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Oxysterols: From redox bench to industry

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ABSTRACT

More and more attention is nowadays given to the possible translational application of a great number of biochemical and biological findings with the involved molecules. This is also the case of cholesterol oxidation products, redox molecules over the last years deeply investigated for their implication in human pathophysiology. Oxysterols of non-enzymatic origin, the excessive increase of which in biological fluids and tissues is of toxicological relevance for their marked pro-oxidant and pro-inflammatory properties, are increasingly applied in clinical biochemistry as molecular markers in the diagnosis and monitoring of several human and veterinary diseases. Conversely, oxysterols of enzymatic origin, the production of which is commonly under physiological regulation, could be considered and tested as promising pharmaceutical agents because of their antiviral, pro-osteogenic and antiadipogenic properties of some of them. Very recently, the quantification of oxysterols of non-enzymatic origin has been adopted in a systematic way to evaluate, monitor and improve the quality of cholesterol-based food ingredients, that are prone to auto-oxidation, as well as their industrial processing and the packaging and the shelf life of the finished food products. The growing translational value of oxysterols is here reviewed in its present and upcoming applications in various industrial fields.

1. Introduction

Oxysterols are cholesterol oxidation products that differ from cholesterol for the presence of an additional ketone, epoxy or hydroxyl group in the A or B rings or for a hydroxyl group in the side chain. The most frequent sites of cholesterol oxidation in pathophysiology are highlighted in Fig. 1. The scientific interest for this family of compounds has increased along the time due to their actual involvement as oxidized lipids in a variety of physiological and pathological processes [1–3]. Moreover, in the last few years new biological and pathological properties have been ascribed to these cholesterol oxidation products, confirming how fascinating is this area of research.

The production of oxysterols could be enzymatically or non-enzymatically driven, the former process being physiologically regulated, the latter occurring in a random and not regulated way. The enzymatic oxidation of cholesterol is mediated by mitochondrial or microsomal enzymes, most of them belonging to the cytochrome P450 family [4]. The oxysterols of enzymatic origin of biomedical interest are 27-hydroxycholesterol (27OHC), 24-hydroxycholesterol (24OHC), 25-hydroxycholesterol (25OHC), 7α-hydroxycholesterol (7αOHC), present in nanomolar amounts in human blood [5,6], cerebrospinal fluid [7,8], maternal colostrum and mature milk [9]. These and other cholesterol oxides of enzymatic origin are recognized to be intermediates of biliary acids and steroid hormones synthesis, good ligands of several membrane and nuclear receptors, thus valid triggers of various cell signaling pathways, almost always implying redox reactions [3,10,11]. In Fig. 2 the main molecular interactions of enzymatic oxysterols are summarized.

The main oxysterols of non-enzymatic origin detectable in human tissues and fluids are 7-ketocholesterol (7KC), 7β-hydroxycholesterol (7βOHC), 5α,6α-epoxide (α-epoxy), 5β,6β-epoxide (β-epoxy), cholestan-3β,5α,6β-triol (triol), but also part of 7αOHC and 25OHC [5,6]. These oxysterols can be produced by light or heat oxidation or excessive storage under air of cholesterol containing food, but also, within tissues and organs, by a variety of oxidant species mainly stemming from inflammatory processes [1,2], thus in any case involving redox reactions.

Comprehensive schemes of cholesterol autoxidation reactions are for

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Abbreviations:

11β-HSD-1	11-β-hydroxysteroid dehydrogenase type 1
20SOHC	20(S)-hydroxycholesterol
22ROHC	22(R)-hydroxycholesterol
22SOHC	22(S)-hydroxycholesterol
24OHC	24-hydroxycholesterol
25OHC	25-hydroxycholesterol
27OHC	27-hydroxycholesterol
7-DHC	7-dehydrocholesterol
7 KC	7-ketocholesterol
7αOHC	7α-hydroxycholesterol
7βOHC	7β-hydroxycholesterol
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ALV-J	avian leukosis virus subgroup J
$A\beta_{1-42}$	amyloid β_{1-42} peptide;
CH25H	cholesterol 25-hydroxylase
COPs	cholesterol oxidation products
COVID-19	coronavirus disease-19
CSF	cerebrospinal fluid
CTX	cerebrotendinous xanthomatosis
CYP11A1	cholesterol side-chain cleavage enzyme
CYP27A1	cholesterol 27-hydroxylase
CYP7A1	cholesterol 7-alpha-hydroxylase
CYP7B1	oxysterol 7-alpha-hydroxylase
DDAB	di-dodecyl-dimethylammonium bromide;
DHCR7	7-dehydrocholesterol reductase
EC50	half maximal effective concentration
EMCV	encephalomyocarditis virus
ERs	estrogen receptors
GC-MS	gas chromatography-mass spectrometry
hACE-2	human angiotensin converting enzyme 2
HIV	human immunodeficiency virus
HT	Huntington disease
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LXRs	Liver X Receptors
MCI	minor cognitive impairment
MFGM	milk fat globule membrane
MS	multiple sclerosis
NF-kB	nuclear factor-kappa B
NOXs	NADPH oxidases
NPB	Niemann Pick disease type B
NPC	Niemann Pick disease type C
Nrf2	nuclear factor erythroid 2-related factor
PBMCs	peripheral blood mononuclear cells
PD	Parkinson's disease
PDLSC	human periodontal ligament stem cells
PEDV	porcine epidemic diarrhea virus
PI3K/Akt	phosphoinositide 3-kinase/Akt
PPARs	peroxisome proliferator-activated receptors
PPARγ	peroxisome proliferator-activated receptor γ
PRRS	porcine reproductive and respiratory syndrome virus
p-tau	phosphorylated tau protein
PUFA	polyunsaturated fatty acids
RORs	retinoic acid receptor related orphan receptors
ROS	reactive oxygen species
SARS-CoV-2	severe acute respiratory syndrome coronavirus-2
SI	selectivity index
Smo	seven-transmembrane protein Smoothened
SMPD1	sphingomyelin phosphodiesterase 1
sNfL	serum neurofilament light chain
SPG5	hereditary spastic paraplegia type 5
SREBPs	sterol regulatory element binding proteins
STAT-1	signal transducer and activator of transcription 1
TGEV	transmissible gastroenteritis virus
TLRs	toll like receptors
triol	cholestan-3β,5α,6β-triol
t-tau	total tau protein
TTC	threshold of toxicological concern
UHT	ultra-high temperature
Wnt	Wingless-Int1
α-epoxy	5α,6α-epoxide;
βCD	β-cyclodextrin
β-epoxy	5β,6β-epoxide.

example those depicted by Cardenia et al. [12] and by Iuliano [13]. The reaction of cholesterol with triplet oxygen first generates the highly unstable 7α-hydroperoxycholesterol and 7β-hydroperoxycholesterol, that rapidly lead to the formation of 7αOHC, 7βOHC and 7KC. The last two compounds are by far the most investigated non-enzymatic oxysterols for their remarkable toxicological and pro-inflammatory properties in different cell models including endothelial and monocytic/macrophage cells, neuroblastoma and glial cells, intestinal cells [14]. At least, with regard to the pro-inflammatory effect exerted on human intestinal cells in vitro (CaCo-2 differentiated cell line), 7βOHC appeared the most harmful one [[15]; Biasi et al. unpublished]. Difficult to ascribe to non-enzymatic oxysterols some physiological role, while this cannot be excluded for instance during time limited inflammatory reactions.

The oxysterols of non-enzymatic origin may be altogether considered harmful compounds. However, also oxysterols of enzymatic source could become noxious when present in excess, due to metabolic disorders or, more frequently, to chronic inflammatory processes. Indeed, disproportionate levels of 27OHC have been found associated with quite a number of human pathological conditions, almost always characterized by a chronic inflammatory status [1,16]. Notably, the key cells of chronic inflammation are the macrophages, that are rich in cholesterol 27-hydroxylase (CYP27A1), the enzyme generating 27OHC [17,18].

In terms of mechanism/s of action, oxysterols of both enzymatic and non-enzymatic origin are in general provided with a pro-oxidant effect, since able to up-regulate expression and activity of various NADPH oxidase isoenzymes (NOXs), a key cellular source of oxidant species [19]. However, in the case of enzymatic oxysterols, their steady-state concentration is more often suitably regulated by substrate and co-factors availability and by negative feedback mechanisms, while in the case of non-enzymatic oxysterols their actual concentration mainly depends on the intensity and duration of the generating stimulus. With regard to oxysterol catabolism and disposition, despite its good definition [4] it is not clear yet how much this would account for their actual level in biological tissues. Indeed, the absorption and the tissue distribution of exogenously added and endogenously generated oxysterols represent an important research area deserving deeper analysis as soon as possible.

Overall, remarkable is the translational potential of oxysterols, namely the possible employment of their properties not only in the laboratory medicine and pharmaceutical fields but also and especially in food industry, as well as in cosmetics industry. These redox-related compounds are increasingly applied in clinical biochemistry as molecular markers in the diagnosis and the monitoring of several human and veterinary diseases. Moreover, some oxysterols of enzymatic origin appear much promising as active pharmaceutical agents because of their antiviral or osteogenic properties. Further, defined oxysterols of non-enzymatic origin are increasingly investigated in a systematic way as

potential quality markers in the processing of food ingredients containing cholesterol and in preparation and storage of related food products. Last but not least, several skincare products are reach in cholesterol, thus prone to autoxidation, with the consequent possible production of harmful oxysterols and simultaneous loss of active ingredients. The growing translational value of cholesterol oxidation products led us to review its present and upcoming applications in the different industrial fields.

2. Non-enzymatic oxysterols as reliable in vivo markers of redox imbalance

A first practical application that is already widely acknowledged is the use of main oxysterols stemming from the autoxidation of cholesterol as accurate and precise markers of redox changes occurring in vivo, in particular in a broad variety of human pathologies.

Among the early studies proposing the adoption of 7KC and 7βOHC as reliable markers of oxidative stress in vivo there are those of Iuliano et al. [20], Yoshida and Niki [21] and of Boaz et al. [22]. In particular, Iuliano and colleagues proposed a relatively rapid isotope dilution gas chromatography-mass spectrometry (GC-MS) method to measure 7KC and 7βOHC in plasma but also in tissues like liver and in atherosclerotic plaques [20]. Moreover, Larsson and colleagues confirmed the reliability of 7 KC and 7βOHC as markers of oxidative stress in humans, despite some interconversion between the two oxysterols operated by the enzyme 11-β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1) [4], and also showed that 7KC and 7βOHC have similar half-life in the blood stream [23].

More recently a comprehensive review was provided on the detection of 7KC and 7βOHC in a great variety of human pathologies characterized, even if not necessarily caused, by a redox imbalance [24]. Apart from the already considered neurodegenerative diseases it is worth quoting the value of quantifying plasma/serum 7KC, 7βOHC and in some cases 7αOHC levels to depict the redox imbalance in patients with type II diabetes [25], with type II diabetes and obesity [26], with metabolic syndrome [27] or to check in atherosclerotic patients in treatment with statins the efficacy of such a therapy [28]. Patients with diagnosed autoimmune thyroiditis and mild hypothyroidism (n = 41), not clinically evident, showed a marked increase of plasma 7KC and triol concentrations, as measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in comparison to healthy controls (n = 45) [29].

Further, the quantification of plasma 7KC in patients with stable coronary artery disease led to unveil high concentrations of this oxysterol as a reliable marker of increased risk of cardiovascular complications and even death [30]. Very recently, a significant increase of both 7KC and 7βOHC serum levels was reported to occur in patients with moderate (n = 36) or severe coronavirus disease-19 (COVID-19) (n = 81), while in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pauci- and a-symptomatic individuals (n = 27) the two oxysterols showed serum values similar to those of control subjects (n = 123) [31]. The rise of serum 7KC and 7βOHC levels in patients with COVID-19 was a clear indication that a sustained redox imbalance, dependent on the inflammatory state, is consistently present during the clinical expression of SARS-CoV-2 infection, on the contrary absent in infected but pauci- or a-symptomatic subjects.

3. Medical laboratory industry: oxysterols as possible diagnostic markers

Accurate and precise quantification of oxysterols in biological tissues and fluids has been made possible since reliable measurements by GC-MS or LC-MS/MS became available [32,33]. Such gold standard methods have been adopted to measure and monitor the level of defined oxysterols in a variety of human pathologies and its application in clinical biochemistry showed in the last years a steady increase. Hereafter, the main applications of oxysterols' quantification in the diagnosis and profiling of various human diseases are critically reviewed.

3.1. Inborn errors in cholesterol synthesis and metabolism

An autosomal recessive mutation of the gene coding for the last enzyme of the cholesterol synthetic pathway, namely 7-dehydrocholesterol reductase (DHCR7), is the genetic basis of the rare Smith-Lemli-Opitz syndrome, a disorder of development characterized by multi-organ malformations and cognitive impairment of different degree of severity. The main biochemical consequence is the accumulation in tissues and blood of 7- dehydrocholesterol (7-DHC), the further metabolism of which generates peculiar oxysterols that are not detectable in control blood, like 24- or 25- or 26-hydroxy-8-dehydrocholesterol [34]. In addition, in all examined cases elevated plasma levels of 7KC and 7βOHC, were measurable, generated by the action of the enzyme

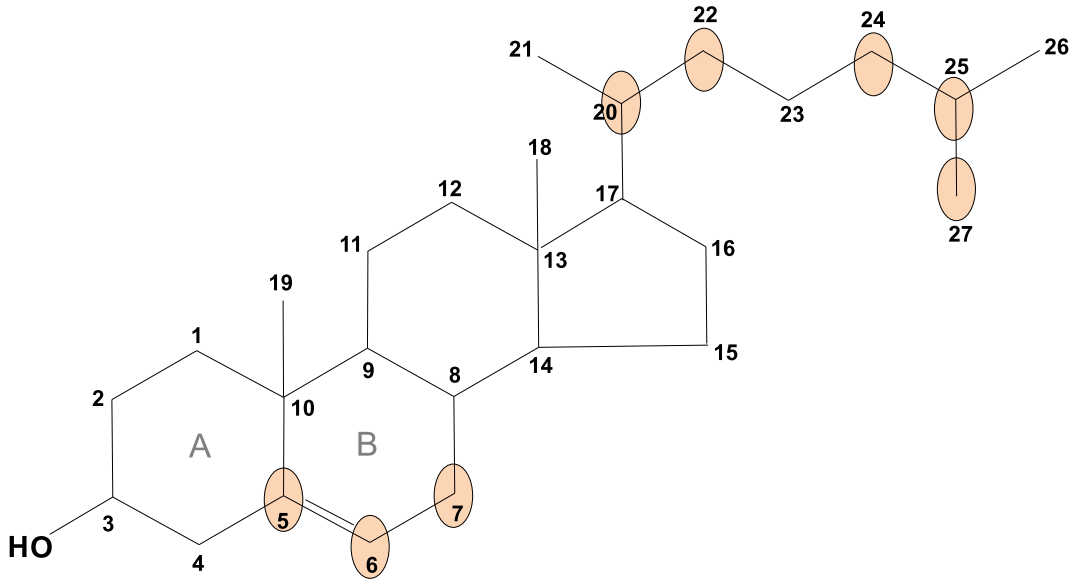

Fig. 1. Molecular structure of cholesterol and main sites of steroid's oxidation.
The most frequent sites of addition of a ketone, epoxy or hydroxyl group to the A, B rings or of a hydroxyl group to the side chain are highlighted.

cholesterol 7α-hydroxylase (CYP7A1) on the excess of 7-DHC [34,35]. The actual role of all these compounds in the embryonic and fetal development has to be clarified yet, but the already available tool to detect and monitor abnormal oxysterol profiles points to clear laboratory and promising biotechnological applications.

This is also the case of cerebrotendinous xanthomatosis (CTX), Niemann Pick disease types B (NPB) and C (NPC), quite different genetic diseases but all characterized by heavily altered cholesterol metabolism and consequently by a pathological oxysterol profile. CTX is a rare autosomal recessive disease, characterized by the aberrant accumulation of lipids at the level of various organs and tissues, including brain, liver and tendons, and a wide and variable symptomatology not rarely with neuropsychiatric implications. The CTX genetic defect is at the level of the expression and synthesis of CYP27A1, a mitochondrial cytochrome P450 enzyme operating the synthesis of 27OHC [17] but also the catabolism of various oxysterols, including 7KC, 7αOHC and 7βOHC [4]. The latter role of CYP27A1 explains why in blood plasma/serum of CTX patients a low level of 27OHC is accompanied by elevated concentrations of the three non-enzymatic oxysterols 7αOHC, 7βOHC and in particular 7KC, all reaching clear pathological levels [36]. Very recently, a comprehensive LC-MS analysis of the oxysterols present in serum and cerebrospinal fluid of CTX patients has proposed the peculiar increase of 7α,12α-dihydroxycholest-4-en-3-one together with the very low level of 27OHC as a couple of very sensitive markers of this genetic disorder [37].

Type B and type C Niemann-Pick diseases are very rare genetic disorders respectively involving the sphingomyelin phosphodiesterase 1 (SMPD1) gene, coding for the enzyme sphingomyelinase and the NPC1 or NPC2 genes, coding for a protein that regulates the traffic of cholesterol at lysosomal level, thus in all cases resulting in an excessive intra-lysosomal accumulation of sphingomyelin and cholesterol. Even if with distinct multiorgan symptomatology (no neurological changes are present in type B disease) both diseases show an abnormal blood oxysterol profile, again with a prominent and marked increase of 7KC [36] but also with a net elevation of another cholesterol oxide of non-enzymatic origin, namely the triol, another candidate selective biomarker of these two genetic disorders [36,38]. The inclusion of serum 7KC quantification among the diagnostic markers of NPC already appears highly recommended [39].

3.2. Alzheimer's disease

Particularly promising and expanding appears the industrial inclusion of oxysterols like 24OHC, produced by neuronal cells only [7], and 27OHC, able to cross the blood-brain barrier [7], in the diagnosis and monitoring of Alzheimer's disease (AD) and relate cognitive disorders, the incidence of which is increasing in parallel to the rise of the average lifetime calculated from birth. As a consequence, the synthesis of oxysterols of analytical grade, the achievement of MS equipment and relevant items and reagents, including the mono/polyclonal antibodies for immunochemical detection of oxysterol generating enzymes, will be further promoted.

In general terms, with regard to cholesterol metabolism, the most suitable substrate for GC/MS or LC/MS analyses are blood serum or plasma; on the contrary, to evaluate neurodegeneration disease processes the cerebrospinal fluid (CSF) is more likely the elective biological

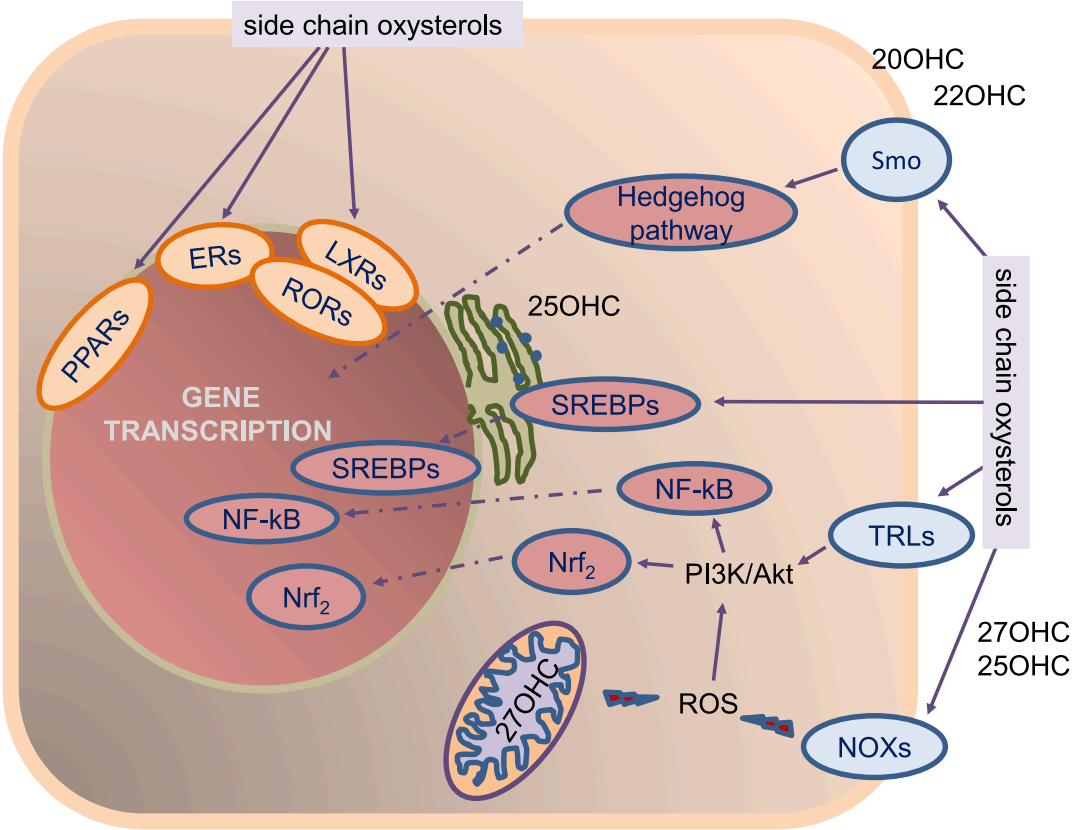

Fig. 2. Main membrane and nuclear receptors and main intracellular signaling pathways activated by side-chain oxysterols.
Side-chain oxysterols can be of both endogenous and exogenous origin. 20OHC:20-hydroxycholesterol; 22OHC:22-hydroxycholesterol; 24OHC:24-hydroxycholesterol; 25OHC:25-hydroxycholesterol; 27OHC:27-hydroxycholesterol; LXRs:Liver X Receptors; RORs:retinoic acid receptor related orphan receptors; ERs:extrogen receptors; PPARs:peroxisome proliferator-activated receptors; SREBPs:sterol regulatory element binding proteins; Smo:seven-transmembrane protein Smoothened; TLRs:toll like receptors; NOXs:NADPH oxidases; NF-kB:nuclear
factor-kappa B; Nrf2:nuclear factor erythroid 2-related factor; PI3K/Akt:phosphoinositide 3-kinase/Akt; ROS:reactive oxygen species.

fluid [40,41], without certainly excluding the peripheral blood sampling. The CSF concentrations of 24OHC and 27OHC, normally ranging between 0.5 and 2.5 ng/ml, increase in the presence of neurodegeneration. 24OHC is released by dying neuronal cells, while the rise of 27OHC is likely a consequence of its reduced metabolism due to loss of the neuronal enzyme oxysterol 7-alpha-hydroxylase (CYP7B1) and higher flux form circulation into CSF because of a damaged blood-brain barrier [42].

In Alzheimer's disease, a direct correlation between CSF concentration of phosphorylated tau protein (p-tau) and 24OHC was observed by Leoni et al. [43] and also confirmed by Lutjohann group [44]. In 2013, a report from the Björkhem's laboratory provided a comparative measurement of CSF levels of total tau protein (t-tau), p-tau, cholesterol and amyloid $\beta_{1\text{-}42}$ peptide ($A\beta_{1\text{-}42}$). The CSF level of 24OHC directly correlated with those of tau and p-tau not only in the patients with dementia but even more clearly in recruited individuals with mild or advanced cognitive impairment, enough to indicate the CSF 24OHC as a sensitive marker in the early phases of the disease's development [45].

A meta-analysis of the results stemming from different studies correlating altered cholesterol metabolism with AD reported that an increase of 24OHC but also, to a minor extent, of 27OHC was almost consistently observed in the CSF of minor cognitive impairment (MCI) and AD patients [46]. A strong indication not to limit the laboratory diagnosis of MCI and AD to the measurement of CSF levels of t-tau, p-tau and $A\beta_{1\text{-}42}$, but to include also oxysterol biomarkers. As far as 24OHC plasma concentration in neurodegenerative diseases is concerned, this mirrors the amount of metabolically active neurons in the brain, thus it decreases in proportion to the extent of brain atrophy [47].

The useful adoption of 24OHC and 27OHC measurements in CSF was further confirmed as an additional reliable biomarker of AD [48], and their quantification also in blood serum/plasma suggested [42,49,50].

Importantly, even if the research trying to elucidate the actually existing link between oxysterols and neurodegenerative diseases focused so far on 24OH and 27OHC, it does not mean that only these two cholesterol oxidation products are detectable in the brain of normal and MCI/AD individuals and patients. Recently, a quite comprehensive quantification of oxysterols was carried out on autoptic specimens from frontal and occipital cortex of AD brains, samples being subdivided in early-stage and late-stage disease, on the basis of the cerebral localization of immunostained neurofibrillary tangles (Braak staging classification system). All the main oxysterols commonly detectable in human blood were shown to be present both in control and AD brains, and all but one compounds showed a marked increase at least in the late-stage AD. Of note, a significant increase of both 7αOHC and 7βOHC was evident in the AD brains, most likely due to progressively increased brain cholesterol autoxidation triggered then sustained by expanding inflammatory reactions [51]. Only 24OHC content showed a decreasing trend, especially marked in late-stage AD; a fact well explainable by its unique origin from neurons, cells that are progressively dying during the development of the disease [51].

Hence, also in the case of AD, the oxysterols of non-enzymatic origin appear reliable candidates, together with 24OHC and 27OHC, to mark the progression of MCI and AD. Recently, also 7KC was considered as possibly involved in AD pathogenesis, because of its significant interaction with the white matter microstructure [52], by this way favoring the association with $A\beta_{1\text{-}42}$ [53].

3.3. Oxysterols and Parkinson's disease

With regard to a potential role of plasma oxysterols as biomarkers of Parkinson's disease (PD), an increased level of 7KC, 7βOHC and 27OHC but not of 24OHC was detected in a cohort of 61 cases of PD in comparison to an identical number of age-matched healthy individuals [54]. Moreover, Björkhem and colleagues, measuring by GC-MS the CSF levels of 24OHC and 27OHC, observed that the concentration level of the first oxysterol, unlike that of the second one, was indicative of the duration of the disease process [55]. A statistically significant increase of 24OHC in CSF from early PD was afterwards confirmed by the same group, that also showed a correlation between this oxysterol and tau levels, most likely a secondary effect of the ongoing brain damage [56].

3.4. Other neurodegenerative and neuropsychiatric diseases

In early studies, an increase of 27OHC and in particular of 24OHC was shown in the CSF of multiple sclerosis (MS) patients, ascribed to altered blood-brain barrier alteration and neuronal death respectively [40]. The quantification of these two oxysterols has been suggested as a reliable marker of altered cholesterol metabolism in multiple sclerosis, but also an useful index to monitor the progression of this autoimmune disease process [57], since both cholesterol oxides may display remarkable redox-mediated pro-inflammatory properties [1].

As regards the hematic concentration of these and other oxysterols, a net decrease of both serum 24OHC and 27OHC levels was reported in a large group of MS patients ($n = 105$) including all the different clinical subtypes [58]. Consistent findings were obtained later on by a different group [59]. The observation of a marked alteration of cholesterol metabolism was then expanded against the time in a five years longitudinal study. At the end of the follow-up, 24OHC, 25OHC and 27OHC plasma levels significantly increased in the patients with progressive MS ($n = 39$), while 7KC and 7αOHC showed a decrease in the group with relapsing/remittent disease ($n = 61$), in comparison to control individuals ($n = 39$) [60].

In relation to the plasma level of 7KC in multiple sclerosis, a direct correlation of this marker with the serum amount of neurofilament light chain (sNfL), specific index of neuroaxonal injury, was very recently demonstrated [61]. Even if further investigation is needed to properly characterize the long-term behavior of plasma/serum oxysterols in the different sub-types of MS, the adoption of some cholesterol oxides both of enzymatic and non-enzymatic source as biomarkers of multiple sclerosis evolution seems attractive [62].

Beyond MS, other neurodegenerative diseases in which defined oxysterols appear to be good candidate markers are hereditary spastic paraplegia type 5 (SPG5), and amyotrophic lateral sclerosis (ALS). SPG5 is a rare autosomal recessive disease characterized by loss of function of the gene coding for cytochrome P450 isoform CYP7B1, important in the catabolism of 25OHC and 27OHC. Indeed, the SPG5 patients show a remarkable increase of both oxysterols in plasma and CSF. The heterozygous carriers show a similar but more attenuated alteration of the oxidative catabolism of these two oxysterols [63]. As expected, the CSF and plasma levels of 24OHC, which follows a different metabolic pathway, resulted to be similar in patients and controls. Indeed, plasma/serum peak levels of 25OH, in particular, and 27OHC should be considered as pathognomonic markers of SPG5.

The three side chain oxysterols, measured by LC-MS, were all increased in the CSF of ALS patients as to control subjects. However, in the blood serum only 25OHC showed a significant increase in comparison to controls, a rise that well correlated with the disease's severity [64]. A previous isotope dilution GC-MS study from a different laboratory quantified a decrease of plasma 27OHC in fifty-two ALS patients versus forty controls and did not much support the prognostic value of the three side chain oxysterols in ALS [65]. Hence, a possible validation of the prognostic value of oxysterols markers in ALS must be supported by further analytical studies [66].

An altered cholesterol metabolism both in the central and peripheral nervous system is a consistent feature of Huntington disease (HT), an autosomal dominant pathology due to mutations in the Huntingtin gene that cause neuromotor impairment, cognitive and psychiatric disorders. Particular attention was drawn by the constant decrease of plasma 24OHC in HT patients, the level of which was proportional to the progression of motor and neuropsychiatric alterations. However, as in the case of ALS, the actual prognostic value of this oxysterol in HT needs further validation [67]. Even in the complex scenario of autism, of

which the pathomechanisms are far from being elucidated, the plasma measurement of oxysterols, in particular of 24OHC, that resulted consistently increased, could represent a useful tool to deeper investigate especially the early development of related mental disorders [68].

The present and outcoming utilization of defined oxysterols as diagnostic and disease monitoring markers is recapitulated in Table 1. It clearly appears that the serum/plasma pathological increase of defined oxysterols, in particular 7KC and 7βOHC, or the decrease of 27OHC, may contribute to monitor the progression of the majority of neurodegenerative disorders and of all the reported diseases non affecting the brain. In the case of Alzheimer's and Parkinson's diseases, the measurement of altered CSF level of 24OHC and 27OHC, more reliably contributes to make a diagnosis or monitor the disease development.

4. Pharma industry: oxysterols as pharmaceutical drugs

While the scientific knowledge accumulated so far on oxysterols has already been applied to Laboratory Medicine, to contribute to diagnosis and monitoring of quite a big number of disease processes, the use of some components of this family of compounds in the pharmaceutical industry has been so far mainly envisaged. Still, their perspective therapeutical application appears promising in certain medical fields, thus we deemed useful to analyze this translational aspect of oxysterols' biochemistry in some details.

4.1. Oxysterols for bone regeneration

The in vitro challenge of mouse marrow multipotent mesenchymal cells with the natural oxysterols 20(S)- and 22(S)-hydroxycholesterol specifically up-regulated the Hedgehog pathway, by this way favoring differentiation of bone mesenchymal cells into osteoblasts. The osteogenic effect of the two oxysterols was prevented when the selective Hedgehog pathway inhibitor cyclopamine was previously added to the cell culture [69]. Indeed, among the signaling pathways that regulate bone development and repair, a key role is played by that governed by Hedgehog family of proteins [70]. Smoothened (Smo), a G protein coupled receptor primarily involved in Hedgehog signaling, recognizes oxysterols, in particular 20(S)-hydroxycholesterol (20SOHC), as the only natural ligand [71].

Thus, at least defined natural cholesterol oxides are becoming promising candidate agents to trigger and promote bone repair. In parallel to a deeper characterization of such peculiar activity, the design of synthetic analogs of 20SOHC took place and some potent new molecules that strongly direct mouse bone marrow stromal cells towards osteoblastic differentiation were produced. These compounds confirmed their pro-osteogenic effect in the rat spinal fusion model [72]. The 20SOHC analogs also exert their osteogenic effects through the up-regulation of Hedgehog signaling pathway.

The Hedgehog-mediated enhancing effect on osteogenesis exerted by 20SOHC, 22(R)-hydroxycholesterol (22ROHC), and 22(S)-hydroxycholesterol (22SOHC), three oxysterols generated by cholesterol side-chain cleavage enzyme (CYP11A1), as well as by some synthetic

Table 1

Oxysterol ls as in vivo markers of redox imbalance in the diagnosis of human diseases. Pathological variations (⇧⇩) as to the normal range.

Disease process	Oxysterols detected	Biological fluid	As to normal range	Normal range* (ng/ml)	References
Atherosclerosis progression	7KC, 7βOHC	plasma/serum	⇧	(p/s 7KC 7–35)	[20,23,28]
Type II diabetes	7KC, 7βOHC	plasma/serum	⇧	(p/s 7βOHC 3–13)	[25,26]
Metabolic syndrome	7KC, 7βOHC	plasma/serum	⇧		[27]
End-stage renal disease	7KC, 7βOHC	plasma/serum	⇧		[22]
Hypothyroidism, autoimmune thyroiditis	7KC, triol	plasma/serum	⇧	(p/s triol 8–10)	[29]
Coronary artery disease with increased risk	7KC	plasma/serum	⇧		[30]
Moderate and severe COVID-19	7KC, 7βOHC	plasma/serum	⇧		[31]
	27OHC	plasma/serum	⇩	(p/s 27OHC 100–249)	[31]
Smith-Lemli-Opitz syndrome	7KC, 7βOHC	plasma/serum	⇧		[34,35]
Cerebrotendinous xanthomatosis	7KC, 7αOHC, 7βOHC	plasma/serum	⇧	(p/s 7αOHC 48–58)	[36]
	27OHC	plasma/serum	⇩		[36,37]
Niemann Pick disease types B and C	7KC, triol	plasma/serum	⇧		[36,39]
CI and Alzheimer's dementia	24OHC, 27OHC	CSF	⇧	(CSF 24OHC, 27OHC 0.5–2.5)	[44–47]
Parkinson's disease	7KC, 7βOHC, 27OHC	plasma/serum	⇧		[54]
	(CSF) 24OHC	CSF	⇧		[55,56]
Multiple sclerosis	(CSF) 24OHC, 27OHC	CSF	⇧		[40]
in progressive disease	24OHC, 25OHC, 27OHC	plasma/serum	⇧	(p/s 24OHC 34–94, 25OHC 3–15)	[60]
in relapsing disease	7KC, 7αOHC	plasma/serum	⇩		[60]
Spastic paraplegia type 5	25OHC, 27OHC	plasma/serum	⇧		[63]
Amyotrophic lateral sclerosis	25OHC	plasma/serum	⇧		[64]
	27OHC	plasma/serum	⇩		[65]
Huntington disease	24OHC	plasma/serum	⇩		[67]
Autism	24OHC	plasma/serum	⇧		[68]

CI:cognitive impairment; CSF:cerebrospinal fluid; 7KC:7-ketocholesterol; 7αOHC:7α-hydroxycholesterol; 7βOHC:7β-hydroxycholesterol; triol:cholestan-3β,5α,6β-triol; 24OHC:24-hydroxycholesterol; 25OHC:25-hydroxycholesterol; 27OHC:27-hydroxycholesterol.

*Normal range values of serum/plasma 7KC, 7βOHC, 27OHC, 24OHC, 25OHC [31]; normal range values of serum/plasma of triol [29] and 7αOHC [60]. Normal values for CSF 24OHC and 27OHC [42].

oxysterols (namely Oxy34, Oxy49, Oxy133) has been further investigated in deep and eventually confirmed in different experimental animal models [73].

Bone tissue engineering exploiting the osteoinductive properties of natural oxysterols as well as that of synthetic analogs is definitely an attractive field. Different biomaterial scaffolds to vehiculate such pro-osteogenic molecules are presently under active development and testing [74,75].

4.2. Oxysterols as antiadipogenic drugs

While investigating the potential use of 20SOHC and related molecules in bone generation and repair, using mouse bone marrow multipotent mesenchymal cells, another important effect was discovered, still mediated through an agonistic action on Hedgehog signaling pathway, namely a robust inhibition of the differentiation of mesenchymal cells into adipocytes [72,76].

As expected, if the multi-lineage potentiality of bone stem cells is significantly directed/diverted towards a specific differentiation pathway, the activation of other pathways like the chondrogenic and adipogenic ones will be consequently affected. In addition, adipogenesis and osteogenesis pathways are differently regulated and at least in part inversely related. The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), that is known to be a key factor in adipocyte differentiation, inhibits osteogenesis, while the Wingless-type (Wnt) signal transduction pathway, a recognized osteogenesis inducer, inhibits PPARγ signaling [77,78].

The antiadipogenic effect combined to the pro-osteogenic activity displayed by some oxysterols appears attractive indeed and of high therapeutical potential, in particular in osteoporosis. In fact, in this chronic and progressive process, the differentiation of bone marrow mesenchymal cells into osteoblasts or adipocytes is markedly shifted towards adipocytes, by this way making the bone more prone to fractures [79]. Besides the osteoinductive and antiadipogenic effects of 20SOHC, 22ROHC, 22SOHC and synthetic analogs, recently reviewed in a comprehensive way [80], an antiadipogenic effect was described as exerted by the oxysterol of non-enzymatic origin 7KC on stem cells obtained from the abdominal adipose tissue of human subjects [26]. 25OHC as well was reported to inhibit adipogenesis in C3H10T1/2 mouse embryonic stromal cells, in this case through a not Hedgehog-dependent pathway [81].

Another relevant and just emerging issue is the modulation of the adipocyte de-differentiation process exerted by some oxysterols. A paradigmatic example of the plasticity of this type of cells is their conversion into alveolar cells in the mammary gland during lactation [82]. In this direction goes the investigation of a pro-chondrogenic effect of 22ROHC, 22SOHC, or 25OHC on human adipose stem cells isolated from abdominal subcutaneous adipose tissue, pointing to a particularly efficient chondrogenic activity of 22ROHC [83]. Certainly, the possibility to suitably de-differentiate and re-direct certain cytotypes represents a great challenge in the near future and oxysterols could likely provide their own contribute to reach this goal.

4.3. Oxysterols with broad antiviral effects

In the last decade a widely increasing interest has been generated by the discovered capacity of some oxysterols to efficiently inhibit the replication of quite a number of human pathogenic viruses, belonging to different families and of different strains, with or without an external envelope made of phospholipids, proteins and glycoproteins, namely enveloped and naked viruses. The scientific evidence that mainly opened this new field of application of biological effects of oxysterols was the demonstration that interferon up-regulates expression and activity of the almost ubiquitous microsomal enzyme cholesterol 25-hydroxylase (CH25H), with the consequent increased production of 25OHC, that showed an inhibiting effect against several enveloped viruses, at least up-regulating the transcription factor signal transducer and activator of transcription 1 (STAT1) [84,85]. The simultaneous report by two independent research groups highlighted the ability of 25OHC to hamper the entry and the subsequent replication within target cells of H1N1 Influenza virus, Varicella zoster virus, Herpex simplex virus, Vesicular stomatitis virus, Human immunodeficiency virus (HIV), Ebola virus, Rift Walley rift virus, Russian Spring-Summer encephalitis virus, and Nipah virus. Following studies extended the range of pathogenic viruses the replication of which was inhibited by 25OHC, including Hepatitis B and C viruses [86].

Another side-chain oxysterol, of enzymatic origin, namely 27OHC showed as well remarkable antiviral properties, with a half maximal effective concentration (EC50) and a selectivity index (SI) in the same range of those of 25OHC, with a spectrum of activity, as for 25OHC, extended to naked viruses, namely to various strains of Rhinovirus and Rotavirus and to Papillomavirus [87–89].

With regard to an inhibitory action of oxysterols against the agent of COVID-19, that is the SARS-CoV-2, 25OHC was proven by different research groups to be effective at low micromolar concentrations and with a good SI. This oxysterol resulted to be one of the most powerful out of 22 broad spectrum antiviral substances tested in vitro on VeroE6 cells [90]. The efficacy of 25OHC in blocking the replication of the COVID-19 virus was confirmed both in VeroE6 infected cells and in the mouse infected with a SARS-CoV-2 pseudo-virus [91]. In the same study, the serum 25OHC concentration in mice made transgenic for the human angiotensin converting enzyme 2 (hACE-2) was reported to increase after infection with SARS-CoV-2 [91], actually with a relatively wide data dispersion.

25OHC and its generating enzyme CH25H were disclosed to act as potent inhibitors of SARS-CoV-2 replication by preventing the virus from using the endosomal pathway to invade the targeted cell, most likely through an impairment of the intracellular cholesterol distribution and flux [92]. Indeed, a depletion of plasma membrane cholesterol induced by the 25OH, stemming from an overexpressed CH25H in cells but also in SARS-CoV-2 infected patients, was demonstrated to inhibit the fusion of the virus envelope with the cell membrane [93].

The increasing bulk of evidence of a potential anti-SARS-CoV-2 activity of 25OHC definitely encourages the development of suitable oxysterol-based drug products. In this regard, nanoparticles of 25OHC and di-dodecyl-dimethylammonium bromide (DDAB) showed to efficiently populate the lung of male ICR mice and, in parallel, to significantly down-regulate interleukin-1β production by peripheral blood mononuclear cells (PBMCs) isolated from COVID-19 patients [94].

As far as the interference of 25OHC with the physiological intracellular cholesterol traffic is concerned, Civra et al. earlier showed that 25OHC as well as another side chain oxysterol, namely 27OHC, were able to sequester human rotavirus within the cell late endosomes actually by hampering the normal cholesterol flux among the different intracellular compartments [89].

The potential activity of 27OHC also against the COVID-19 virus was then investigated, and this oxysterol complexed to β-cyclodextrin (βCD) was demonstrated to fully inhibit the replication of SARS-CoV-2 in Vero E6 (monkey) and Huh7 (human) cell lines [31]. In addition, such 27OHC-βCD complex blocked the replication of another coronavirus, responsible for part of the cases of common cold, that is HCoV-O43, in human fibroblast MRC-5 [31].

In the same report, the results of GC-MS quantification of serum 24OHC, 25OHC and 27OHC in SARS-CoV-2 infected individuals and patients were provided. In comparison to control non infected subjects (n = 123), serum 24OHC level showed a slight decrease in moderate (n = 36) and severe COVID-19 patients (n = 81), while 25OHC serum level showed a significant drop in severe COVID-19 patients only, likely dependent upon an impairment of interferon 1 activity occurring at that disease's stage [95,96]. Notably, 27OHC was the only side chain oxysterol examined the concentration of which was already reduced in pauci-symptomatic individuals (n = 27) and progressively dropped

down to almost half normal concentration in the severe COVID-19 group of patients [31], most likely because of a progressive derangement of mitochondria, the production site of this oxysterol [7]. These findings together with the later observation of a drop of 25OHC plasma concentration in severe as to moderate COVID-19 patients, in particular over 70 years of age [94] further underline that an impaired cholesterol metabolism consistently occurs in COVID-19, and, at the same time, point to a possible replacement of defined oxysterols especially in the severe forms of such a disease [97].

In addition, some non-enzymatic oxysterols, such as 7KC and 7βOHC, were reported to exert an antiviral in vitro effect [86], but modest and afforded at relatively high concentrations, known to be toxic for a variety of cytotypes.

4.4. Replacement therapy with oxysterols

A further application in Medicine of oxysterols, of enzymatic source mainly, stems from the recognized potential role of some components of this family with remarkable antiviral and/or osteoinductive properties.

As just mentioned, 27OHC and 25OHC, given as single or in combination, could help in restoring, at least partially, the antiviral defenses in COVID-19 patients or even contribute to prevent worsening of the clinical condition. Similarly, compounds like 20SOHC could be utilized in a variety of osteoplasty intervention. In support of this latter usage, a 1:1 mixture of 20SOHC and 22SOHC was demonstrated to strongly stimulate the osteogenic differentiation in vitro of human periodontal ligament stem cells (PDLSC), involving the Hedgehog signaling pathway [97]. Comparable conclusions were reached by another group still testing these oxysterols on human mesenchymal stem cells [98]. Thus, some oxysterols could soon compete with the bone morphogenetic proteins, the growth factors widely used in bone reconstruction, also because of likely much lower toxic adverse effects.

Finally, 27OHC replacement could be considered in the treatment of cerebrotendinous xanthomatosis, characterized by a deficit of 27-cholesterol hydroxylase and very low or even absent amount of this cholesterol oxide in the patients' blood serum. Actually, such a deficiency is not properly corrected by the presently adopted oral therapy with chenodeoxycholic acid, that just secures a sufficient stabilization of the disease [99].

4.5. Oxysterols in veterinary medicine

Similar to the possible application of oxysterols to human medicine is their likely use in veterinary medicine. Just a limited survey will be made here, quoting only some indicative and very recent reports.

The pro-osteogenic and anti-adipocytic properties of some side chain oxysterols, in particular 20SOHC, are for instance very attractive to markedly improve the healthy growth of poultry, favoring the development of bone and muscle, at the same time hindering fat accumulation. The attainment of this target would be particularly convenient in the broiler chicken industry. Indeed, 20SOHC appears strongly effective in directing the differentiation of mesenchymal cells obtained from chick compact bones towards the generation of osteoblasts and myoblasts, by this way also exerting an anti-adipocytic effect [100]. As regards the mechanism underlying the overall observed effect as induced by this oxysterol of enzymatic origin, the involvement of the Hedgehog signaling pathway was once again confirmed [100].

Even more focused on the welfare in general are the attempts to adopt a wide spectrum antiviral action exhibited by certain oxysterols, by 25OHC above all, to prevent or treat a number of viruses affecting various types of animals. In addition to becoming an extremely important tool to understand viral oncogenesis, the wide complex of avian leukoses has represented and still represents an enormous veterinary problem [101].

With regard to the possible use of 25OHC in this setting, its generating enzyme, namely CH25H, was proven to be an effective inhibitor of chicken infection by the avian leukosis virus subgroup J (ALV-J), a very dangerous oncogenic retrovirus for poultry [102]. The anti-viral property of the type 1 interferon-inducible CH25H was confirmed also against different strains of the porcine epidemic diarrhea virus (PEDV), an alpha coronavirus. In fact, this enzyme's overexpression, as well as 25OHC supplementation, inhibited PEDV replication in Vero cells [103]. This oxysterol was hampering the in vitro replication of another coronavirus, namely the porcine transmissible gastroenteritis virus (TGEV) [103]. Still with regard to the enzyme CH25H, its knock-out was shown to lose the ability of inhibiting in vitro replication of the encephalomyocarditis virus (EMCV) [104]. Moreover, the 25OHC treatment of piglets challenged with the Porcine reproductive and respiratory syndrome (PRRS) virus, which induces a severe respiratory failure in infected pigs, provided a significant reduction of the necrotic and inflammatory lesions of the lungs, so that the clinical symptoms of the syndrome resulted to be mild [105].

The potential use of enzymatic side chain oxysterols as therapeutical agents is resumed in Table 2.

5. Food industry

Starting from the 1970s, the potential role of oxysterols as reliable markers of foodstuffs processing, quality and storage has gained interest and has demonstrated several practical applications in the food industry world [106]. This is particularly true, considering the double valence of these compounds. From one side, in fact, the nutritional applications that see oxysterols, both of non- and of enzymatic origin, potentially linked to several diseases or discovered to have strong antiviral properties, respectively, have attracted nutritionists in the field. From the other side, food technologists have taken advantage of the fact that such compounds have displayed a differential pattern of accumulation and formation depending on the processing and storage procedures applied to foodstuff [107].

5.1. Main sources of oxysterols: from raw materials to finished products

Cholesterol can be obtained from two main sources. It can either be

Table 2
Enzymatic side chain oxysterols as potential future therapeutical agents.

Oxysterols	Pharmaceutical action	References
	Human Medicine	
20(S)-hydroxycholesterol 22(S)-hydroxycholesterol 22(R)-hydroxycholesterol	pro-osteogenic and anti-adipogenic effects	[69–71, 76]
Oxy34, Oxy49, Oxy133	pro-osteogenic and anti-adipogenic effects	[72,73,80]
7KC	anti-adipogenic effect	[26]
25OHC	anti-adipogenic effect	[81]
22(R)-hydroxycholesterol 22(S)-hydroxycholesterol	pro-chondrogenic activity	[83]
25OHC	broad spectrum antiviral effect	[84–86]
25OHC	anti-SARS-COV-2 in vitro	[90–94]
27OHC	broad spectrum antiviral effect	[86–89]
27OHC	anti-SARS-COV-2 in vitro	[31]
20(S)-hydroxycholesterol 22(S)-hydroxycholesterol	bone replacement	[96]
27OHC	this oxysterol replacement in CTX	[97]
	Veterinary Medicine	
20(S)-hydroxycholesterol 22(S)-hydroxycholesterol	pro-osteogenic and anti-adipogenic effects	[98]
25OHC	broad spectrum antiviral effect	[101–105]

CTX:cerebrotendinous xanthomatosis; 7KC:7-ketocholesterol; 25OHC:25-hydroxycholesterol; 27OHC:27-hydroxycholesterol.

synthesized *de novo* mainly by hepatocytes (endogenous), or derive from the diet (exogenous) [108]. Regarding the latter, because of the presence of Δ^5 unsaturated bonds, cholesterol is prone to oxidation, especially when exposed to processing and/or storage procedures that can generally compromise the nutritional properties of foodstuffs [109]. Dietary cholesterol is one of the main steroids that makes up animal tissues and is therefore found in a greater extent in animal-based foods such as egg yolk, shrimp, beef, pork, poultry, cheese and butter. The main food sources and the amount of cholesterol intake heavily depend on the cultural/geographic context and, of course, vary based on the country of examination. As a comparison, the first five main food sources of cholesterol intake in the US population are, in order, eggs and mixed egg dishes, chicken, beef and beef mixed dishes, burgers, and cheese [110]. In the EU, conversely, the main food sources of cholesterol intake are meat, eggs, dairy products, fish and cakes [111]. The above-mentioned examples represent simply a general overview and example of cholesterol intake differences as in both cases, regional, sub-country levels, individuals, and particular sub-populations may have a different exposure.

Of the total cholesterol amount, total cholesterol oxidation products (COPs) in foods account, on average, for 1%. This number, however, may be as high as or even above 10%, depending on the processing and storage conditions employed [112]. COPs have been extensively quantified in a variety of foodstuffs; however, it is often problematic to compare different studies, mainly because of notable discrepancies in the reported quantified values. Such can be due, at least in part, to the lack of a generally accepted standardized method for determining COPs in different food matrices, with indicators such as linearity range, detection limits, repeatability, being the recovery rate of the various analytical methods developed for quantifying COPs in different foods extremely variable. A need of a standardized, generally accepted and harmonized method of detection of COPs in different food matrices is therefore needed [113].

Several studies have focused on the quantification of oxysterols in a broad range of dairy ingredients and products including, but not limited to, dairy and cheese spreads, butter, butter oil, cheese (both aged and fresh, whole and grated), milk (both raw and processed, liquid, powdered, condensed, and evaporated), buttermilk, infant formula, and yogurt [114–119]. In these kinds of products, total non-enzymatic (or partially enzymatic) oxysterols were found to be in the broad range of 0–366 μg/g, depending on several factors such as the type of analyzed matrix, the age of the tested product, the processing conditions employed. While most of the studies present in literature have quantified 7βOHC and 7KC, fewer studies analyzed the broader array of these compounds together, such as 7αOHC, α-epoxy, β-epoxy, 25OHC, 27OHC and triol (Table 3). Regarding enzymatic oxysterols, to our knowledge so far only a single study recorded and quantified the presence of 27OHC in dairy and dairy products [117].

Egg and egg products have also been extensively studied and the quantification of oxysterols in these food matrices has been reported in pasteurized and spray dried egg, fresh and dried egg pasta products. Non-enzymatic oxysterols quantified in the above-mentioned products include 7αOHC, 7βOHC, 7KC, α-epoxy, β-epoxy, 25OHC, and triol and have been reported in a broad range of 0–311 μg/g in egg ingredients, and 0.27–9.33 μg/g in composite products, depending on the type and intensity of the process employed [112,118,120–122,125]. 27OHC, to our knowledge, has not been measured in these products so far (Table 3).

Meat and fish are other food products that have been deeply studied under different cooking and storage conditions and considering different varieties of animal sources. To quote a few, oxysterol presence in meat has been quantified in pork, beef, chicken, veal, ham, turkey and sausage [112,123]. Similarly, in fish, oxysterols have been reported in shrimp, salmon, squid, mackerel, flounder, turbot, plaice, bream, crucian, anchovy, cod whiting, saury, herring, and perc [123,124]. The analyzed oxysterols include 7αOHC, 7βOHC, 7KC, α-epoxy, β-epoxy, 25OHC, and triol (Table 3). Total COPs in these matrices have been reported in the trace-191 μg/g range.

Perhaps surprisingly, and contrary to the common belief, cholesterol is also present in plant-based ingredients, although in much lower amounts compared to animal-based ones [128,129]. Consequently, COPs can also be found in plant-based products. However, only a handful of studies has quantified oxysterols in such products. More specifically, one study analyzed 7αOHC, 7βOHC, 7KC, α-epoxy, β-epoxy, 25OHC, and triol in a variety of common edible oils including camellia, olive, sesame, peanut, rapeseed, rice, soybean, and corn. The amount of 27OHC still remains to be investigated in these matrices. The total reported values in these kinds of matrices are generally low and span from 0.05 to 1.35 μg/g [127]. Similarly, another study analyzed the amounts of COPs in cocoa butter, cocoa paste, and soy lecithin, reporting total non-enzymatic oxysterol values in the range of 0.09–0.16 μg/g. In addition, 27OHC was found to be present in these products in concentrations 0.0001–0.004 μg/g [126].

Lastly, oxysterols have been quantified, although at a minor extent, in composite products as well. These include biscuits, snacks, pasta, and milk chocolate [122,123,129,130, 141]. Once again, the main oxysterols tested in such products were 7αOHC, 7βOHC, 7KC, α-epoxy, β-epoxy, 25OHC, and triol and the highest concentrations were reached in biscuits (as high as 3.98 μg/g) and milk chocolates (as high as 0.54 μg/g) (Table 3). 27OHC has been, so far, only recorded in milk chocolates in concentrations as high as 0.07 μg/g [126].

5.2. Main factors affecting non-enzymatic oxysterols generation

The very first factor that affects the quantity of oxysterols in foods, both ingredients and finished products, of animal origin, comes from the source of such products. More specifically, the feed that is given to the animal plays an important role in shaping the baseline amounts of COPs in the fresh product. Indeed, non-negligible amounts of cholesterol have been detected in feedstuffs and can influence the lipid portion of the derived foods. The presence and quantity of cholesterol-derived compounds in animal-based products may therefore be affected by the sterol profile of the animal feed [132]. Supplementing animal feed with antioxidants, namely vitamin E and linseed oil, has been shown to reduce total COPs in beef meat [133]. Similarly, milk produced by cows fed with feed fortified with oxidation-protective agents, such as vitamin E, polyunsaturated fatty acids (PUFA) or high phytosterol levels, has been shown to contain lower levels of cholesterol and/or total non-enzymatic COPs [134,135].

Fresh foods contain very low levels of non-enzymatic COPs because of their low oxygen content and their presence and generation has been attributed mainly to exposure to oxygen, pH decrease, and to processing and storage procedures [109]. Food processing, more in general, has been shown to dramatically trigger COPs accumulation in meats, eggs, dairy products, fish and poultry [123]. In milk and dairy products this may be due to cholesterol in milk being located mainly in the milk fat globule membrane (MFGM) that, during dairy processing, is prone to substantial damage, which increases its susceptibility to oxidation [136]. Although very early studies showed how no or only limited amounts of oxysterols are formed during milk processing, more recent publications have highlighted how, instead, industrial processes and storage procedures may significantly impact the oxysterol profile of milk and dairy products [116,117]. This is likely due to the technological methodologies and more sensible methods for COPs determination that have been developed in more recent times, although there is still a need for a harmonized analytical method to quantify cholesterol oxides using the same standards, reagents, protocols, and calibration materials to reduce interlaboratory variations and sources of errors [113,118].

Among the factors shown to influence the formation of oxidized cholesterol compounds there are pasteurization and ultra-high temperature (UHT) treatments of milk (both bovine and caprine), that increase the level of COPs significantly, up to 58% in bovine milk and 42% in caprine milk respectively [116]. Pasteurization at high temperatures

Table 3
COP levels (μg/g) in milk and dairy, egg, meat, poultry, fish, and composite products.

Food category	Type	Total COPs	7αOHC	7βOHC	α-epoxy	β-epoxy	7KC	25OHC	27OHC	triol	References
Milk and dairy											
Ghee		366	71	na	na	na	na	295	na	na	[119]
Dairy spreads		2.59	0.33	0.2	0.22	0.16	1.68	nd	na	0	[119]
Butter, butter oil		9–18.6	10.8	nd-2	nd-7	na	7.3	na	na	0.5	[119]
Parmesan	Grated	1.73	0.53	0.6	na	0.6	na	nd	na	nd	[119]
Yogurt	Vanilla	13	2	1	2	3	4	nd	na	1	[119]
Whole, skimmed, semi-skimmed milk	Raw, pasteurized, UHT	0.001–0.82	0.13–0.2	<LOQ-0.17	<LOQ-0.06	0.06–0.08	0.004–0.15	<LOQ-0.16	0.01–0.02	<LOQ-0.001	[114,116,117]
Evaporated milk		0.024	na	0.018	0.005	na	na	<LOQ	na	<LOQ	[114]
Condensed milk		0.029	na	0.026	0.002	na	na	<LOQ	na	<LOQ	[114]
Whole, skimmed milk powder	Fresh	0.08–0.19	na	0.04–0.12	na	na	0.04–0.07	na	0.05–0.14	na	[117]
Whole, skimmed milk powder	5–6 months	0.19–0.23	na	0.13–0.17	na	na	0.05–0.06	na	0.04–0.18	na	[117]
Whole, skimmed milk powder	11–12 months	0.34–0.74	na	0.27–0.57	na	na	0.08–0.17	na	0.05–0.14	na	[117]
Infant formula		2.13	0.58	0.75	0.02	0.02	0.16	0.1	na	0.12	[115]
Egg products											
Egg	Fresh; pasteurized; spray-dried;	<LOD-311	<LOD-9.69	<LOD-18.6	<LOD-13.71	<LOD-10.5	<LOD-6	na	na	0.17–2.7	[112,118,120,121,122]
Meat, poultry, fish											
Beef	Raw, cooked	0.21–16	na	na	na	na	0.21–16	na	na	na	[112,123]
Minced veal	Raw, cooked	1.22–1.23	0.18–0.64	na	na	na	0.2–0.92	0.13–0.38	na	na	[123]
Chicken	Cutlet, smocked, freeze-dried	5.79–22	2.04–2.43	na	na	na	2–22	1.75–2.19	na	na	[123]
Turkey meat	Raw	22.1	3.6	4.8	1.6	7	5.1	na	na	1.5	[112]
Pork	Raw, Boiled, cutlet, roast, bacon, cooked	1.24–8.15	0.19–2.28	na	na	na	0.92–8.15	0.13–2.12	na	na	[123]
Ham	Raw, roast	10.49–12.04	na	na	na	na	10.49–12.04	na	na	na	[123]
Sausage		tr-16.6	2.24	na	na	na	0.2–8.9	2.16	na	na	[112,123]
Fish	Raw, Salted-dried, Boiled-dried, Grilled, Smoked	4.6–191	0.36–0.77	0.5–58.8	tr-18	tr-43.4	0.78–60.6	tr-10.8	na	tr-39.1	[123,124]
Composite products											
Pasta with egg	Pasteurization + 2nd treatment	6.81–9.33	na	1.65–2.15	0.38–0.55	1–1.6	2.97–4.31	na	na	na	[125]
Biscuit with egg		3.98	0.63	1.53	0.17	0.34	0.05–1.32	na	na	na	[120,122]
Milk chocolate		0.27-0.54	na	0.09-0.25	0.01-0.02	0.01-0.03	0.05-0.15	0.03-0.07	0.03-0.07	0.00-0.01	[126]
Plant-based ingredients											
Vegetable oils		0.05–1.35	0.05–0.05	0.05–0.05	0.08–0.12	0.2–0.33	0.4–1.35	na	na	0.07–0.68	[127]
Cocoa paste		0.09	na	0.04	0.003	0.003	0.02	0.002	0.002	0.0001	[126]
Cocoa butter		0.16	na	0.08	0.006	0.005	0.04	0.003	0.004	0	[126]
Soy lecithin		0.1	na	0.05	0.004	0.009	0.04	0.001	0.0001	0.0002	[126]

COPs:cholesterol oxidation products; 7KC:7-ketocholesterol; 7αOHC:7α-hydroxycholesterol; 7βOHC:7β-hydroxycholesterol; α-epoxy:5α,6α-epoxide; β-epoxy:5β,6β-epoxide; triol:cholestan-3β,5α,6β-triol; 25OHC:25-hydroxycholesterol; 27OHC:27-hydroxycholestero; tr = trace; na: not available; nd: not detectable.

(85 °C) affects the generation of oxysterols differently, depending on the presence of fat in the product. More specifically, while their cholesterol ratios increased significantly after skimming and processing of skimmed milk and milk products, this was not observed after processing whole milk and milk cream [117]. In general, other factors that drive COPs formation include radiation, presence of unsaturated fatty acids, metal ions (like Fe, Cu, Co, Mg, Ni), natural dyes (for example, chlorophyll) and presence of free radicals/peroxides [109,118]. Moreover, storage conditions have been shown to have a crucial role on the formation of oxysterols. A storage time as short as 10 h affects the generation of COPs and the decrease of the antioxidant potential of the product [136]. Oxysterols in foodstuffs, in particular in powdered and/or frozen goods, possessing in general long shelf-lives of one year or even longer, have been shown to accumulate in high amounts, reaching concentrations that have been demonstrated potentially toxic *in-vitro*. This has been recorded, for example, in spray-dried whole eggs, whole and skimmed milk powders, frozen meat and meat products [117–120]. Therefore, the choice of packaging plays a fundamental role in prevention and therefore in the reduced formation of oxidized compounds. Among these, the ones that allow better prevention performance are those that act as a barrier with respect to light-induced oxidation and the ones that act against the oxygen such as multilayer gas barrier bags or modified atmosphere packaging [107].

5.3. Oxysterols as markers of food quality and ingredients' freshness

Taking into account what discussed above, in light of the fact that oxysterols tend to accumulate in food matrices after certain processing and storage procedures, COPs have been proposed as potential valuable markers to increase the commercial value, nutritional and beneficial properties of foodstuffs, to be monitored during the production chain [117,120,136]. 7KC, more specifically, has been identified as a biomarker for infant formula manufacturing being a direct derivative of cholesterol autooxidation, enhanced by food processing [115]. Cholesterol oxides, 7KC and 7βOHC in particular, have been shown to carry a considerable negative effect on human metabolism and, at relatively high concentrations, have major cytotoxic properties, having been implicated in a series of biological consequences such as cardiovascular diseases, retinopathies, neurodegenerative disorders and inflammatory bowel diseases [14,123,137]. No toxicity limits have been specified for these compounds, however, the threshold of toxicological concern (TTC) for each unclassified compound (0.15 μg per person per day) is currently utilized as reference upper limit of intake in an attempt of containing their intake [12]. An accurate, detailed COPs exposure intake from the diet is currently lacking either in the EU or in the US. However, a simulated dietary intake for total COPs has been performed for the first year of infants, finding how infant formula consumption can possibly lead to an increase of 7KC plasma concentration [115]. Therefore, it is still unknown whether the current intake level in different populations is safe or not. For this reason, in a precautionary approach, authors have recommended to limit the intake of these compounds and reduce their presence in foodstuff [12,118,123,138]. To this purpose, several methods have been suggested that include the reduction of their production during the food chain by the application of a shorter shelf-life, the fortification of foodstuffs or animal feed with antioxidants, the processing of food at lower temperatures, the food storage in the dark and the packaging improvement with more efficient materials to exclude or limit the amount of O_2 [12,118,123,138]. All in all, oxysterols represent innovative parameters to be exploited to better characterize and assess the nutritional composition and overall quality of both food ingredients and finished products, having the potential of being implemented as routine markers to quantify and monitor the oxidative status of foodstuffs undergoing different processing and storage procedures.

6. Cosmetic industry

Many skin care products contain lanolin, a wax secreted by sebaceous glands of the sheep and spread in its wool. Lanolin is a natural product very rich in cholesterol [139], thus susceptible to the non-enzymatic generation and possibly harmful excessive deposition of oxysterols. Definitely innovative appears the inclusion of the quantitative analysis of these compounds of toxicological relevance in the management of quality and shelf life of cosmetics products.

Only few studies are presently available in literature dealing with the GC-MS measurement of COPs, and absolutely surprising is the very high concentration that these compounds may reach in products like creams (0.001–0.2 mg/g), fat ointments (0.09–23 mg/g), lip sticks (0.6–9 mg/g) [140], amounts that are several orders of magnitude higher than those detectable in the various foodstuffs.

As expected, all main oxysterols of non-enzymatic origin were detected in the cosmetic products analyzed so far. Of interest, 7KC was by far the most represented oxysterol found in fat ointments (15 mg/g), lip-care products (2.1 mg/g), fatty creams (2.3 mg/g) [141].

7. Conclusions

In this effort of comprehensively review the actual and upcoming industrial utilization of oxysterols' redox biochemistry, a net distinction between oxysterols of enzymatic and oxysterols of non-enzymatic origin came out as quite evident and important. Moreover, the great advantage of oxysterols detection by GC/LC-MS is represented by the high sensitivity, the extreme accuracy and precision of such a technical procedure. Of the various practical applications, the use of oxysterols as reliable markers of the presence and progression of a large variety of diseases, will be most likely further expanded in the near future. Clearly, their quantification has to be matched with other instrumental and/or laboratory evaluations and often it is useful to measure a certain oxysterols' pattern rather than single compounds, as proven for serum/plasma 7KC+7βOHC and maybe triol in conditions of altered lipid and glucose metabolism, or thyroid metabolism, and for CSF levels 24OHC and 27OHC in various brain degenerative disorders. Notably, already promising and extremely important from both healthy and economic reasons appear the transfer of oxysterols from the basic redox bench to food industry. Indeed, there is a toxicological concern regarding some oxysterols when present in foodstuff in relatively high amounts, so it is desirable to adopt routine analysis of oxysterols content in cholesterol-containing food ingredients and finished products as soon as possible. Moreover, in this way the most suitable shelf-life of such finished products as well as their content of beneficial oxysterols of enzymatic origin could be reliably determined. The same concepts apply to other manufacturing sectors, certainly including cosmetics, which often include the cholesterol-rich natural product, lanolin.

Author contributions

Conceptualization, G.P.; writing—original draft preparation, G.P., V. L., D.R., F.B., F.C.; writing—review and editing, G.P., F.C.; visualization, G.P., F.C.; supervision, G.P. and R.M. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

D.R., F.C., R.M. are employed by Soremartec Italia Srl, Alba (CN, Italy). The above-mentioned authors were involved in the writing of this article and the decision to submit it for publication. G.P. has a scientific consultancy contract with Soremartec Italia Srl. All authors declare no other competing interests.

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