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Review

Metabolites in vertebrate Hedgehog signaling

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ABSTRACT

The Hedgehog (HH) signaling pathway is critical in embryonic development, stem cell biology, tissue homeostasis, chemoattraction and synapse formation. Irregular HH signaling is associated with a number of disease conditions including congenital disorders and cancer. In particular, deregulation of HH signaling has been linked to skin, brain, lung, colon and pancreatic cancers. Key mediators of the HH signaling pathway are the 12-pass membrane protein Patched (PTC), the 7-pass membrane protein Smoothened (SMO) and the GLI transcription factors. PTC shares homology with the RND family of small-molecule transporters and it has been proposed that it interferes with SMO through metabolites. Although a conclusive picture is lacking, substantial efforts are made to identify and understand natural metabolites/sterols, including cholesterol, vitamin D3, oxysterols and glucocorticoides, that may be affected by, or influence the HH signaling cascade at the level of PTC and SMO. In this review we will elaborate the role of metabolites in HH signaling with a focus on oxysterols, and discuss advancements in modern analytical approaches in the field.

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Contents

1. Introduction

While the Hedgehog (HH) signaling pathway plays a role in embryonic development, stem cells, cellular metabolism, axon targeting, synapse formation and nociception [1–5], it is also involved in human disorders and diseases, including developmental abnormalities and several forms of cancer [6–12]. The HH signaling cascade is unusual as it appears to involve metabolites while it alters the sub-cellular localization of pathway components that are closely entangled with the primary cilium [2]. Key players in this pathway, the morphogen Hedgehog, the trans-membrane proteins Patched and Smoothened and the zinc finger transcription factor Cubitus interruptus were predominantly identified through genetic screens in Drosophila in the late 1970's and early 1980's [13–17], with the subsequent discovery of the vertebrate homologs Sonic Hedgehog, Indian Hedgehog, Desert Hedgehog (SHH, IHH, DHH), Patched (PTC), Smoothened (SMO) and GLI [18–24]. As the interactions in this pathway were mapped, substantial similarities between Drosophila and vertebrates became apparent, reflecting deep evolutionary roots [2,25–28]. However, also differences exist between species; in particular many of the HH pathway components localize to the cell's primary cilium in vertebrates, an organelle that is not present in Drosophila [28–31].

In cells that produce the HH morphogen, HH undergoes cleavage, and its N-terminal peptide is dual-lipidated by cholesterol and palmitic acid (Fig. 1a). HH is then released by the resistance-nodulation division (RND) protein dispatched (DISP) either as monomeric particles, as multimeric particles or as exo-vesicles [32,33]. The form in which HH is released appears to define its signaling range [2]. It has been proposed that lipidation of HH promotes the association of HH with sterol-rich membrane areas in

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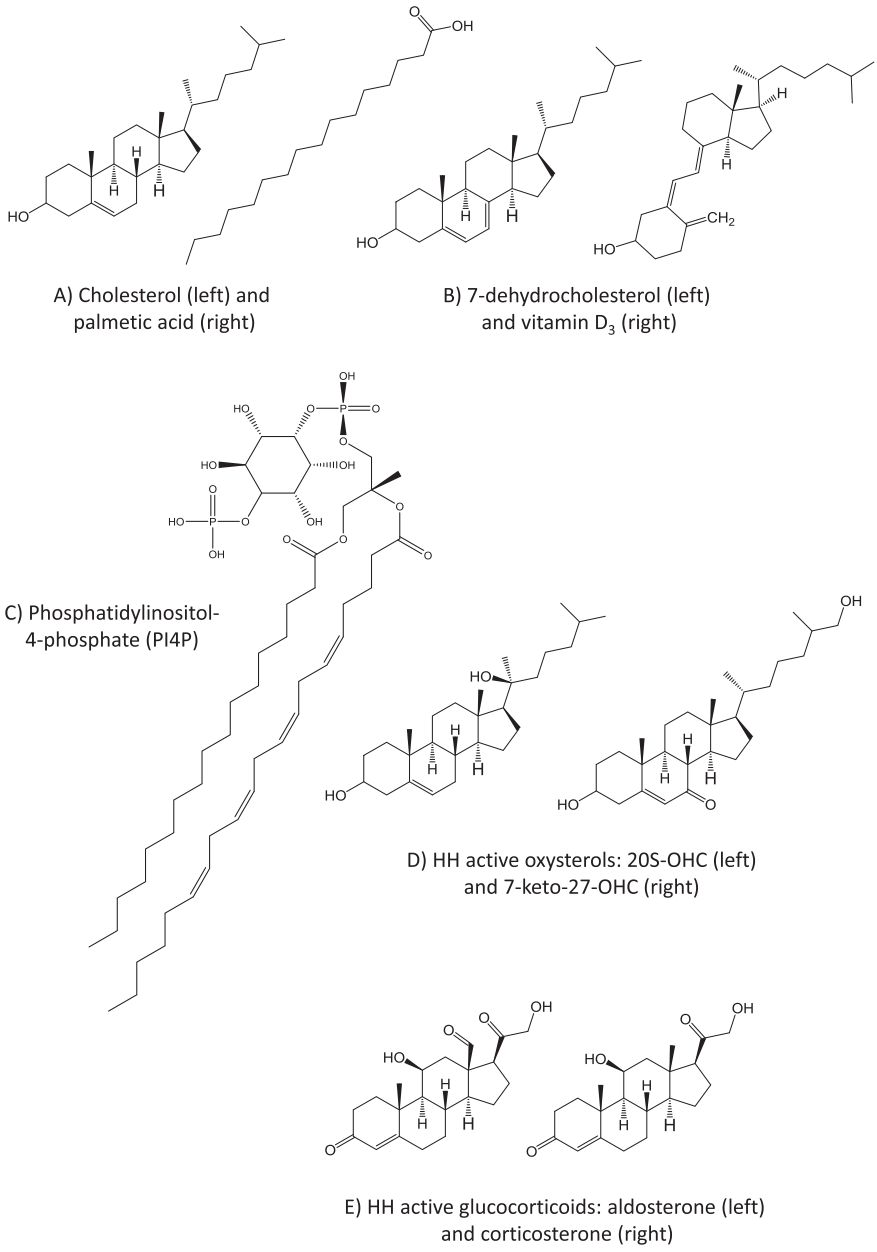

Fig. 1. Structures of selected Hedgehog pathway-modulating natural metabolites.

receiving cells [2,34,35] where HH binds to a number of membrane proteins including its canonic receptor the 12 pass transmembrane protein PTC, that similar to DISP, is a member of the RND family of proteins [21,23,36,37]. The binding of HH to PTC is promoted by the membrane co-receptor proteins CDO (CAM-related/downregulated by oncogens); BOC (brother of CDO) and GAS1 (growth arrest-specific 1) [2,26,38]. In the absence of the HH morphogen, PTC constitutively inhibits the signaling cascade [36,39]. However, upon HH binding, PTC releases the inhibition of the pathway.

How HH signaling is progressed beyond PTC is not entirely settled and there are curious differences between Drosophila and vertebrates [28]. PTC regulates the sub-cellular localization and activity of SMO, but PTC does not appear to directly interact with SMO. When the RND domain of PTC is mutated, it is no longer capable to inhibit SMO, suggesting that metabolites may be involved in the interaction between PTC and SMO [40,41]. What these metabolites are *in vivo* remains to some extent unclear and will be discussed below.

In vertebrates several sub-cellular alterations occur upon activation of the HH signaling pathway. In the absence of HH, the PTC receptor is enriched at the basis and in the primary cilium, a specialized organelle at the cellular surface that depends on the intraflagellar transport system (IFT) and that has been implied in various sensing functions [31]. When HH binds PTC, PTC leaves the primary cilium and enters the endocytotic pathway to be degraded. As PTC exits the primary cilium, also the G-protein coupled receptor GPR161, a rhodopsin family GPCR protein, is transported out of the primary cilium [42]. GPR161 negatively regulates HH signaling in the primary cilium through enhancing PKA activity by increasing cAMP levels. PKA is involved in phosphorylating the HH dependent zinc finger transcription factors GLI2 and GLI3 as described below [42]. Importantly, upon HH binding to PTC, the 7 transmembrane protein SMO, a Frizzled (FZD) class G-protein-coupled receptor with an unusually complex structure, is activated and moves in association with β-arrestin and the microtubule motor KIF3A into the primary cilium [29,30,35,43,44]. This

movement originates predominantly from exocytotic SMO containing vesicles [45] but also from the plasma membrane [46]. For the translocation of SMO to the primary cilium and its activation, SMO is phosphorylated at its carboxy-terminal cytoplasmic tail by CK1 and GRK2 [47,48] leading to a conformational switch [25].

One of the roles of vertebrate cilia is to control the proteolytic processing of members of the Ci/GLI family of zinc finger transcription factors [2]. In the inactive state of HH signaling two members of the GLI family, GLI2 and GLI3 pass through the primary cilium [31,49] in a process that leads to their partial proteolytic processing into transcriptional repressors. GLI3 contains an N-terminal repressor domain that, in the absence of HH signaling, is sequentially phosphorylated by protein kinase A (PKA), casein kinase1 (CK1) and glycogen synthase kinase 3 (GSK3). Phosphorylated GLI3 is then recognized by βTrCP leading to ubiquitinatation of GLI3 and a partial degradation of the C-terminal trans-activation domain in the proteasome. This leads to a repressor form of GLI3 [50]. The ciliar SUFU protein assists the process by immobilizing GLI3 [51,52]. Similar to GLI3, GLI2 is also proteolytically processed, but to a lesser extent [2,53,54].

When SMO enters the primary cilium it associates, phosphorylation dependent, with the EVC and EVC2 proteins (Ellis-van Creveld Syndrome protein) [55–57]. It is thought that SMO/EVC/ EVC2 interact with SUFU thereby resolving the SUFU/ GLI3 inhibitory complex [57]. In consequence, GLI3 proteolytic processing is prevented leading to a weak activator form of GLI3 that translocates to the nucleus [58]. Active GLI3 up-regulates the expression of the transcriptional activator GLI1 that, although apparently not involved in the initial Hh dependent response of the pathway, is a strong potentiator of the signal [54,59].

2. Metabolites, PTC and SMO

The roles of metabolites in HH signaling remain only partially understood although multiple lines of evidence suggest that PTC and SMO communicate via metabolites. PTC has been proposed to be involved in sterol trafficking. PTC contains two main domains, a RND domain that is related to bacterial proteins implied in proton transport and permease activity [60], and a related sterol-sensing domain (SSD) that has been linked to sensing sterols [26,61]. Other members of the PTC-family of RND proteins include the cholesterol transporter Niemann–Pick disease type C1 protein (NPC1) and dispatched (DISP) [26,61]. It has been shown that cholesterol binds to, and is transported by PTC [62]. Furthermore, Bijlsma et al. imply that PTC pumps the 3β-hydroxysteroids 7-dehydrocholesterol and vitamin D3 (Fig. 1b) into the extracellular space [63]. In Drosophila it has been suggested that Ptc recruits lipophorin and thereby changes the lipid composition of endosomes through which Smo passes [2,64]. Furthermore, Drosophila Smo is stabilized in the plasma membrane by phospholipid phosphatidylinositol-4-phosphate (PI4P) (Fig. 1c) leading to increased Hh signaling whereby Ptc functions directly or indirectly to suppress accumulation of PI4P. In particular, it has been shown that Hh binding to Ptc de-represses PI4P levels. A similar PI4P dependency appears to be present in mouse fibroblasts [65]. Evidence suggests that side chain-hydroxylated oxysterols (Fig. 1d) and glucocorticoides (Fig. 1e) play a role in HH signaling, however, direct evidence that PTC is either involved in controlling the intra- or extracellular levels of these sterols or that they directly affect PTC activity is currently lacking.

Also SMO is sensitive to sterols and the fine mapping of sterol/metabolite, and drug-binding sites in SMO has advanced considerably. Two major functional sites have been identified in SMO: the heptahelical domain (7TM) a long, narrow cavity within the membrane passing helical core of SMO [66,67], and a flexible cysteine-rich extra cellular domain (CRD) [68–72]. It has been suggested that both domains are in an allosteric interaction whereby the CRD domain, that in its native state is not activating the HH pathway, can positively regulate high level HH signaling by flipping into the 7TM domain in the presence of CRD binding metabolites [71–73]. A plethora of inhibitory and activating metabolites have been identified that may either bind to the CRD domain, to the 7TM domain, or possibly bridge both domains [68–70,72]. The 7TM domain has been proposed to be a binding site for a ligand that is controlled by PTC [74] although critical mutations in the 7TM domain that disenable binding of the 7TM binder cyclopamine, still allow a regulation of SMO through HH/PTC [68]. In contrast, although it is sensitive to multiple sterols, the current understanding is that the CRD domain appears to be dispensable for a regulation of SMO through PTC [68].

The crystal structure of the 7TM domain of SMO was recently elucidated [67] and numerous inhibitors including cyclopamine, SANT-1 and vismodegib (GDC-0449) have been shown to bind the 7TM pocket [42,66,75–77]. Also vitamin D3 may bind as a negative regulator to the 7TM pocket of SMO as it competes with cyclopamine, and has been proposed to be a metabolite or highly similar to a metabolite by which PTC may constitutively inhibit SMO both in a cell-autonomous and possibly in a non-cell autonomous manner in the absence of HH signaling [63]. In accordance, Tang et al. showed that external vitamin D3 exposure decreases Gli1 mRNA expression in BCC cell lines [78]. However, vitamin D3 while active on SMO in a reasonably low dose, needs UV light to be processed from its precursor 7-dehydrocholesterol. 7-dehydrocholesterol in turn only inhibits SMO at very high concentrations [63]. Small molecules that have been proposed to bind to the 7TM domain may not only be negative regulators of SMO. Substances such as SAG and puromorphamine that compete with BODIPY-cyclopamine are SMO activators [66,77,79].

Several metabolites and in particular oxysterols that bind to the CRD domain have an activating effect on SMO[68–70,80]. Curiously, binding of oxysterols is dependent on a stretch of amino acids in vertebrate SMO that is not shared by Drosophila Smo rendering Drosophila Smo insensitive to oxysterols [70]. In particular the binding of oxysterol 20S-OHC (Fig. 1d) to the CRD domain has been extensively studied. Binding was found to be stereo-specific, as the S-isomer of 20-hydrocholesterol is docking to the CDR domain while the R-isomer is not [69,80]. However, 20S-OHC may not be the natural SMO ligand as it has not been detected in HH responsive cells that are oxysterol sensitive [68,81]. Another natural oxysterol that activates SMO, although less efficient, is 25-OHC [80,82]. Recently, 7-keto-27-OHC (Fig. 1d), but also 7-keto-25-OHC has been proposed to bind to the CRD domain of SMO *in vivo* [68]. In summary, the following set of SMO-CRD binding and activating rules have been proposed for oxysterols. Oxysterols that possess a (saturated) side chain at least 6 atoms long and the presence of a 3ß hydroxy-group can activate SMO [70]. Oxysterols can also contain additional/alternative side groups with maintained or augmented activity e.g. a 7-keto group [68], or an alkyne group instead of an iso-butyl group at the end of the iso-octyl chain (synthetic) [69]. Furthermore, an activation effect of oxysterols is not influenced by the absence of the oxysterol Δ5 double bond [70]. In contrast, the ring-hydroxylated oxysterols 7β-OHC and 19-OHC do not activate HH signaling [80,82].

A second group of natural metabolites that bind SMO are glucocorticoids and it has been shown that aldosterone and corticosterone (Fig. 1e) activate HH signaling and stimulate SMO accumulation in the primary cilium [73,83]. The exact binding site of natural glucocorticoides on SMO has not been mapped, but fluorinated glucocorticoides have been shown to displace BODIPY-cyclopamine [83,84]. Surprisingly, two synthetic molecules that

share the core glucocorticoide scaffold but contain bulky hydrophobic groups at positions 16 and 17, Budesonide and Ciclesonide, show inhibition of HH dependent activation of the pathway [84]. Budesonide was recently shown by co-crystallography to bind to both the Drosophila and vertebrate SMO CRD domain [72].

The binding of metabolites to either the 7TM or the CRD domain is not always clear-cut. It has been proposed that cyclopamine in addition to binding the 7TM domain with high affinity, may also bind at lower affinity to the CRD domain [69], rendering the precise biochemical mapping of the binding site of a metabolite through cyclopamine competition assays problematic.

There may be further interference points between SMO and metabolites. Neither of the tested activating oxysterols shares a binding site with intraconazole, a potent HH inhibitor which also retains activity on known resistance-conferring SMO mutants [85,86]. Since intraconazole does not dock at the cyclopamine-binding site [86] it has been postulated that SMO may directly or indirectly have further sites at which metabolites and/or drugs can attenuate its function [68].

While no proof has been presented that PTC is directly involved in transporting oxysterols, it is conceivable that PTC mediated metabolites, such as vitamin D3 or 7-dehydrocholesterol, indirectly, regulate the impact of oxysterols on HH signaling through interfering with the 7TM site [58]; When PTC is deactivated through HH binding, it will not distribute such metabolites to SMO allowing CRD-based activation of SMO by oxysterols or other metabolites. This interaction may occur both intracellular, and at the extracellular space around the primary cilium, as Hh-active oxysterols are not intracellularly confined [87]. In accordance, Myer et al. speculate that SMO senses and interacts with a cocktail of excreted activators [68].

In summary, although some light has been shed on metabolites that are capable of regulating the HH pathway at the level of PTC and SMO, numerous questions related to metabolites and in particular sterols remain unresolved (i) Which metabolites if any interact with the PTC sterol sensing domain *in vivo* and how does that impact HH signaling, (ii) What are the natural metabolites that are transported by PTC, (iii) which natural metabolites or hormones interfere with the 7TM domain of SMO, (iv) which natural oxysterols or other metabolites act through the SMO CRD domain.

Further work is required to gain a comprehensive understanding of the interface between PTC and metabolites in the context of HH signaling.

3. Analytical challenges for monitoring and discovering endogenous HH-active oxysterols and other metabolites

The uncertainty of the roles that metabolites play in HH signaling can be met to a large degree with sensitive and selective analytical methodology, which can be applied to e.g. rule out possible candidates, but also for discovering new ones. As mentioned above, one of the most potent oxysterols examined so far that activates SMO *in vitro* is 20S-OHC. 20S-OHC has been speculated as being the prominent activator *in vivo* [80], however, our group and others [68,81,88] have not been able to securely identify this particular oxysterol in HH sensitive reporter cells. To our knowledge, 20S-OHC has been proposed as present *in vivo* in just two studies (rat brain and human placenta [89,90]). Considering the current state methodology and the lack of known natural enzyme responsible for creating this particular oxysterol, further work is required to establish whether 20S-OHC plays a role in HH regulation in some context. Other oxysterols have been suggested as core endogenous SMO activators (e.g. 7-keto-27-OHC [68]), but there is nonetheless a need for extended studies on the role and context of endogenous sterols/oxysterols in HH signaling.

The analysis of sterols/oxysterols and related compounds can be an especially "slippery slope" due to a number of analytical challenges, which can also result in misinterpretations [91].

For small molecules, mass spectrometry (MS) based methodology is often a necessity for analysis as e.g. immunoassays can suffer from poor selectivity and quantitation capabilities. In addition, MS can be used for discovering and identifying metabolites, often in combination with gas chromatography (GC–MS). GC–MS has also been traditionally used for oxysterol analysis [92,93]. GC–MS typically uses electron ionization (EI) to prepare the analytes for mass spectrometric detection, which results in extensive molecular fragmentation, which in many cases is useful for establishing a "mass fingerprint" of an oxysterol. However, this fragmentation may in some cases provide limited structural information compared to complementary "softer" ionization techniques used in liquid chromatography (LC)–MS [94]. GC–MS methods also require a derivatization process to make oxysterols volatile enough for gas chromatographic separation. Such a step may be avoided when employing LC coupled with e.g. atmospheric pressure chemical ionization (APCI) [95] at the inlet of the mass spectrometer (LC–APCI–MS). However, due to the mass sensitive nature of APCI and incompatibility with narrow LC columns (which provide enhanced sensitivity) [96], LC coupling with the more common electrospray ionization (ESI) may be appropriate to reach low detection limits. To detect oxysterols satisfactory using ESI, they can be "charge-tagged" with a derivatization reagent [81,97,98] or by exploiting adduct formation by adding e.g. ammonia to the mobile phases [88]. The charge-tagging LC–MS approach, although more tedious due to derivatization steps, can provide low femtogram detection levels [98] and/or can be beneficial for identification purposes when using a sterol-specific charge-tag, e.g. the Girard T reagent [99]. 7-dehydrocholesterol can be measured simultaneously with oxysterols in serum, following selective reduction of their 3β-hydroxy group with cholesterol oxidase followed by Girard T derivatization [97]. Derivatization (i.e. esterification of phosphate groups) is again important for sensitive LC–ESI–MS analysis of phosphatidylinositol phosphates such as PI4P [100], which is implied in SMO regulation. LC-atmospheric pressure photo ionization (APPI)–MS can be a suitable option for derivatization-free determination of vitamin D3 and metabolites, as an APPI based approach has significantly higher sensitivity compared to APCI [101]. Glucocorticoides such as aldosterone and corticosterone can be measured more straightforward, using LC–ESI–MS without derivatization [102,103]. Another interesting approach is based on matrix assisted laser desorption ionization (MALDI) imaging [104], which can potentially be used for e.g. mapping oxysterols and other metabolites at the surface of intact cells.

In the analysis of oxysterols, an important pitfall to consider is possible non-enzymatic oxidation of cholesterol into oxysterols. Such autoxidation effects are especially important concerning oxysterols with hydroxy-groups placed in the ring structure (most notably the 7-position, where the carbon-hydrogen bond is relatively weak [93,105]). Cholesterol is present in much higher concentrations in cells than oxysterols (10^3 fold), and autoxidation can therefore result in poor quantification or even mistaken identification of natural/non-natural occurring oxysterols. Autoxidation can in particular occur if the sample is subjected to light or heat. Attempts to avoid *in vitro* autoxidation have been made through time, such as adding anti-oxidant agents (e.g. butyl hydroxytoluene), or purging all reagents and vials with N_2 [93]. Others insist that cholesterol should be removed from the sample immediately before further sample preparation [93]. However, removal of cholesterol often requires a number of manual steps, which may not be optimal for handling of limited samples. Also, the similarity in structure and hydrophobicity of cholesterol and oxysterols makes them arguably difficult to separate on methods

like solid phase extraction (SPE) with good reproducibility and high recovery which can affect comparative assessments of oxysterols that do not have a dedicated internal standard [88].

Autoxidation monitoring can be enabled by adding heavy cholesterol into all samples and standards [81,99]. If autoxidation occurs during sample preparation, heavy cholesterol is oxidized into heavy oxysterols. These heavy oxysterols can be spotted by mass spectrometry and the sample can be excluded. Although side-chain hydroxylated oxysterols are considered not to be susceptible to autoxidation effects, this has been observed by us in a limited number of samples, evident from detection of heavy oxysterols, and a 2–3 times increase in concentration of the corresponding "natural" oxysterols.

References

[1] P.W. Ingham, A.P. McMahon, Hedgehog signaling in animal development: paradigms and principles, Gene Dev. 15 (2001) 3059–3087.
[2] J. Briscoe, P.P. Thérond, The mechanisms of Hedgehog signalling and its roles in development and disease, Nat. Rev. Mol. Cell Biol. 14 (2013) 416–429.
[3] D.T. Babcock, S. Shi, J. Jo, M. Shaw, H.B. Gutstein, M.J. Galko, Hedgehog signaling regulates nociceptive sensitization, Curr. Biol. 21 (2011) 1525–1533.
[4] J. Courchet, F. Polleux, Sonic hedgehog, BOC, and synaptic development: new players for an old game, Neuron 73 (2012) 1055–1058.
[5] R. Teperino, S. Amann, M. Bayer, S.L. McGee, A. Loipetzberger, T. Connor, C. Jaeger, B. Kammerer, L. Winter, G. Wiche, Hedgehog partial agonism drives Warburg-like metabolism in muscle and brown fat, Cell 151 (2012) 414–426.
[6] J.L. Mullor, P. Sánchez, Pathways and consequences: hedgehog signaling in human disease, Trends Cell Biol. 12 (2002) 562–569.
[7] D. Amakye, Z. Jagani, M. Dorsch, Unraveling the therapeutic potential of the Hedgehog pathway in cancer, Nat. Med. 19 (2013) 1410–1422.
[8] S. Teglund, R. Toftgård, Hedgehog beyond medulloblastoma and basal cell carcinoma, BBA-Rev. Cancer 2010 (1805) 181–208.
[9] M.T. Barakat, E.W. Humke, M.P. Scott, Learning from Jekyll to control Hyde: hedgehog signaling in development and cancer, Trends Mol. Med. 16 (2010) 337–348.
[10] M.M. Cohen Jr., Hedgehog signaling update, Am. J. Med. Genet. A 152 (2010) 1875–1914.
[11] R.L. Johnson, A.L. Rothman, J. Xie, L.V. Goodrich, J.W. Bare, J.M. Bonifas, A.G. Quinn, R.M. Myers, D.R. Cox, E.H. Epstein, Human homolog of patched, a candidate gene for the basal cell nevus syndrome, Science 272 (1996) 1668–1671.
[12] L. Yang, G. Xie, Q. Fan, J. Xie, Activation of the hedgehog-signaling pathway in human cancer and the clinical implications, Oncogene 29 (2009) 469–481.
[13] C. Nüsslein-Volhard, E. Wieschaus, Mutations affecting segment number and polarity in Drosophila, Nature 287 (1980) 795–801.
[14] J. Alcedo, M. Ayzenzon, T. Von Ohlen, M. Noll, J.E. Hooper, The Drosophila *smoothened* gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal, Cell 86 (1996) 221–232.
[15] G. Tiniakow, E. Terentieva, Cubitus interruptus, a new genovariation of the fourth chromosome of *Drosophila melanogaster*, Genetics 18 (1933) 117–120.
[16] T. Orenic, D. Slusarski, K. Kroll, R. Holmgren, Cloning and characterization of the segment polarity gene cubitus interruptus Dominant of Drosophila, Gene Dev. 4 (1990) 1053–1067.
[17] M. van den Heuvel, P.W. Ingham, Smoothened encodes a receptor-like serpentine protein required for hedgehog signalling, Nature 382 (1996) 547–551.
[18] Y. Echelard, D.J. Epstein, B. St-Jacques, L. Shen, J. Mohler, J.A. McMahon, A.P. McMahon, Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity, Cell 75 (1993) 1417–1430.
[19] R.D. Riddle, R.L. Johnson, E. Laufer, C. Tabin, *Sonic hedgehog* mediates the polarizing activity of the ZPA, Cell 75 (1993) 1401–1416.
[20] S. Krauss, J.-P. Concordet, P. Ingham, A functionally conserved homolog of the Drosophila segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos, Cell 75 (1993) 1431–1444.
[21] V. Marigo, R.A. Davey, Y. Zuo, J.M. Cunningham, C.J. Tabin, Biochemical evidence that patched is the Hedgehog receptor, Nature 384 (1996) 176–179.
[22] J.-P. Concordet, K.E. Lewis, J.W. Moore, L.V. Goodrich, R.L. Johnson, M.P. Scott, P.W. Ingham, Spatial regulation of a zebrafish patched homologue reflects the roles of sonic hedgehog and protein kinase A in neural tube and somite patterning, Development 122 (1996) 2835–2846.
[23] D.M. Stone, M. Hynes, M. Armanini, T.A. Swanson, Q. Gu, R.L. Johnson, M.P. Scott, D. Pennica, A. Goddard, H. Phillips, The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog, Nature 384 (1996) 129–134.
[24] H. Akiyama, C. Shigeno, Y. Hiraki, C. Shukunami, H. Kohno, M. Akagi, J. Konishi, T. Nakamura, Cloning of a mouse smoothened cDNA and expression patterns of hedgehog signalling molecules during chondrogenesis and cartilage differentiation in clonal mouse EC cells, ATDC5, Biochem. Biophys. Res. Commun. 235 (1997) 142–147.
[25] Y. Zhao, C. Tong, J. Jiang, Transducing the Hedgehog signal across the plasma membrane, Fly 1 (2007) 333–336.
[26] G. Hausmann, C. von Mering, K. Basler, The hedgehog signaling pathway: where did it come from?, PLoS Biol 7 (2009) e1000146.
[27] J. Fan, K. Jiang, Y. Liu, J. Jia, Hrs promotes ubiquitination and mediates endosomal trafficking of smoothened in drosophila hedgehog signaling, PLoS One 8 (2013) e79021.
[28] P.W. Ingham, Y. Nakano, C. Seger, Mechanisms and functions of Hedgehog signalling across the metazoa, Nat. Rev. Genet. 12 (2011) 393–406.
[29] D. Huangfu, A. Liu, A.S. Rakeman, N.S. Murcia, L. Niswander, K.V. Anderson, Hedgehog signalling in the mouse requires intraflagellar transport proteins, Nature 426 (2003) 83–87.
[30] K.C. Corbit, P. Aanstad, V. Singla, A.R. Norman, D.Y. Stainier, J.F. Reiter, Vertebrate Smoothened functions at the primary cilium, Nature 437 (2005) 1018–1021.
[31] S.C. Goetz, K.V. Anderson, The primary cilium: a signalling centre during vertebrate development, Nat. Rev. Genet. 11 (2010) 331–344.
[32] R. Burke, D. Nellen, M. Bellotto, E. Hafen, K.-A. Senti, B.J. Dickson, K. Basler, Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells, Cell 99 (1999) 803–815.
[33] T. Caspary, M.J. García-Garcıa, D. Huangfu, J.T. Eggenschwiler, M.R. Wyler, A.S. Rakeman, H.L. Alcorn, K.V. Anderson, Mouse dispatched homolog1 is required for long-range, but not juxtacrine, Hh signaling, Curr. Biol. 12 (2002) 1628–1632.
[34] A. Rietveld, S. Neutz, K. Simons, S. Eaton, Association of sterol-and glycosylphosphatidylinositol-linked proteins with Drosophila raft lipid microdomains, J. Biol. Chem. 274 (1999) 12049–12054.
[35] M.-H. Chen, Y.-J. Li, T. Kawakami, S.-M. Xu, P.-T. Chuang, Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates, Gene Dev. 18 (2004) 641–659.
[36] J.E. Hooper, M.P. Scott, The Drosophila *patched* gene encodes a putative membrane protein required for segmental patterning, Cell 59 (1989) 751–765.
[37] N. Denef, D. Neubüser, L. Perez, S.M. Cohen, Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened, Cell 102 (2000) 521–531.
[38] P.A. Beachy, S.G. Hymowitz, R.A. Lazarus, D.J. Leahy, C. Siebold, Interactions between Hedgehog proteins and their binding partners come into view, Gene Dev. 24 (2010) 2001–2012.
[39] Y. Nakano, I. Guerrero, A. Hidalgo, A. Taylor, J. Whittle, P. Ingham, A protein with several possible membrane-spanning domains encoded by the Drosophila segment polarity gene patched, Nature 341 (1989) 508–513.
[40] K.E. Ryan, C. Chiang, Hedgehog secretion and signal transduction in vertebrates, J. Biol. Chem. 287 (2012) 17905–17913.
[41] J. Taipale, M. Cooper, T. Maiti, P. Beachy, Patched acts catalytically to suppress the activity of Smoothened, Nature 418 (2002) 892–896.
[42] S. Mukhopadhyay, X. Wen, N. Ratti, A. Loktev, L. Rangell, S.J. Scales, P.K. Jackson, The ciliary G-protein-coupled receptor Gpr161 negatively regulates the Sonic hedgehog pathway via cAMP signaling, Cell 152 (2013) 210–223.
[43] K.J. Lavine, A. Kovacs, D.M. Ornitz, Hedgehog signaling is critical for maintenance of the adult coronary vasculature in mice, J. Clin. Invest. 118 (2008) 2404–2414.
[44] J.J. Kovacs, E.J. Whalen, R. Liu, K. Xiao, J. Kim, M. Chen, J. Wang, W. Chen, R.J. Lefkowitz, β-Arrestin–mediated localization of smoothened to the primary cilium, Science 320 (2008) 1777–1781.
[45] Y. Wang, Z. Zhou, C.T. Walsh, A.P. McMahon, Selective translocation of intracellular Smoothened to the primary cilium in response to Hedgehog pathway modulation, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 2623–2628.
[46] L. Milenkovic, M.P. Scott, R. Rohatgi, Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium, J. Cell Biol. 187 (2009) 365–374.
[47] T. Evron, M. Philipp, J. Lu, A.R. Meloni, M. Burkhalter, W. Chen, M.G. Caron, Growth Arrest Specific 8 (Gas8) and G protein-coupled receptor kinase 2 (GRK2) cooperate in the control of Smoothened signaling, J. Biol. Chem. 286 (2011) 27676–27686.
[48] Y. Chen, N. Sasai, G. Ma, T. Yue, J. Jia, J. Briscoe, J. Jiang, Sonic Hedgehog dependent phosphorylation by CK1α and GRK2 is required for ciliary accumulation and activation of smoothened, PLoS Biol. 9 (2011) e1001083.
[49] A. Liu, B. Wang, L.A. Niswander, Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors, Development 132 (2005) 3103–3111.
[50] D. Tempé, M. Casas, S. Karaz, M.-F. Blanchet-Tournier, J.-P. Concordet, Multisite protein kinase A and glycogen synthase kinase 3β phosphorylation leads to Gli3 ubiquitination by SCFβTrCP, Mol. Cell. Biol. 26 (2006) 4316–4326.
[51] E.W. Humke, K.V. Dorn, L. Milenkovic, M.P. Scott, R. Rohatgi, The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins, Gene Dev. 24 (2010) 670–682.
[52] H. Tukachinsky, L.V. Lopez, A. Salic, A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu–Gli protein complexes, J. Cell Biol. 191 (2010) 415–428.

[53] Y. Pan, C.B. Bai, A.L. Joyner, B. Wang, Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation, Mol. Cell. Biol. 26 (2006) 3365–3377.
[54] J. Li, C. Wang, Y. Pan, Z. Bai, B. Wang, Increased proteolytic processing of full-length Gli2 transcription factor reduces the hedgehog pathway activity in vivo, Dev. Dyn. 240 (2011) 766–774.
[55] K.V. Dorn, C.E. Hughes, R. Rohatgi, A Smoothened–Evc2 complex transduces the Hedgehog signal at primary cilia, Dev. Cell 23 (2012) 823–835.
[56] C. Yang, W. Chen, Y. Chen, J. Jiang, Smoothened transduces Hedgehog signal by forming a complex with Evc/Evc2, Cell Res. 22 (2012) 1593–1604.
[57] J.A. Caparrós-Martín, M. Valencia, E. Reytor, M. Pacheco, M. Fernandez, A. Perez-Aytes, E. Gean, P. Lapunzina, H. Peters, J.A. Goodship, The ciliary Evc/Evc2 complex interacts with Smo and controls Hedgehog pathway activity in chondrocytes by regulating Sufu/Gli3 dissociation and Gli3 trafficking in primary cilia, Hum. Mol. Genet. 22 (2013) 124–139.
[58] Y. Wang, A.P. McMahon, B.L. Allen, Shifting paradigms in Hedgehog signaling, Curr. Opin. Cell Biol. 19 (2007) 159–165.
[59] S.X. Atwood, M. Li, A. Lee, J.Y. Tang, A.E. Oro, GLI activation by atypical protein kinase C ι/λ regulates the growth of basal cell carcinomas, Nature 494 (2013) 484–488.
[60] T.-T. Tseng, K.S. Gratwick, J. Kollman, D. Park, D.H. Nies, A. Goffeau, M.H. Saier Jr., The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins, J. Mol. Microbiol. Biotechnol. 1 (1999) 107–125.
[61] P.E. Kuwabara, M. Labouesse, The sterol-sensing domain: multiple families, a unique role?, Trends Genet 18 (2002) 193–201.
[62] M. Bidet, O. Joubert, B. Lacombe, M. Ciantar, R. Nehmé, P. Mollat, L. Brétillon, H. Faure, R. Bittman, M. Ruat, The hedgehog receptor patched is involved in cholesterol transport, PLoS One 6 (2011) e23834.
[63] M.F. Bijlsma, C.A. Spek, D. Zivkovic, S. van de Water, F. Rezaee, M.P. Peppelenbosch, Repression of smoothened by patched-dependent (pro-) vitamin D3 secretion, PLoS Biol. 4 (2006) e232.
[64] H. Khaliullina, D. Panáková, C. Eugster, F. Riedel, M. Carvalho, S. Eaton, Patched regulates Smoothened trafficking using lipoprotein-derived lipids, Development 136 (2009) 4111–4121.
[65] A. Yavari, R. Nagaraj, E. Owusu-Ansah, A. Folick, K. Ngo, T. Hillman, G. Call, R. Rohatgi, M.P. Scott, U. Banerjee, Role of lipid metabolism in smoothened derepression in hedgehog signaling, Dev. Cell 19 (2010) 54–65.
[66] J.K. Chen, J. Taipale, M.K. Cooper, P.A. Beachy, Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened, Gene Dev. 16 (2002) 2743–2748.
[67] C. Wang, H. Wu, V. Katritch, G.W. Han, X.-P. Huang, W. Liu, F.Y. Siu, B.L. Roth, V. Cherezov, R.C. Stevens, Structure of the human smoothened receptor bound to an antitumour agent, Nature 497 (2013) 338–343.
[68] B.R. Myers, N. Sever, Y.C. Chong, J. Kim, J.D. Belani, S. Rychnovsky, J.F. Bazan, P.A. Beachy, Hedgehog pathway modulation by multiple lipid binding sites on the smoothened effector of signal response, Dev. Cell 26 (2013) 346–357.
[69] S. Nachtergaele, D.M. Whalen, L.K. Mydock, Z. Zhao, T. Malinauskas, K. Krishnan, P.W. Ingham, D.F. Covey, C. Siebold, R. Rohatgi, Structure and function of the Smoothened extracellular domain in vertebrate Hedgehog signaling, eLife 2 (2013).
[70] D. Nedelcu, J. Liu, Y. Xu, C. Jao, A. Salic, Oxysterol binding to the extracellular domain of Smoothened in Hedgehog signaling, Nat. Chem. Biol. 9 (2013) 557–564.
[71] P. Aanstad, N. Santos, K.C. Corbit, P.J. Scherz, L.A. Trinh, W. Salvenmoser, J. Huisken, J.F. Reiter, D.Y. Stainier, The extracellular domain of Smoothened regulates ciliary localization and is required for high-level Hh signaling, Curr. Biol. 19 (2009) 1034–1039.
[72] R. Rana, C.E. Carroll, H.-J. Lee, J. Bao, S. Marada, C.R. Grace, C.D. Guibao, S.K. Ogden, J.J. Zheng, Structural insights into the role of the Smoothened cysteine-rich domain in Hedgehog signalling, Nat. Commun. 4 (2013), http://dx.doi.org/10.1038/ncomms3965 (Article number: 2965).
[73] Y. Wang, A.P. McMahon, A novel site comes into sight, eLife (2013) (2:e01680).
[74] R.L. Yauch, G.J. Dijkgraaf, B. Alicke, T. Januario, C.P. Ahn, T. Holcomb, K. Pujara, J. Stinson, C.A. Callahan, T. Tang, Smoothened mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma, Science 326 (2009) 572–574.
[75] J.K. Chen, J. Taipale, K.E. Young, T. Maiti, P.A. Beachy, Small molecule modulation of Smoothened activity, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 14071–14076.
[76] K.D. Robarge, S.A. Brunton, G.M. Castanedo, Y. Cui, M.S. Dina, R. Goldsmith, S.E. Gould, O. Guichert, J.L. Gunzner, J. Halladay, GDC-0449—a potent inhibitor of the hedgehog pathway, Bioorg. Med. Chem. Lett. 19 (2009) 5576–5581.
[77] M. Frank-Kamenetsky, X. Zhang, S. Bottega, O. Guicherit, H. Wichterle, H. Dudek, D. Bumcrot, F. Wang, S. Jones, J. Shulok, Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists, J. Biol. 1 (2002) 10.
[78] J.Y. Tang, T.Z. Xiao, Y. Oda, K.S. Chang, E. Shpall, A. Wu, P.-L. So, J. Hebert, D. Bikle, E.H. Epstein, Vitamin D3 inhibits hedgehog signaling and proliferation in murine basal cell carcinomas, Cancer Prev. Res. 4 (2011) 744–751.
[79] S. Sinha, J.K. Chen, Purmorphamine activates the Hedgehog pathway by targeting Smoothened, Nat. Chem. Biol. 2 (2006) 29–30.
[80] S. Nachtergaele, L.K. Mydock, K. Krishnan, J. Rammohan, P.H. Schlesinger, D.F. Covey, R. Rohatgi, Oxysterols are allosteric activators of the oncoprotein Smoothened, Nat. Chem. Biol. 8 (2012) 211–220.
[81] H. Roberg-Larsen, M.F. Strand, A. Grimsmo, P.A. Olsen, J.L. Dembinski, F. Rise, E. Lundanes, T. Greibrokk, S. Krauss, S.R. Wilson, High sensitivity measurements of active oxysterols with automated filtration/filter backflush-solid phase extraction-liquid chromatography–mass spectrometry, J. Chromatogr. A 1255 (2012) 291–297.
[82] R.B. Corcoran, M.P. Scott, Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 8408–8413.
[83] J. Wang, J. Lu, M.C. Bond, M. Chen, X.-R. Ren, H.K. Lyerly, L.S. Barak, W. Chen, Identification of select glucocorticoids as Smoothened agonists: potential utility for regenerative medicine, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 9323–9328.
[84] Y. Wang, A.C. Arvanites, L. Davidow, J. Blanchard, K. Lam, J.W. Yoo, S. Coy, L.L. Rubin, A.P. McMahon, Selective identification of hedgehog pathway antagonists by direct analysis of smoothened ciliary translocation, ACS Chem. Biol. 7 (2012) 1040–1048.
[85] J. Kim, B.T. Aftab, J.Y. Tang, D. Kim, A.H. Lee, M. Rezaee, J. Kim, B. Chen, E.M. King, A. Borodovsky, Itraconazole and arsenic trioxide inhibit hedgehog pathway activation and tumor growth associated with acquired resistance to smoothened antagonists, Cancer Cell 23 (2013) 23–24.
[86] J. Kim, J.Y. Tang, R. Gong, J. Kim, J.J. Lee, K.V. Clemons, C.R. Chong, K.S. Chang, M. Fereshteh, D. Gardner, Itraconazole, a commonly used antifungal that inhibits Hedgehog pathway activity and cancer growth, Cancer Cell 17 (2010) 388–399.
[87] A.J. Brown, W. Jessup, Oxysterols: sources, cellular storage and metabolism, and new insights into their roles in cholesterol homeostasis, Mol. Aspects Med. 30 (2009) 111–122.
[88] J.G. McDonald, D.D. Smith, A.R. Stiles, D.W. Russell, A comprehensive method for extraction and quantitative analysis of sterols and secosteroids from human plasma, J. Lipid Res. 53 (2012) 1399–1409.
[89] Y.Y. Lin, M. Welch, S. Lieberman, The detection of 20 (S)-hydroxycholesterol in extracts of rat brains and human placenta by a gas chromatograph/mass spectrometry technique, J. Steroid Biochem. Mol. Biol. 85 (2003) 57–61.
[90] J.E. Pie, K. Spahis, C. Seillan, Cholesterol oxidation in meat products during cooking and frozen storage, J. Agric. Food Chem. 39 (1991) 250–254.
[91] I. Björkhem, Five decades with oxysterols, Biochimie 95 (2013) 448–454.
[92] S. Dzeletovic, O. Breuer, E. Lund, U. Diczfalusy, Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry, Anal. Biochem. 225 (1995) 73–80.
[93] W.J. Griffiths, P.J. Crick, Y. Wang, Methods for oxysterol analysis: past, present and future, Biochem. Pharmacol. (2013).
[94] K. Karu, M. Hornshaw, G. Woffendin, K. Bodin, M. Hamberg, G. Alvelius, J. Sjövall, J. Turton, Y. Wang, W.J. Griffiths, Liquid chromatography–mass spectrometry utilizing multi-stage fragmentation for the identification of oxysterols, J. Lipid Res. 48 (2007) 976–987.
[95] K. Raith, C. Brenner, H. Farwanah, G. Müller, K. Eder, R.H. Neubert, A new LC/APCI–MS method for the determination of cholesterol oxidation products in food, J. Chromatogr. A 1067 (2005) 207–211.
[96] E. Lundanes, L. Reubsaet, T. Greibrokk, Chromatography: Basic Principles, Sample Preparations and Related Methods, John Wiley & Sons, 2013.
[97] W.J. Griffiths, M. Hornshaw, G. Woffendin, S.F. Baker, A. Lockhart, S. Heidelberger, M. Gustafsson, J. Sjovall, Y. Wang, Discovering oxysterols in plasma: a window on the metabolome, J. Proteome Res. 7 (2008) 3602–3612.
[98] A. Honda, K. Yamashita, T. Hara, T. Ikegami, T. Miyazaki, M. Shirai, G. Xu, M. Numazawa, Y. Matsuzaki, Highly sensitive quantification of key regulatory oxysterols in biological samples by LC–ESI–MS/MS, J. Lipid Res. 50 (2009) 350–357.
[99] K. Karu, J. Turton, Y. Wang, W.J. Griffiths, Nano-liquid chromatography–tandem mass spectrometry analysis of oxysterols in brain: monitoring of cholesterol autoxidation, Chem. Phys. Lipids 164 (2011) 411–424.
[100] J. Clark, K.E. Anderson, V. Juvin, T.S. Smith, F. Karpe, M.J. Wakelam, L.R. Stephens, P.T. Hawkins, Quantification of PtdInsP3 molecular species in cells and tissues by mass spectrometry, Nat. Methods 8 (2011) 267–272.
[101] J. Adamec, A. Jannasch, J. Huang, E. Hohman, J.C. Fleet, M. Peacock, M.G. Ferruzzi, B. Martin, C.M. Weaver, Development and optimization of an LC–MS/MS-based method for simultaneous quantification of vitamin D2, vitamin D3, 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3, J. Sep. Sci. 34 (2011) 11–20.
[102] J.G. Van Der Gugten, J. Dubland, H.-F. Liu, A. Wang, C. Joseph, D.T. Holmes, Determination of serum aldosterone by liquid chromatography and tandem mass spectrometry: a liquid–liquid extraction method for the ABSCIEX API-5000 mass spectrometry system, J. Clin. Pathol. 65 (2012) 457–462.
[103] Y. Nakamura, J. Rege, F. Satoh, R. Morimoto, M.R. Kennedy, C.N. Ahlem, S. Honma, H. Sasano, W.E. Rainey, Liquid chromatography–tandem mass spectrometry analysis of human adrenal vein corticosteroids before and after adrenocorticotropic hormone stimulation, Clin. Endocrinol. 76 (2012) 778–784.
[104] D.F. Cobice, C.L. Mackay, R.J. Goodwin, A. McBride, P.R. Langridge-Smith, S.P. Webster, B.R. Walker, R. Andrew, Mass spectrometry imaging for dissecting steroid intracrinology within target tissues, Anal. Chem. 85 (2013) 11576–11584.
[105] L. Iuliano, Pathways of cholesterol oxidation via non-enzymatic mechanisms, Chem. Phys. Lipids 164 (2011) 457–468.