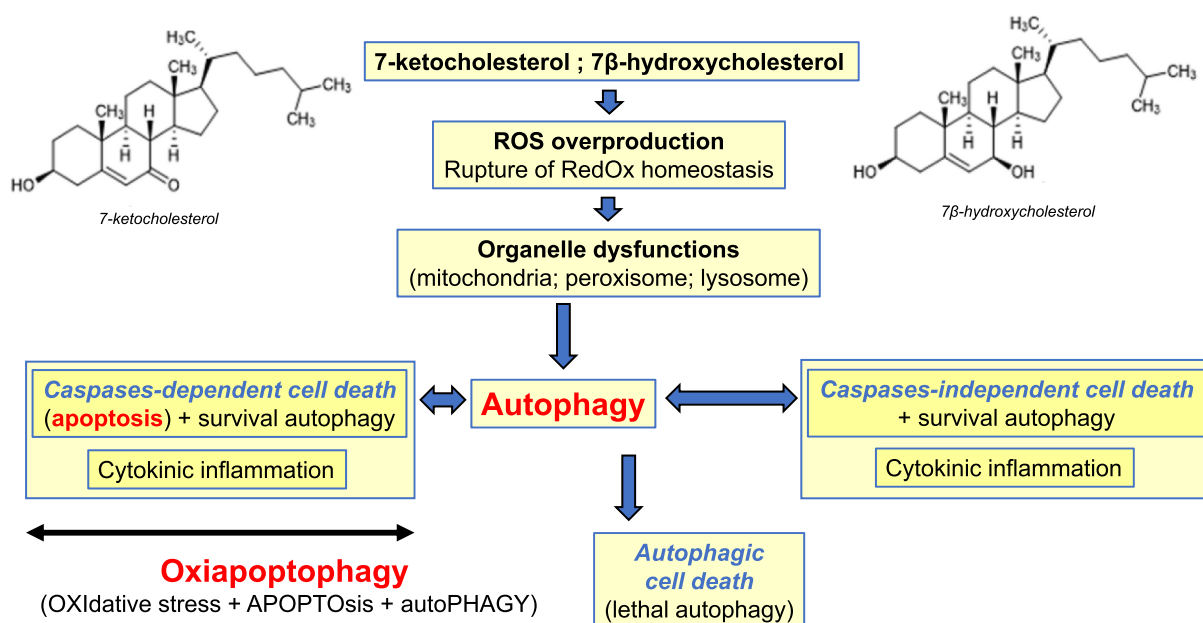


Fig. 2. Caspase-dependent and -independent mode of cell death induced by 7-ketocholesterol and 7β-hydroxycholesterol. Various types of cell death have often been described as caspase-dependent and -independent [182]. 7KC and 7β-OHC are strong inducers of cell death, and the mode of cell death induced is always associated with an increase production of reactive oxygen species (ROS) and a rupture of RedOx homeostasis, as well as with organelle dysfunctions (mitochondria, peroxisome and/or lysosome). The signalling pathways associated with 7KC- and 7β-OHC-induced oxiaptophagy (a caspase-dependent mode of cell death associated with oxidative stress, apoptosis and autophagy) are well described by Vejux *et al.* and Nury *et al.* [95,183]. Oxiaptophagy is currently considered as an hybrid cell death type [93].

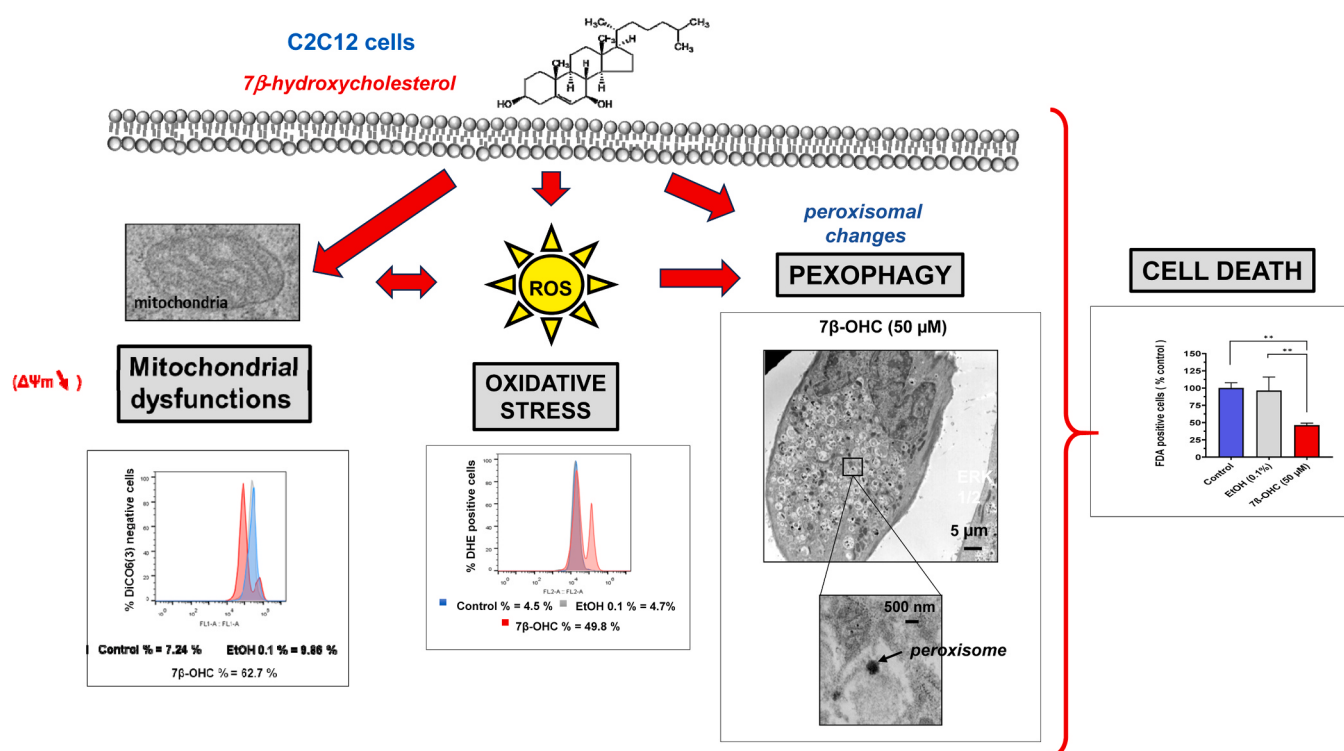


Fig. 3. Main characteristics of 7β-hydroxycholesterol-induced cell death in the immortalized mouse myoblast C2C12 cell line. 7β-OHC -induced cell death in C2C12 cells, which is not an apoptotic mode of cell death, is associated with an increase of depolarized mitochondria, overproduction of ROS at both whole-cell and mitochondrial levels, and the presence of altered peroxisomes leading to a particular type of autophagy: pexophagy. This figure illustrates the main cytotoxic effects of 7β-OHC and 7KC observed whatever the type of cell used and the type of cell death induced: ROS overproduction, mitochondrial and peroxisomal dysfunctions.

3.2. Prevention of 7-ketocholesterol- and 7 β -hydroxycholesterol-induced cytotoxic effects with major nutrients of the Mediterranean diet and edible oils: *in vitro* studies

To prevent 7KC- and 7 β -OHC-induced cytotoxic effects (oxidative stress, inflammation, and cell death induction), several strategies can be used. Currently, three strategies are possible consisting of either i) promoting the degradation of 7KC with bacterial enzymes [116], ii) selectively removing 7KC from the cells with UDP-003, which belongs to a new class of cyclodextrin dimers [117] or iii) acting on the signaling pathways involved in the cytotoxicity of this oxysterol [5]. For this latter strategy, molecules or mixtures of molecules (edible oils), which are efficient on 7KC, are also most often efficient on 7 β -OHC [5,86,106]. Interestingly, among natural molecules and edible oils counteracting 7KC- and 7 β -OHC-induced cytotoxic effects, several of them are representative nutrients of Mediterranean diet known for its benefits on human health, to prevent cardiovascular diseases, and to favor ageing in good health [118,119].

Up to now, only a few synthetic molecules attenuate 7KC- and 7 β -OHC-induced cytotoxic effects. These are dimethyl fumarate (DMF), also known under the name of Tecfidera which is commercialized by Biogen and used in the treatment of remitting-relapsing multiple sclerosis, and one of its metabolites, monomethyl fumarate (MMF) [120,121]. Interestingly, sulfo-N-succinimidyl oleate (SSO, a synthetic derivative of oleic acid (C18:1 n-9)) (SSO) has been shown to prevent 7KC-induced oxidative stress and cell death on 158 N and ARPE-19 cells, without lipid droplet formation induction as observed in the presence of fatty acids and edible oils [122].

Among natural molecules ω 3 and ω 9 fatty acids such as docosahexaenoic acid (DHA, C22:6 n-3) present in high amount in some fishes (sardine, mackerel, tuna) present in Mediterranean cuisine [123] and fish oils [124], as well as oleic acid (C18:1 n-9) present in high amount in several Mediterranean oils, especially olive and argan oils [125,126], strongly attenuate 7KC- and 7 β -OHC-induced cytotoxic effects [5]. Alpha-tocopherol (a component of vitamin E comprising four tocopherols (α -, β -, γ -, and δ -tocopherol) and four tocotrienols (α -, β -, γ -, and δ -tocotrienol)) [127] is also present in several edible oils associated with the Mediterranean diet, and can be considered as a strong inhibitor of 7KC- and 7 β -OHC-induced cell death: it is efficient whatever the cell type considered and the type of cell death induced [5,86,128–131]. In 7KC-treated cells, α -tocopherol prevents the accumulation of 7KC in lipid rafts [132,133]. However, whereas 7 β -OHC does not accumulate in lipid rafts, α -tocopherol also prevents its cytotoxicity [133]. Besides its accumulation in lipid raft and its antioxidant role, α -tocopherol has also important effects on cell signaling and gene expression which could explain its cytoprotective activity [134]. There is also evidence that several polyphenols found in high amount in the Mediterranean diet (*trans* resveratrol, quercetin, apigenin) [135] attenuate 7KC-induced oxiaapoptophagy on neuronal N2a cells, Human Monocyte-Derived M1 and M2 Macrophages or ARPE-19 cells [99,109,136]. Other nutrients present in the Mediterranean diet as γ -tocopherol, sterculic acid, lycopene, epicatechin, epigallocatechin-3-gallate, hydroxytyrosol, and tyrosol are also potent inhibitors of 7KC- and 7 β -OHC-induced cytotoxic effects [5,35,137–144]. Protective effects of biotin (also known as Vitamin B8, identified in seeds and oleaginous fruits) have only been observed on 7 β -OHC-treated cells [145]. However, epidemiological studies revealed that isolated bioactive phytochemicals (polyphenols, tocopherols) are not as effective as fruits and vegetables containing these substances whereas they are of interest for the functional food industry [146].

As several Mediterranean oils are a mixture of nutrients (fatty acids, polyphenols, tocopherols), which counteract 7KC- and/or 7 β -OHC-induced cytotoxic effects, the cytoprotective activities of these edible oils (olive and argan oils, *Pistacia lentiscus* seed oil, pomegranate seed oil, and milk thistle seed oil) were evaluated *in vitro* in 7KC- and/or 7 β -OHC-treated cells.

In a recent study conducted by Ghzaïel *et al.* [147], it was proven that with Tunisian *Pistacia lentiscus* seed oil (PLSO) (100 μ g/mL), the 7 β -OHC-induced cytotoxic effects in murine C2C12 myoblasts were strongly attenuated. The cytoprotection was comparable to that seen with 400 μ M of α -tocopherol, which was utilized as a positive control. This cytoprotective effect was characterized by decreased oxidative stress (reduction in ROS overproduction in whole cells and at the mitochondrial level; decrease in the formation of lipid and protein oxidation products; and normalization of antioxidant enzyme activities: glutathione peroxidase (GPx) and superoxide dismutase (SOD)), as well as prevention of cell death and organelle dysfunctions (restoration of cell adhesion, cell viability, and plasma membrane integrity; prevention of mitochondrial and peroxisomal damages). These findings demonstrate that PLSO comprises a combination of bioactive compounds that counteract the cytotoxic effects of 7 β -OHC on C2C12 myoblasts and has antioxidant qualities comparable to α -tocopherol used at high concentration. The cytoprotective properties of Mediterranean oils against the toxicity induced by 7KC (25 μ M; 50 μ M) and 7 β -OHC (12.5 μ M; 25 μ M) were also confirmed by Ksila *et al.* [148] in N2a murine neuronal cells when using pomegranate seed oil (100–200 μ g/mL). Additionally, it was observed that argan oil and milk thistle seed oil prevented ROS overproduction induced by 7KC (25 μ M) in murine oligodendrocytes (158 N) (measured by flow cytometry after staining with DHE) and improved plasma membrane permeability to propidium iodide (PI), which is a cell death criterion [149,150]. It is widely known that 7KC causes oxidative stress, cell death, and organelle malfunction (mitochondria, lysosomes, endoplasmic reticulum, and peroxisomes), which are the hallmarks of neurodegeneration. It was proven that the 7KC-induced adverse effects are significantly reduced by olive oil which is frequently utilized in the Mediterranean diet for cooking. The cytoprotective effects obtained with this oil on 158 N oligodendrocytes and BV-2 microglial cells further support its potential to protect against neurodegenerative illnesses. Indeed, on 158 N and BV-2 cells, 7KC (25 μ M)-induced loss of plasma membrane esterase activity measured after staining with FDA, which is also a cell death criterion, is prevented by olive and argan oil [151,152]. These data support that olive oil could be a helpful supplement to pharmaceutical treatments or to prevent central nervous system (CNS) dysfunction [153]. Based on animal models and clinical investigations, some polyphenols of olive oil, such as hydroxytyrosol, may induce their protective effects through the potentiation of neurotrophins, which are molecules known to favor neuron growth, proliferation, survival and differentiation, and which also have antioxidant properties [154,155].

In a fibroblast model of patients with Smith-Lemli-Opitz syndrome (SLOS, an inborn error of cholesterol biosynthesis characterized by diminished cholesterol and increased 7-dehydrocholesterol (7-DHC) levels), a mix of antioxidants showed a decrease in 7-DHC-derived oxysterol, 3 β ,5 α -dihydroxycholest-7-en-6-one levels but also a normalization of the changes observed in gene expression [156]. The antioxidant mix was composed of vitamin A, coenzyme Q10, vitamin C, and vitamin E. According to the results of this study, the effects of the antioxidant mix were mainly due to vitamin E [156].

There are also several studies supporting that the cytoprotective effects of edible oils could be due to tocopherols (α - and γ -tocopherol), and oleic acid (C18:1 n-9), which strongly reduce 7KC- and 7 β -OHC-induced cytotoxicity [109,137,157,158]. Interestingly, some of them could cross the blood-brain barrier. This is well established for tocopherols, long-chain fatty acids (carbon chain of 13–22 carbons) as well as polyphenols [159–161]. Bioactive compounds counteracting 7KC and 7 β -OHC-induced cytotoxicity are listed respectively in Table 1 and Table 2.

3.3. Prevention of age-related diseases associated with increased levels of 7-ketocholesterol and 7 β -hydroxycholesterol: *in vivo* arguments in favor of nutritherapy on animal models

Numerous evidences of the contribution of 7KC and 7 β -OHC in age-

Table 1
Bioactive compounds counteracting 7-ketocholesterol-induced cytotoxicity (oxidative stress, inflammation and/or cell death induction).

Oxysterol	Cytoprotective compounds (nutrients and edible oils)	Cells	7KC concentrations	References
7-ketocholesterol (7KC)	Tocopherols	U937 cells; A7r5 cells; 158 N cells; N2a cells; BV-2 cells	25–50 µM	[39,111, 113, 114–116, 140]
	Fatty acids	BV-2 cells	50 µM	[114]
		N2a cells	50 µM	[111]
		N2a cells	50 µM	[111]
		158 N cells; BV-2 cells	50 µM	[111,114,133,140]
	Polyphenols	N2a cells	12 µM	[123,124]
		ARPE-19 cells	20 µM	[125]
		J774A.1 cells	50 µM	[126]
		Human angiosarcoma cells (ISO-HAS)		
		N2a cells; Human Monocyte-Derived M1 and M2 Macrophages; ARPE-19 cells	15–150 µM	[93,103,120]
		N2a cells	50 µM	[93]
		N2a cells	50 µM	[93]
		Caco-2 cells	1875 µM	[127,128]
		Caco-2 cells	1875 µM	[127,128]
	Edible oils	158 N cells	50 µM	[114; 133, 140]
		158 N cells	50 µM	[133]
		158 N cells	50 µM	[134]
		N2a cells	25 µM; 50 µM	[132]

ALA: Alpha Linoleic Acid; **EPA:** Eicosapentaenoic acid; **OA:** Oleic Acid.
Nerve cells: murine neuroblast N2a cells; rat oligodendrocyte 158 N cells; murine microglial BV-2 cells; **human colorectal adenocarcinoma cells:** CaCo-2; **Human angiosarcoma cells:** ISO-HAS; **Pro-Monocytic cells:** human monocytes derived from M1 and M2 macrophages; human pro-monocytic leukemia cells U937; murine macrophages J774A.1; **Rat smooth muscle cells** A7r5; **Human retinal pigment epithelial cells:** ARPE-19.

related diseases are reported in cardiovascular diseases, ocular diseases and sarcopenia. In this context, convincing data are described on various animal models [162]. Dietary 7-ketocholesterol exacerbates myocardial ischemia–reperfusion injury in mice through monocyte/macrophage-mediated inflammation [86,163] and in humans [68,164,165]. In cardiovascular diseases, several compounds representative of the Mediterranean diet known to counteract 7KC- and 7β-OHC-induced *in vitro* cytotoxic effects, can also preserve a normal vascular status. Thus, phytosterols (β-sitosterol, stigmasterol), which are abundant in several vegetables, are beneficial in improving lipoprotein profile and aortic function in hamster [166]. Less 7KC- and 7β-OHC were also identified in the meat from chicken fed α-tocopherol- and β-carotene-supplemented diets supporting a potential benefits of these nutrients in sarcopenia[167]. Although studies have shown that people who eat diets rich in vitamin E are less likely to suffer from cardiovascular diseases, other studies on vitamin E supplements are rather contradictory [168,169]. In a model of hamsters fed hypercholesterolemic diets, it was shown that antioxidant supplementation (selenium, α-tocopherol) led to a drop in plasma cholesterol concentrations, a reduction in lipid peroxidation in tissues, and an increase in liver oxysterol concentrations (inhibition of free radical-mediated oxysterol catabolism) [170]. In a mouse model of SLOS (Dhcr7-heterozygous), pregnant females were fed a vitamin E-enriched diet. This supplementation reduced the formation of oxysterols in the brain and liver of the newborn Dhcr7-knockout pups [156]. Currently, several benefits of *trans* resveratrol have been shown to be effective in the prevention and / or treatment of patients with age-related macular degeneration or cataract [171]. The PREDIMED (“PREvención con Dieta MEDiterránea”) study has also shown that the incidence of major cardiovascular events was lower among the patients assigned to a Mediterranean diet supplemented with extra-virgin olive oil or nuts than among those assigned to a reduced-fat diet [172]. Red wine polyphenolic compounds also preserve a normal vascular reactivity by acting at different stages of the cascade that leads to lipid oxidation, endothelium dysfunctions and vasospasm [173]. Extended lifespan was also observed in obese mice fed a diet supplemented with a polyphenol-rich plant extract (PRPE) [174] which also favors the differentiation of myocytes in myotubes supporting also potential benefits in patients with sarcopenia [175]. Altogether, these *in vivo* data support benefits of nutritherapy with representative compounds of the Mediterranean diet to prevent and / or cure age-related diseases associated with increased levels of 7KC and 7β-OHC.

3.4. Prevention of age-related diseases associated with increased levels of 7-ketocholesterol and 7β-hydroxycholesterol: *in vivo* arguments in favor of nutritherapy based on human studies

Clinical studies have been carried out to assess the effects of various dietary compounds on oxysterol-induced toxicity. These studies were mainly carried out in the context of cardiovascular diseases identified very early on as involving oxysterols. Most clinical studies focus on the involvement of oxysterols in pathology, notably cardiovascular pathology, but also in neurodegenerative diseases such as Alzheimer’s disease. Few studies on nutritherapy applied to oxysterols have published results, but their number is increasing. Some are still in the recruitment phase, while others have completed their recruitment very recently. Here are a few interesting studies: Cholesterol and Antioxidant Treatment in Patients With SLOS (ClinicalTrials.gov ID NCT01773278) and Evaluation of Consuming Olive Extract on Total Cholesterol Levels (OLICOL) (ClinicalTrials.gov ID NCT06490133).

In healthy subjects, the changes in lipid species relevant to cardiovascular disease (fatty acids, sterols, and oxysterols) were studied after drinking 400 mL coffee a day for 8 weeks [176]. Two coffees were tested containing 787 mg or 407 mg of chlorogenic acid. In the “coffee” group, subjects saw their levels of oxysterols and free fatty acids fall, while in the control group there was an increase [176]. At the same time, the

Table 2
Bioactive compounds counteracting 7β-hydroxycholesterol-induced cytotoxicity.

Oxysterol	Cytoprotective compounds (nutrients and edible oils)		Cells	7 β-OHC concentrations	References
7β-hydroxycholesterol (7β-OHC)	Tocopherols	α-tocopherol; 10–400 μM	158 N cells; U937 cells	50 μM 30 μM	[112,117]
	Polyphenols	Epigallocatechin–3-gallate; 1 μM	CaCo–2 cells	4.4 μM	[122]
		Resveratrol; 1 μM	ARPE- 19 cells	75 μM	[103]
	Edible oils	Pomegranate seed oil; 100 μg/mL; 200 μg/mL	N2a cells	12.5 μM; 25 μM	[132]
		<i>Pistacia lentiscus</i> L. seed oil; 100 μg/mL	C2C12 cells	50 μM	[131]

Human Retinal Pigment Epithelial Cells: ARPE-19, Nerve Cells: N2a (mouse neuroblasts); 158 N (rat oligodendrocytes), Human Pro-Monocytic Cells: U937, Epithelial Intestinal Cells: CaCo-2; Mouse Myoblasts: C2C12.

antioxidants in coffee were tested on a foam cell model (human THP-1 monocytic cells treated with oxidized LDL) and a decrease in the presence of oxysterols and arachidonic acid was observed [176]. In 68 postmenopausal women with hypercholesterolemia, Korean red ginseng consumption (2 g once a day) improves cholesterol metabolism by decreasing cholesterol and 7-hydroxycholesterol levels [177]. However, further studies are needed to corroborate these results. The effect of vitamin E supplementation was tested in candidates for carotid endarterectomy for whom plasma levels of 7β-OHC, 7KC, cholesterol, and vitamin E were measured [178]. In parallel with an increase in plasma vitamin E levels, this supplementation showed a decrease in 7β-OHC levels. This improvement in oxidative status via the drop in 7β-OHC was only observed in plasma, with no difference observed in the content of oxysterol and vitamin E in plaques. Twenty healthy Italian subjects were recruited to study the bioavailability of vitamin E in relation to dietary intake and the effect on plasma lipid peroxide-scavenging activity and on 7β-OHC and 7KC as markers of oxidative stress [179]. Vitamin E was administered either with food or directly on an empty stomach. Plasma vitamin E levels increased significantly with food intake. The lipid peroxide-scavenging activity of plasma increased significantly in the group where vitamin E was given with food. A reduction in 7β-OHC and 7KC levels was observed, but without statistical differences [179]. The authors emphasize that oxysterol levels were initially low, probably related to the Mediterranean diet. In view of these results, the authors therefore recommend using vitamin E added to meals for greater effectiveness. Altogether, the clinical studies presented tend to demonstrate that nutritherapy can be a possible way to help slow the progression of cardiovascular diseases associated with increased levels of 7β-OHC and/or 7KC, but that it is important to consider the form of this nutritional intake.

4. Conclusion

Oxysterols, 7KC and 7β-OHC, are often present at an increased level in biological fluids, tissues and/or organs of patients affected by age-related diseases: cardiovascular diseases, ocular diseases (age-related macular degeneration, cataract), neurodegenerative diseases (Alzheimer's disease in particular), and sarcopenia. In the light of these considerations, 7KC and 7β-OHC may be considered as biomarkers of these diseases specially to evaluate the local and / or systemic oxidative stress level. However, there is still a need to better define how to optimize the use of 7KC and 7β-OHC alone or in combination with other biomarkers in terms of diagnosis, prognosis, and evaluation of treatments efficiencies. In addition, a better knowledge of the cytotoxic activities of 7KC and 7β-OHC will provide a better understanding of the associated signaling pathways, enabling pharmacological targets to be identified for better treatment of the diseases. Interestingly, the ability of several nutrients from the Mediterranean diet (fatty acids, polyphenols and tocopherols) to counteract 7KC- and 7β-OHC-induced cytotoxic effects brings additional arguments supporting the importance of nutrients to prevent age-related diseases. The marked cytoprotective activities observed with ω3 and ω9 fatty acids, polyphenols and α-tocopherol highlight a new concept relying on oxysterols, nutrition

and age-related diseases. Consequently, diet or functional foods rich in nutrients (ω3 and ω9 fatty acids, polyphenols and α-tocopherol) that counteract the toxicity of 7KC and 7β-OHC could therefore reduce the incidence of age-related diseases, the frequency of which is increasing as life expectancy is longer. As well as being of scientific and medical interest, the use of nutritherapy to prevent and / or cure 7KC- and 7β-OHC-induced age-related diseases can be considered of economic and social interest.

CRediT authorship contribution statement

El Midaoui Adil: Writing – original draft. Meziane Smail: Investigation. Atanasov Atanas G.: Writing – original draft. Hammami Sonia: Writing – original draft. Ksila Mohamed: Investigation. Zarrouk Amira: Investigation. Nury Thomas: Investigation. Brahmi Fatiha: Investigation. Mackrill John J: Writing – original draft. Ghzaïel Imen: Writing – original draft, Investigation. Dias Irundika H K: Writting - original draft. Rezig Leila: Writing – original draft. Vejux Anne: Writing – original draft, Supervision, Conceptualization, Investigation. Latruffe Norbert: Writting - original draft. Jouanny Pierre: Writting - original draft. Lizard Gérard: Writing – original draft, Conceptualization, Investigation.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Data availability

No data was used for the research described in the article.

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