Drug effects on melanoma

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Chapter 4

Chemoprevention of melanoma

Chemopreventive drugs and their pharmacological mechanism of action, efficacy, safety and tolerability

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Submitted

Abstract

Background: In most countries, despite sun protection measures, the burden of melanoma is increasing. Therefore, melanoma chemoprevention may be a promising approach for high risk target populations. However, it is unclear which candidate drugs for chemoprevention of cutaneous melanoma have the potential to be useful and safe. Our aim was to systematically search the literature to identify candidate drugs for melanoma chemoprevention and to critically review their possible mechanism(s) of action, the existing evidence for their chemopreventive efficacy, as well as their safety and tolerability.

Methods: We conducted a systematic literature search in Medline, Embase, Web of Science and The Cochrane Library. Subsequently, we conducted a qualitative review on the potential chemopreventive drugs for which human data from clinical trials or observational research were available.

Results: Considerable evidence exists to suggest that melanoma development may be prevented or delayed by aspirin, NSAIDs and statins. Less evidence is available for other potential chemopreventive drugs, such as fibrates, retinoids, imiquimod, dehydroepiandrosterone, and acetaminophen. Long-term safety data in suitable chemopreventive dosages are not available for most these candidate drugs.

Conclusion: Although considerable preclinical evidence is available for aspirin, NSAIDs, and statins, in our opinion, there are still not sufficient (clinical) efficacy data and long-term safety data in chemopreventive dosages to perform a formal risk-benefit ratio and justify melanoma chemoprevention to move forward to current practice.

Abbreviations

ACTH	adrenocorticotropin
AJCC	American Joint Committee on Cancer
AK	actinic keratoses
APL	acute promyelogenous leukemia
APPROVe	Adenomatous Polyp Prevention on Vioxx
BCC	basal cell carcinoma
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitors
CI	confidence interval
CK	creatinine kinase
CNS	central nervous system
COX	cyclooxygenase
DAIS	Diabetes Atherosclerosis Intervention Study
DHEA	dehydroepiandrosterone
EMEA	European Medicines Agency
ERK	extra cellular signal-regulated kinase
FAMMM	Familial atypical multiple mole-melanoma
FDA	Food and Drug Administration
FFP	farnesyl pyrophosphate
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
FTI	farnesyl transferase inhibitors
GFR	glomerular filtration rate
GGP	geranylgeranyl pyrophosphate
GGTI	geranyl geranyl transferase inhibitors
GI	gastrointestinal
GPRD	General Practitioners' Research Database
GSH	glutathione
G-6-PD	glucose-6-Phosphate Dehydrogenase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme-A
HR	hazard ratio
IFN	interferon
IL	interleukin
LFA1	lymphocyte function-associated antigen 1
LM	lentigo maligna
LMM	lentigo maligna melanoma

LSR	local skin reactions
MC1R	melanocortin-1 receptor
MEK	mitogen-activated protein kinase
NAC	N-acetylcysteine
NF-κB	nuclear factor-κB
NMSC	nonmelanoma skin cancer
NSAID	non-steroidal anti-inflammatory drug
OR	odds ratio
OTC	over the counter
PPAR	peroxisome proliferator-activated receptor
RA	retinoid acid
RAR	retinoic acid receptor
RCT	randomized clinical trial
ROS	reactive oxygen species
RR	relative risk
RXR	retinoid X receptor
SCC	squamous cell carcinoma
SCID	severe combined immunodeficient mice
SIR	standardized incidence rate
Th1	T helper cell type 1
TLR	toll-like receptor
TNF	tumor necrosis factor
TXA_2	thromboxane A_2
VIN	vaginal intraepithelial neoplasia
VITAL	Vitamins and Lifestyle
WHO	World Health Organization

Introduction

Melanoma incidence is rising steadily in most European countries as well as in Australia and in the US. [1] Although melanoma of the skin is usually diagnosed while confined to the local site / skin (AJCC stage I or II) and melanoma mortality rates seem to be stabilizing or even slightly decreasing in countries with high melanoma incidence rates [2], safe and effective treatment options for advanced stages of melanoma are still lacking making the prognosis for patients with advanced melanoma (AJCC stage III or IV) poor. [3] Thus, the burden of cutaneous melanoma is increasing. [4] Consequently, melanoma

prevention has high potential benefit and is increasingly the focus in melanoma research. Cancer prevention can be categorized into: 1) primary prevention of the initial cancer; 2) secondary prevention of invasive cancer in patients with premalignant conditions; and 3) tertiary prevention of second primary cancers. [5] As preventive measures for melanoma several strategies, mostly sun protection measures, have been suggested. However, even in countries where comprehensive sun protection programs have been in place for more than a decade and the use of sun screen is widely promoted, the incidence of melanoma is still rising. [6] Therefore, alternative approaches should also be considered and one of these alternatives could be chemoprevention.

Several definitions for the term 'chemoprevention' have been proposed. The term was first used in 1976 by Sporn and colleagues. They defined 'chemoprevention' as 'the use of natural or synthetic drugs to reverse, suppress, or prevent premalignant molecular or histological lesions from progressing to invasive cancer'. This also includes preventing in situ lesions to progress to invasive melanoma. [7]

Over the last decades, chemoprevention of cancer in general has gained interest and has resulted in a few first successes, such as tamoxifen in breast cancer, the first Food and Drug Administration (FDA)-approved chemopreventive drug, celecoxib for familial adenomatous polyposis and diclofenac and imiquimod for actinic keratosis. [8] Despite this 'proof of principle', adverse results appeared in chemoprevention trials hampering progress in cancer chemoprevention. For example, beta carotene has been associated with an increase rather than a reduction of the incidence of lung cancers [9], oral alfa-tocopherol supplementation resulted in an excess second primary head and neck cancers [10], and rofecoxib (Vioxx®, Merck) was withdrawn from the market after thrombotic cardiovascular events were observed in the APPROVe (Adenomatous Polyp Prevention on Vioxx) trial. [11] Indeed, these examples highlight the need for sound preliminary evidence of chemopreventive efficacy and also for a critical review of safety issues and the assessment of the overall risk-benefit ratio.

Specifically, chemoprevention of melanoma has gained interest in the recent years. Several epidemiological studies and clinical trials from different clinical settings may provide evidence for the chemopreventive efficacy of cutaneous melanoma. Associations between drug use and melanoma incidence from observational studies may help to test the hypotheses on chemopreventive activity. Clinical trials that may be of interest include: 1) cancer chemoprevention trials among healthy high risk individuals, 2) clinical trials in the non-oncology setting if incident cancers including melanomas were recorded as a secondary end point, 3) surrogate marker trials and 4) adjuvant melanoma trials. [8] Due to this broad range of sources of evidence, we believe the form of a true systematic review in this particular field would be restrictive and even inappropriate.

The aim of this qualitative review was to systematically search the literature to identify candidate drugs for chemoprevention of cutaneous melanoma, to critically review their possible mechanisms of action and to summarize the existing evidence for their chemopreventive efficacy, as well as safety and tolerability.

Methods

We define chemoprevention of melanoma as the use of natural or synthetic drugs to prevent, reverse, suppress or delay premalignant lesions from progressing into invasive cutaneous melanoma. This includes preventing in situ lesions from progressing to invasive melanoma.

Literature search

We searched Medline, Embase, Web of Science and The Cochrane Library (January 1st 1991 until April 12th 2008) using the search terms 'melanoma', 'chemoprevention', 'melanoma/prevention and control', 'chemoprophylaxis', 'chemicals and drugs category' and 'drug'. The complete search strings can be issued on request. Only manuscripts in English were included.

We selected scientific papers on drugs aimed for chemoprevention of cutaneous melanoma. Papers were excluded if they did not include cutaneous melanoma, did not meet the definition of chemoprevention, if there was no drug intervention (e.g., a non-pharmacological intervention) or if it was a non-scientific publication type.

Papers identified through cross referencing were as yet included if the studies concerned clinical trials or epidemiological research (meta-analyses, cohort studies or case control studies) generating evidence for chemopreventive activity in humans.

Drugs

We restricted our review to drugs for which human data were available from (randomized) clinical trials (RCT) or observational research, (i.e., meta-analyses, cohort studies or case-control studies).

Results

Search results

Our initial literature search resulted in 1158 references from Medline, Embase, Web of Science and The Cochrane Library (Fig. 1). In total, 1112 of these references were excluded; 619 because they focused on a non-pharmacological intervention (such as sun protection measures, vaccines or counseling), 152 because they did not include cutaneous melanoma, 300 because they did not meet the definition of chemoprevention, 32 because they were of one the following publication types: editorial, case report, letter or commentary, 4 because they were not published in English and 5 because no studies with human data were available on this (group of) drug(s). Additionally, 131 papers were identified through cross referencing, were as yet included.

General remarks

Potential Chemopreventive Drug Classes

The potential chemopreventive drugs that resulted from our systematic literature search were: non-steroidal anti-inflammatory drugs (NSAIDs, including selective cyclooxygenase-2-inhibitors and aspirin), statins, fibrates, retinoids, imiquimod, dehydroepiandrosterone (DHEA), acetaminophen, apomine, capsaicin, urokinase receptor antagonists, N-acetylcysteine, farnesyl transferase inhibitors (FTIs), and geranyl geranyl transferase inhibitors (GGTIs).

For apomine, capsaicin, urokinase receptor antagonists, N-acetylcysteine, FTIs, GGTIs, we did not find any human efficacy data on melanoma chemoprevention from observational research or clinical trials. Consequently, this review focused on NSAIDs, statins, fibrates, retinoids, imiquimod, DHEA, and acetaminophen.

Prerequisites

Prerequisites and requirements for research in melanoma chemoprevention and for a valid melanoma strategy have been defined earlier by Demierre, Nathanson, Merlino and Sondak (Table 1). [8;12-14]

From the clinical viewpoint, it requires:

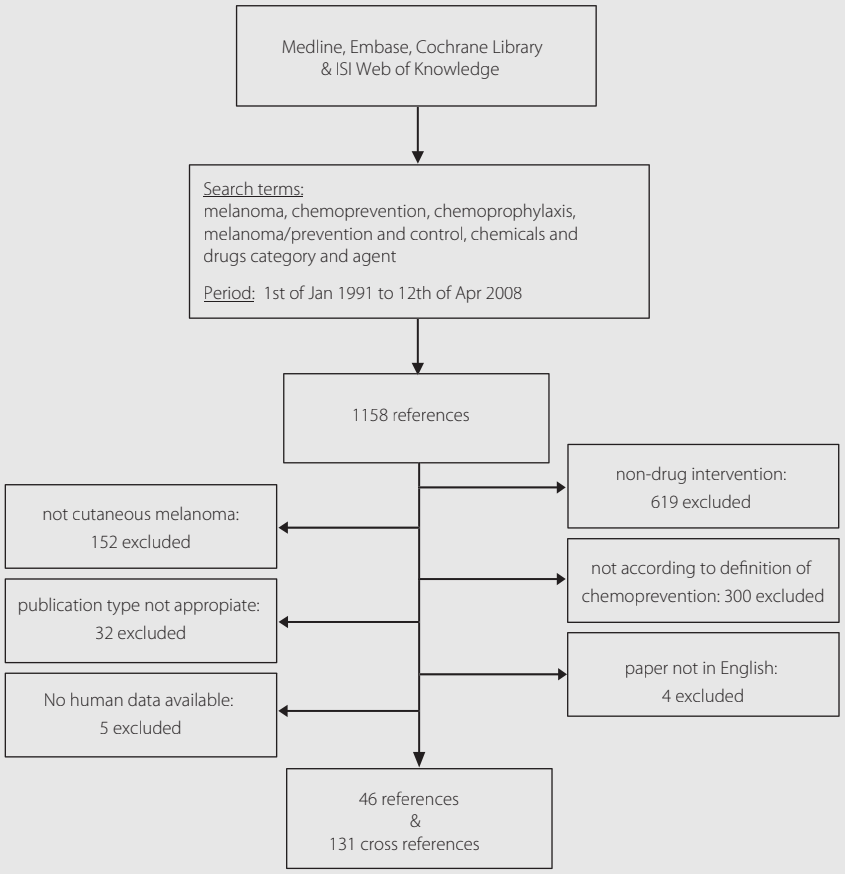
(1) chemopreventive drug efficacy;

(2) acceptable safety & tolerability;

(3) effectiveness in clinical practice, and

(4) a large potential benefit for the chemoprevention target population.

Figure 1



Total exluded:	1112	(100%)
- non-pharmacological intervention	619	(55.7%)
- not cutaneous melanoma	152	(13.7%)
- definition of chemoprevention	300	(27.0%)
- publication type	32	(2.9%)
- paper not in English	4	(0.4%)
- no human data available	5	(0.4%)

Table 1 Prerequisites for progress in cancer chemoprevention research

Prerequisite	Requirements
Elements of a strong scientific rationale	(i) Determination of the underlying molecular mechanisms of carcinogenesis (ii) Discovery of genetic markers that identify the early events in the carcinogenic process (iii) Availability of drugs that can target the molecular mechanism of carcinogenesis
Long-term safety of candidate drugs	(i) Availability of long-term human safety data (ii) Availability of animal tumor models that permit preclinical trials of evaluation of drug toxicity
Critical elements of a rigorous chemoprevention clinical trial design	(i) Availability of animal tumor models that permit preclinical trials of evaluation of drug efficacy (ii) Compilation of data from epidemiologic, basic science, and cancer research literature that can yield candidate prevention drugs for in-vitro or in-vivo testing (iii) Availability of molecular or histologic markers of the carcinogenic process to be used as endpoints and to obviate the need for prolonged and costly trials (iv) Access to defined groups at very high risk for the disease

From: Demierre MF. What about chemoprevention for melanoma? Curr Opin Oncol 2006 Mar;18(2):180-4.

Ad 1. Obviously, a strong scientific rationale and proven efficacy of the chemopreventive drug is required. As Demierre and Nathason described earlier [8], efficacy should be demonstrated in *in vitro* research, validated animal models, such as transgenic murine models. Additionally, efficacy must be observed in humans at (high) risk of a (second) invasive melanoma. Human efficacy data should include well designed phase I and II chemoprevention studies, and finally full-scale phase III trials. [15-17] These phase III trials should be designed to include endpoints to evaluate both expected and unexpected adverse events to allow full evaluation of the risk-benefit ratio.

Ad 2. In melanoma chemoprevention, healthy individuals at high risk of developing melanoma are the target population. Thus, there is no direct therapeutic effect. Moreover, chemopreventive drugs are frequently given for at least 5 years during which adherence to the drug regimen must be maintained. Little-to-no toxicity is, therefore, an absolute prerequisite to ensure both long-term safety and compliance.

A well-established safety profile may exist for drugs already marketed for alternative indications. However, higher drug dosages and longer treatment durations may be required for (melanoma) chemoprevention. Moreover, the distribution of risk factors for potential adverse events may differ between the target populations of these indications. Thus, a drug that appears to be safe for one indication may not be considered sufficiently safe for the use in cancer chemoprevention. Ideally, a chemopreventive drug would have additional major health benefits on high-prevalent diseases or health outcomes.

Ad 3. Efficacious drugs may not be effective in clinical practice. A possible explanation is lack of adherence to the drug regimen. Important prerequisites for adherence are likely to be little-to-no toxicity of the drug and a sufficiently motivated target population

Ad 4. It should be clear-cut for which patients the chemopreventive drug would be indicated. Because the absolute risk of getting a melanoma is small, chemoprevention should be targeted at patients at high risk of developing an invasive melanoma. To define the high risk populations that would benefit from chemoprevention, validated prediction models are warranted.

Target population

Well-established risk factors for melanoma are history of sun burns, older age, clinical atypical nevi, prior melanoma, family history of melanoma (FAMMM) or mutational status (CDKN2A/p16^{INK4A} mutations, CDK4 mutations, MC1R variants), and phenotypic traits, such as fair skin type, freckles, light eye color and photosensitivity. Among these, the validated and strongest predictors of melanoma incidence are likely to be suitable for the selection of a chemoprevention target population.

Possible high risk populations to target could be patients with prior melanoma, individuals with a family history of melanoma and clinical atypical nevi, individuals with multiple clinical atypical nevi and/or patients with atypical mole syndrome. [18-21] Future advances in research on validated prediction models and biomarkers, will hopefully increase possibilities for more specific definitions of high risk groups on whom melanoma chemoprevention should target.

Non-steroidal Anti-inflammatory Drugs

NSAIDs are traditionally prescribed because of their analgesic, antipyretic and anti-inflammatory effects. NSAIDs inhibit the cyclooxygenase (COX) enzyme reversibly leading to reduced synthesis of prostaglandins and thromboxane.

Based upon their pharmacological effects, NSAIDs can be subdivided in three groups.

First, traditional NSAIDs, e.g. diclofenac, naproxen, sulindac, indomethacin, and piroxicam, reversibly inhibit both the constitutively expressed COX-1 and the inducible COX-2 isoform of the enzyme (i.e, nonselective COX-inhibitors). Secondly, the selective COX-2-inhibitors, e.g. celecoxib, etoricoxib, and rofecoxib, in regular doses, inhibit only the COX-2-isoform. Aspirin forms the third group because it irreversibly inactivates COX-1 by acetylating a serine residue in its active site and, therefore, reduces thromboxane A_2 (TXA_2) in platelets. Due to the fact that platelets cannot synthesize new enzyme, TXA_2 synthesis does not recover until new platelets arise after 7-10 days.

Mechanism of action

Overexpression of COX, especially COX-2, has been demonstrated in human cancer cells of several tumor types. Based upon these observations, the COX-pathway is hypothesized to be involved in carcinogenesis. Indeed, the *ras* oncogene stimulates and *p53*, a tumor suppressor, down-regulates COX-2 expression. Moreover, COX-2 expression also seems to enhance metastatic potential of colon cancer cells and may be involved in resistance to chemotherapeutic drugs. [22] Thus, the primary potential mechanism of action of NSAIDs in cancer chemoprevention is considered to be COX inhibition (Table 2). [23]

Increased COX-2 expression has been noted in the majority, but not all, melanoma cell lines. [24-26] Denkert *et al.* showed that five melanoma cell lines (A375, MeWo, SK-Mel-13, SK-Mel-28, and IGR-37) and 26 out of 28 (93%) patient derived primary melanomas showed COX-2 expression, whereas benign nevi (n=4) and epithelial cells were negative. After introduction of a COX-2 blocking agent, NS-398, cell line growth and invasive potential were inhibited. [24] Similarly, in a series of 101 ex vivo melanoma, 96 (95%) showed COX-2 expression. More importantly, in this study, the level of COX-2 expression was also negatively associated with disease-specific survival ($p = 0.046$). [25] Increasing evidence suggests that NSAIDs inhibit tumor growth and invasion [24;27;28] and can induce apoptosis [28;29]. Roh and colleagues demonstrated an inhibitory effect of both celecoxib and indomethacin on melanoma cell growth in a murine B16F10 melanoma model. [30] Also, in a study of human A-375 melanoma cells, incubations for 72-hour of 50 and 100 μM of celecoxib showed reduced proliferation. Additionally, in a Toxilight TU-cytotoxicity assay, 100 μM celecoxib was toxic to the cancer cells. In this experiment, indomethacin (240 and 480 μM) also inhibited cell proliferation, but was only slightly toxic. Neither aspirin nor piroxicam exhibited cytostatic or cytotoxic effects. Thus, of the tested NSAIDs (aspirin, indomethacin, piroxicam and celecoxib), only celecoxib and indomethacin reduced proliferation. Because these NSAIDs all inhibit COX-2 in these concentrations, the authors suggested

Table 2 Chemopreventive drugs, their potential mechanism of action, side effects and safety profile

Drug	Chemopreventive Mechanism(s)	In vitro effects	Side Effects	Health benefits	Improvement of risk-benefit ratio
NSAIDs[1]	*COX dependent*: • inhibited COX-2 expression • inhibition of PG synthesis *COX independent*: • LOX-metabolism • apoptotic genes • activation of caspases • p38 MAP kinase activation • mitochondrial cytochrome c • ceramide pathway activation	▪ inhibition of tumor growth ▪ apoptosis ▪ inhibition of invasiveness	▪ duodenal/gastric ulcers ▪ GI bleeding ▪ decreased renal function ▪ cardiovascular events ▪ cerebrovascular events	▪ no general extra health benefits	▪ *H. pylori* eradication and/or adding PPI to prevent ulcers ▪ exclude patients with decreased renal function / users of ACE inhibitors ▪ exclude patients with cardiovascular risk factors
Aspirin	• see NSAIDs *Additional COX independent*: • thrombocyte-aggregation • NF-κB • DNA-repair • oxidative stress • mitochondrial Ca^{2+}-uptake	▪ inhibition of tumor growth ▪ apoptosis ▪ inhibition of invasiveness	*Low-dose*: ▪ GI bleeding ▪ cerebrovascular bleeding *High dose*: ▪ duodenal/gastric ulcers ▪ GI bleeding ▪ decreased renal function	▪ prevents thrombotic cardiovasular and cerebrovascular events	▪ *H. pylori* eradication and/or adding PPI to prevent ulcers ▪ High dose aspirin: exclusion of patients with decreased renal function / users of ACE inhibitors
Statins	*Inhibition HMG-CoA reductase*: Prevent prenylation of: • RhoA, RhoC and Ras, and other prenylation-dependent proteins *Cholesterol independent*: • binding to LFA1 • inhibition of the proteasome • increased fibrinolytic activity	▪ inhibition tumor growth by cell cycle arrest and apoptosis ▪ reduced invasiveness by inhibiting migrating factors & reducing adhesion molecules ▪ effects on angiogenesis ▪ attenuation of resistance mechanisms	▪ myopathy ▪ elevated CK levels ▪ rhabdomyolysis ▪ anorexia ▪ nausea ▪ diarrhea ▪ fatigue ▪ ulcerative lesions	▪ prevents cardiovascu-lar events ▪ potential positive effects in osteoporosis and Alzheimer's disease	▪ High dosages: contraindicated in presence of relative renal dysfunction (CLcr < 60-70 ml/min) ▪ adding ubiquinone to prevent statin-induced myopathy ▪ prevent concomitant drug use with gemfibrozil, CYP3A4 or CYP2C9 inhibitors[2]

Fibrates	▪ PPAR-α or PPAR-γ agonism ▪ direct toxic effect of low cholesterol on malignant cells	▪ inhibition of tumor growth ▪ apoptosis ▪ antimetastatic effects	▪ abdominal pain/dyspepsia ▪ increased creatinine/urea ▪ myopathy ▪ elevated CK levels ▪ rhabdomyolysis ▪ increased homocysteine ▪ cholelithiasis ▪ (venous thrombosis) ▪ (pulmonary emboli)	▪ prevents cardiovascu-lar events ▪ potentially reduces proteinuria in diabetes patients	▪ adjusted fibrate dosing or contraindication if renal function is decreased (CLcr < 50 ml/min); does not apply for gemfibrozil
Retinoids	▪ RXR or RAR-α, β, or γ binding leading to altered gene transcription *RAR & RXR independent:* ▪ inhibition of mitogen-induced c-fos expression ▪ Rac-dependent ROS increase ▪ increased expression of p16, p21, p27, p53, and bax ▪ MAPK, Bcl-2 down-regulated	▪ inhibition of tumor growth ▪ apoptosis ▪ proangiogeneic effects ▪ antimetastatic effects	*Topical treatment*: ▪ Skin irritation *Oral treatment*: ▪ teratogenicity ▪ bone toxicity ▪ hepatotoxicity ▪ serum lipid abnormalities ▪ cheilitis, xerosis ▪ ocular effects ▪ hair loss	▪ no general extra health benefits	▪ combining topical retinoid with topical hydrocortisone to control skin irritation ▪ contraindicated during pregnancy or lactation ▪ avoid use among women of child bearing age ▪ use contraceptive measures required ▪ pregnancy test prior to start of therapy
Imiquimod	▪ TRL7 stimulation induces a Th1 immune response which results in transformation of naïve T cells ito antigen-specific T cells directed against antigens expressed on potentially immunogenic skin tumors	▪ inhibition of tumor growth ▪ apoptosis	▪ skin irritation ▪ sun sensitivity ▪ allergy ▪ headache ▪ muscle weakness ▪ fever & flu-like symptoms ▪ fungal infection	▪ no general extra health benefits	
Acetaminophen	▪ GSH depletion leading to ROS formation and mitochondrial toxicity ▪ may act as tyrosinase substrate	▪ inhibition of tumor growth ▪ cytotoxic effects in high doses (?)	▪ urticarial rash ▪ allergic reactions ▪ renal failure (chronic use) *Very high doses*: ▪ nausea, vomiting ▪ hyperglycaemia ▪ liver failure	▪ no general extra health benefits	▪ high doses: NAC infusion ▪ exclude patients with G6PD deficiency

NSAID = non steroidal antiinflammatory drug, COX = cyclooxygenase, PG = prostaglandin, LOX = lipoxygenase, MAP = mitogen-activated protein, PPAR = peroxisome proliferator-activated receptor, RAR = retinoic acid receptor, RXR = retinoid X receptor, ROS = reactive oxygen species, TRL = Toll-like receptor, GI = gastrointestinal, *H. pylori* = Helicobacter pylori, PPI = proton pump inhibitor, ACE = Angiotensin Converting Enzyme, CK = creatinine kinase, CLcr = creatinine clearance, NAC = N-acetylcysteine.
[1] Both traditional NSAIDs and COX-2-inhibitors. Note: cardiovascular events are more prevalent among users of selective COX-2-inhibitors and duodenal/gastric ulcers & GI bleedings are less prevalent. [2] For atorstatin, lovastatin, cerivastatin or simvastatin, concomitant use of CYP3A4 inhibitors (e.g., grape fruit juice, itraconazole, ketoconazole, nelfinavir, indinavir, ritonavir, erythromycin, verapamil) should be avoided. For fluvastatin, concomitant use of CYP2C9 inhibitors (e.g., fluconazole, amiodarone) should be avoided. Increased risk of myopathy and rhabdomyolysis if a statin is combined with gemfibrozil.

that the growth inhibitory effect of celecoxib cannot be explained solely by its COX-inhibitory activity. [27]

Additional COX-independent pathways have also been suggested in other cancer types. [31;32] Numerous possible targets, such as lipoxygenase metabolism (ALOX15) [33], the proapoptopic gene *PAWR* [34], the anti-apoptopic gene *BCL2L1* [35], activation of caspases {36], the activation of p38 MAP kinase [37], release of mitochondrial cytochrome c [38], and activation of the ceramide pathway [39], have been suggested to be involved. These COX-independent pathways, however, need further study. For example, some investigators have suggested that only higher aspirin doses lead to these COX-independent molecular mechanisms. [40] Moreover, aspirin may have additional anticancer pathways as compared to other NSAIDs, such as inhibition of thrombocyte-aggregation [41], NF-κB, DNA-repair systems, apoptosis, oxidative stress or mitochondrial calcium uptake [31].

Evidence for efficacy in humans

Although some studies were promising, conflicting results exist on NSAIDs in melanoma prevention (Table 3). Initially, Harris *et al.* reported a small case control study (110 cases, 609 controls, all females) in which regular NSAID use showed a significantly decreased relative risk (RR) of melanoma (RR = 0.45 with a 95% confidence interval (CI) of 0.22 to 0.95). With increasing NSAID use, melanoma risk further decreased (*p*-linear trend <0.05). Estimates for daily use of aspirin were similar (RR = 0.55). [42]

Subsequently, in a small retrospective cohort study of 83 melanoma patients, users of NSAIDs or COX-2-inhibitors, as compared to nonusers, had a lower incidence of new melanoma, recurrence, and metastasis (combined end point; odds ratio (OR) of 0.08, 95% CI = 0.01-0.77). [43] However, we believe guarantee-time bias may have importantly influenced these results. In explanation, NSAID exposure in this study was defined as any prescription after first diagnosis of melanoma and prior to development of a new melanoma, a recurrence or metastatic lesion. Consequently, patients with longer survival are more likely to be categorized as a NSAID user due to the simple fact that their follow-up period was longer. More complex study designs and statistical analyses could have prevented such bias. [44]

In a secondary analysis of the Women's Health Study, Cook and colleagues studied low-dose aspirin (100 mg every other day) versus placebo. Among the 39,885 women included in this RCT, low-dose aspirin was not associated with melanoma risk (RR = 0.97, 95% CI = 0.70-1.36). [45] Similar results were obtained in a secondary analysis of the Cancer Prevention Study II Nutrition Cohort. Although long-term adult-strength

aspirin (≥325 mg for ≥5 years) was associated with lower overall cancer incidence in men and a non-statistically significant lower overall cancer incidence was observed in women, melanoma incidence was not reduced (current daily use, ≥5 years: RR = 1.15, 95% CI = 0.83-1.59, <5 years: RR = 0.99, 95% CI = 0.79-1.25). [46]

Recently, in the Vitamins and Lifestyle (VITAL) cohort study, Asgari *et al.* examined the association between NSAID use and melanoma risk. Among 63,809 men and women, during a 10 year follow-up period, 349 patients with incident melanomas were identified including 157 in situ melanomas. Use of any NSAID for at least 4 days per week as compared to nonuse, did not seem to reduce the melanoma hazard rate (HR; HR = 1.12, 95% CI = 0.84-1.48). Similar results were obtained for any NSAID excluding low-dose aspirin (HR = 1.03, 95% CI = 0.74-1.43), for regular- or extra-strength aspirin (HR = 1.10, 95% CI = 0.76-1.58), and for nonaspirin NSAIDs (HR = 1.22, 95% CI = 0.75-1.99). Additionally, NSAID use was not associated with tumor invasion (*p*-interaction = 0.38), tumor thickness (*p*-linear trend = 0.98), or risk of metastasis (HR = 1.09, 95% CI = 0.32-3.62). [47]

In a large population-based case control study of our group including 1,318 patients with invasive melanoma and 6,786 controls, incident melanoma was not associated with aspirin use (OR = 0.92, 95% CI = 0.76-1.12) or non-aspirin NSAID use (OR = 1.10, 95% CI = 0.97-1.24). However, continuous use of low-dose aspirin was associated with a significant reduction of melanoma risk in women (OR = 0.54, 95% CI = 0.30-0.99) but not in men (OR = 1.01, 95% CI = 0.69-1.47). A significant linear trend ($p = 0.04$) from non use, non-continuous use, to continuous use was observed in women. [48]

Recently, the Harvard Cancer Center performed a case control study among 400 melanoma patients and 600 matched community based controls. After adjusting for confounders, use of any NSAID, at least once weekly for more than 5 years as compared to use for less than 2 years, was associated with an adjusted OR of 0.55 (95% CI = 0.42-0.77). For aspirin and non-aspirin NSAIDs the odds ratios were comparable (OR = 0.51, 95% CI = 0.35-0.75 and OR = 0.64, 95% CI = 0.46-0.89, respectively). If NSAID use was defined as any use versus no use, the results were somewhat less pronounced (*personal communication*).

Specific studies on selective COX-2 inhibitors are lacking. Duke and colleagues have planned a Cochrane review 'COX-inhibitors in the prevention of melanoma'. [49] If enough eligible trials will be pursued, this review will likely provide more insight.

In summary, due to heterogeneity in study design (ascertainment and definition of exposure, type of NSAID, dose, duration, patterns of use, drug adherence, study population etc), conflicting results and the limited number of studies, the efficacy of NSAIDs and aspirin for melanoma prevention remains unclear. The results of *in vitro*

Table 3 Associations between use of potential chemopreventive drugs and incident melanomas

Drug	Design	Numbers	Dose	Duration of use	Follow up	Estimate[1]	95% CI	Primary endpoint	Ref	Remarks
NSAIDs, all	CO	N= 63,809	≥ 4 d/wk	NR	5 y [2], 1-10 y [3]	HR=1.12	0.84 - 1.48	no	1391	MM: N = 348
	CC	N= 400 MM N= 600 C	≥ 1 PPW	>5 y vs. <2 y	n.a.[4]	OR=0.73	0.55 – 0.97	yes	*	
	CC	N= 101 MM N= 609 C	≥ 1 PPD	≥ 2 y	-	OR=0.45	0.22 - 0.92	yes	260	only females
	CC	N= 101 MM N= 609 C	< 1 PPD	≥ 2 y	-	OR=0.77	0.35 - 1.70	yes	260	only females
Non-aspirin	CO	N= 63,809	≥ 4 d/wk	NR	5 y [2], 1-10 y [3]	HR=1.12	0.85 - 1.49	no	1391	MM: N = 348
	CC	N= 1,318 MM N= 6,786 C	No dose limit	≥ 1/2 y	3 y (100%)	OR=1.10	0.97 - 1.24	yes	1487	
	CC	N= 400 MM N= 600 C	≥ 1 PPW	>5 y vs. <2 y	n.a.[4]	OR=0.64	0.46 – 0.89	yes	*	
Aspirin	RCT	N= 19,942 P N= 19,934 A	100 mg qod	NR [5]	10.1 y [2]	RR=0.97	0.70 - 1.36	no	1434	only females MM: N = 138
	CO	N= 146,113	≥ 325 mg qd	max. 11 y	≥ 5 y	RR=1.15	0.83 - 1.59	no	1435	MM: N = 871
	CO	N= 146,113	≥ 325 mg qd	max. 11 y	< 5 y	RR=0.99	0.79 - 1.25	no	1435	MM: N = 871
	CO	N= 63,809	≥ 325 mg ≥ 4 d/wk	NR	5 y [2], 1-10 y [3]	HR=1.10	0.76 - 1.58	no	1391	MM: N = 348
	CC	N= 1,318 MM N= 6,786 C	≤ 100 mg qd	≥ 1/2 y	3 y (100%)	Males: OR=1.01 Females: OR=0.54	Males: 0.69 - 1.47 Females: 0.30 - 0.99	yes	1487	stratified for sex (prespecified)
	CC	N= 1,318 MM N= 6,786 C	> 100 mg qd	≥ 1/2 y	3 y (100%)	OR=1.35	0.96 - 1.92	yes	1487	

	CC	N= 400 MM N= 600 C	≥ 1 PPW	>5 y vs. <2 y	n.a.[4]	OR=0.51	0.35 – 0.75	yes	*	
	CC	N= 101 MM N= 609 C	≥ 1 PPD	≥2 y	-	OR=0.55	NR	yes	260	only females
Retinoids	CO	N= 162,000	≥1.8 vs. <0.4 mg/d	max. 8-14 y	max. 8-14 y	RR=0.39	0.22 – 0.71	no	726	MM: N = 414 only reviewers biopsies blinded
	CC	N= 542 MM N= 538 C	highest vs. lowest quartile	NR	NR	OR=0.57	0.39 – 0.83	yes	138	
Acetami-nophen	CO	N= 39,946	No dose limit	NR	4.7 y [2], 1-9 y [3]	SIR=0.9	0.6-1.2	no	1469	MM: N = 39 NSAID and aspirin use included
	CO	N= 13,482	No dose limit	NR	4.7 y [2], 1-9 y [3]	SIR=0.6	0.2-1.3	no	1469	MM: N = 7 NSAID and aspirin users excluded
	CC	N= 101 MM N= 609 C	≥ 1 PPD	≥ 2 y	-	OR=0.95	0.45-1.98	no	260	only females matched on age and place of residence
Statins	RCT	N= 2,223 P N= 2,221 S	10-40 mg qd	ITT	5.4 y [2]	RR=2.34	0.60 – 9.06	no	16	4S study MM: N=7 S / 3 P
				(5.4 y [2] ITT)	10.4 y	RR=1.28	NR	no	1470	Follow up 4S study MM: N=9 S / 7 P
	RCT	N= 10,267 P N= 10,269 S	40 mg qd	ITT	4.6 y [2]	RR=1.66	0.78 - 3.54	no	1467	HPS study MM: N=17 S / 10 P
	RCT	N= 3,301 P N= 3,304 L	20-40 mg qd	0.2-7.2 y [3]	5.2 y [2]	OR=0.52	0.27 – 0.99	no	1399	AFCAPS study MM: N=14 L / 27 P
	RCT	N= 2,078 P N= 2,081 Pr	40 mg Pr qd	NR [6]	5 y [7]	OR=1.33	0.30 – 5.96	no	35	CARE study MM: N=4 Pr / 3 P
	RCT	N= 4,502 P N= 4,512 Pr	40 mg Pr qd	ITT	6.1 y [2]	OR=1.07	0.64 – 1.79	no	16	LIPID study MM: N=30 Pr / 28 P

Table 3 Continued

Drug	Design	Numbers	Dose	Duration of use	Follow up	Estimate[1]	95% CI	Primary endpoint	Ref	Remarks
Statins	RCT	N= 3,293 P N= 3,302 Pr	40 mg qd	ITT	4.9 y [2]	OR=0.66	0.19 – 2.36	no	16	WOSCOP study males only MM: N=4 Pr / 6 P
	RCT	N= 1,049 P N= 1,045 F	40 mg qd [8]	ITT	5.1 y [2]	RR=0.40	NR	no	1468	ALERT trial MM: N=2 F / 5 P
	CC	N= 79 MM N= ~ 395 C	No dose limit	current use	6.4 y [7]	RR=2.5	0.8 - 7.3	yes	1400	GPRD database
	CC	N= 1,318 MM N= 6,786 C	No dose limit	≥ 1/2 y	3 y (100%)	OR=0.98	0.78 - 1.2	yes	1003	
Fibrates	RCT	N= 1,542 P N= 1,548 B	400 mg qd	ITT	6.2 y [2]	OR=0.33	0.07 – 1.64	no	16	BIP study MM: N=2 B / 6 P
	RCT	N= 785 P N= 783 B	400 mg qd [9]	ITT	4.6 y [7]	OR=1.00	0.06 – 16.1	no	16	LEADER study males only MM: N=1 G / 1 P
	RCT	N= 2,030 P N= 2,051 G	600 mg bid	ITT	5 y [2]	OR=2.97	0.12 – 73.0	no	1461	HHS study males only MM: N=1 G / 0 P
	RCT	N= 1,267 P N= 1,264 G	1200 mg qd	ITT	5.1 y [7]	OR=0.11	0.01 – 0.87	no	1403	VA-HIT study males only MM: N=1 G / 9 P
	RCT	N= 2,789 P N= 1,103 C	1.8 g qd	NR	6.2 y [2]	OR=1.69	0.28 – 10.1	no	16	CDP study MM: N=2 C / 3 P

RCT=Randomized Controlled Trial, CO=Cohort study, CC= Case-Control study, N=number, MM=melanoma cases, C=controls, P=placebo, A=aspirin, S=simvastatin, L=lovastatin, Pr=pravastatin, B=bezafibrate, C=ciprofibrate, G=gemfibrozil, d/wk=days per week, mg=milligram(s), g= gram(s), PPD=pills per day, PPW=pills per week, mg/d = milligram per day, qd= once a day, qod=every other day, bid=twice a day, y=year(s), ITT=intention to treat, RR=relative risk, OR=Odds Ratio, HR=Hazard Ratio, SIR=Standarized Incidence Ratio, CI=confidence interval, NR=not reported, Ref=reference (see referencelist).

* Personal communication on a case control study among 400 melanoma patients and 600 matched community based controls (Harvard Cancer Center, T. Nijsten).

and animal studies, however, are promising. A pivotal unresolved problem is the definition of the temporal and dose-response cause effect relationships between NSAID use and incident invasive melanoma. Thus, additional experimental and observational research is warranted, particularly on required dosages and duration.

Safety, Tolerability & Compliance

Side effects of NSAIDs are gastrointestinal (GI) complaints, such as nausea, vomiting, dyspepsia (10-20%), diarrhea, duodenal or gastric ulcers (10-30%), sometimes even leading to GI bleedings or perforation (± 2%). [50] In addition, skin reactions, cardiovascular and cerebrovascular events, and decreases in renal function also occur. Rare, but serious, side effects are bone marrow disturbances and hepatotoxicity. The prevalence of GI related side effects differs substantially between several traditional NSAIDs, being less pronounced for aspirin and diclofenac compared to piroxicam.

COX-2-inhibitors have been developed to selectively inhibit COX-2 and thus to reduce side effects related to COX-1-inhibition, most importantly duodenal and gastric ulcers. Indeed, duodenal or gastric ulcers are less prevalent (± 2%) for this class of NSAIDs. [50] However, thrombotic cardiovascular events observed in the APPROVe trial, a chemopreventive trial in which patients with a history of colorectal adenomas were randomized to receive rofecoxib or placebo [11], have raised safety concerns regarding the risk-benefit ratio of COX-2-inhibitors in cancer chemoprevention. [51;52] Subsequent epidemiological studies have suggested that these events are also associated with traditional NSAIDs, such as ibuprofen or diclofenac. [53;54] In these studies, naproxen, as an exception, is associated with a reduced cardiovascular event rate. [53;54]

To prevent GI ulcers and bleeds, additional interventions such as *Helicobacter pylori* eradication and concomitant use of a proton pump inhibitor to the chemopreventive strategy could be considered, but this introduces new adverse effects and additional costs. Currently, in the AspECT trial a combination of aspirin plus proton pump inhibitor is studied for the chemopreventive activity on cancer among patients with Barret's esophagus. [55]

Aspirin may also cause bleeding through inhibition of thrombocyte-aggregation. Due to this feature, however, aspirin does not cause an

[1] multivariable adjusted estimates (RR, OR, HR) as reported in original publication.
[2] mean value presented.
[3] range value presented.
[4] not applicable.
[5] compliance, defined as taking ≥ 2/3 of the study drugs, was 76% at 5 years and 67% at 10 years.
[6] in the last year of follow-up, 86% of the placebo group and 94 percent of the treatment group were taking their study medication.
[7] median value presented.
[8] randomized to 40 mg fluvastatin or placebo. In both arms open label prescriptions of an additional 40 mg fluvastatin were allowed if cholesterol levels were too high.
[9] 400 mg qd or 400mg qod if creatinine 135-149 mmol/L.

excess of cardiovascular events and actually has the advantage of protection against cardiovascular disease. Moreover, aspirin may have additional chemopreventive effects as compared to other COX-inhibitors. [31;41] Nevertheless, due to the lack of definitive evidence on (differences in) efficacy, required dosages and duration, it is too early to claim aspirin as the preferential NSAID for cancer chemoprevention.

Conclusion Non-steroidal Anti-inflammatory Drugs

In vitro studies demonstrate COX-2-expression in melanoma and suggest effects of NSAIDs on growth inhibition, invasiveness and apoptosis. COX independent pathways, however, may also be involved in these anti-tumor effects. These pathways should be further investigated in order to disentangle dose-response relationships and identify the most promising NSAIDs. Although promising efficacy data were shown in other cancers, NSAIDs have yet to demonstrate sufficiently convincing evidence for efficacious melanoma chemoprevention. Convincing evidence is lacking and comparing the conflicting results of the limited number of published studies is challenging due to heterogeneity in study design and uncertainties in temporal and dose-response relationships. Moreover, concerns over the long-term safety of COX-2 inhibitors and NSAIDs have tempered the enthusiasm for their use in chemoprevention. Therefore, if sufficient data on efficacious drug dosages and temporal cause effect relationships become available, formal risk-benefit analyses should be performed on different scenarios of chemopreventive strategies.

Statins

Statins, or 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase inhibitors, are widely prescribed to reduce cholesterol levels aiming to prevent cardiovascular events. This drug class consists of atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, simvastatin, pitavastin, pravastatin, and rosuvastatin. Cerivastatin, however, has been withdrawn from the market in 2001 due to reports of rhabdomyolysis, especially with concomitant use of gemfibrozil.

Statins differ in several aspects. For example, lovastatin, simvastatin, and pravastatin were originally derived from fungi, whereas atorvastatin and fluvastatin are synthetically derived. Additionally, some statins are prodrugs, e.g. simvastatin and lovastatin, and have a closed lactone ring that is converted by carboxyesterases to the open-ring acid form that inhibits HMG-CoA reductase. [56]

Historically, an inverse association between cholesterol and the incidence of (smoking-related) cancers has been observed [57], suggesting a link between low cholesterol and cancer. In addition, lovastatin and gemfibrozil were shown to promote development

of liver cancer in rodents. [58] However, subsequent research demonstrated paradoxical results suggesting decreased cancer incidences with use of lipid-lowering drugs.

Mechanism of action

The putative mechanism of action for both the cholesterol lowering and anticancer effects of statins is considered to be inhibition of HMG-CoA reductase, an enzyme upstream in the mevalonate biosynthetic pathway. Inhibition of HMG-CoA reductase leads to reduced synthesis of mevalonate and its downstream products. Farnesylpyrophosphate (FPP), a C_{15}-moiety, is one of these downstream products and is the precursor of both geranylpyrophosphate (GPP), a C_{20}-moiety, and cholesterol. FPP and GPP are also referred to as isoprenoids. They are essential for the activation of a variety of intracellular proteins. In this process, called (iso)prenylation, farnesyl or geranylgeranyl-moieties are coupled to the protein, resulting in a farnesylated or geranylgeranylated protein. These reactions are catalyzed by farnesyltransferase and geranylgeranyl-transferase, respectively. Several proteins involved in signaling are dependent on prenylation for their activity, such as ras, rho, nuclear lamins, transducin c, rhodopsin kinase, and G proteins. Consequently, statins lead to pleiotropic effects. [59]

Several of the proteins dependent on posttranslational prenylation, either farnesylation or geranylgeranylation, such as ras, rhoA and rhoC, have been linked to cancer pathogenesis. For example, *ras* is a known oncogene and ~30% of human tumors harbor *ras* mutations resulting in aberrant ras activity which is dependent on prenylation. [59] Specifically, N-*ras* and B-*raf* mutations are observed in ~30% and ~60% of melanomas, respectively. [60] N-*ras* and B-*raf* mutations both result in activation of the so-called Ras/Raf/MEK/ERK signaling pathway. [13] Raf which is downstream of ras, however, does not require prenylation to achieve full biological activity. [61] Still, in melanomas with a B-*raf* mutation, but no *ras* mutation, possible antineoplastic effects may be mediated through for instance rhoA or rhoC. Potential chemopreventive agents that may interfere in this pathway are: statins, FTIs, GGTIs, apomine, and perillyl alcohol. [13;59]

Furthermore, the rho family is involved in signaling and regulation of cell differentiation and proliferation. [62;63] Moreover, high-throughput screens for transcriptionally regulated targets involved in metastasis have shown that rhoC overexpression is strongly associated with the metastatic potential of inoculated melanoma in mice. [64] Indeed, *in vitro* and animal melanoma studies show a potentially chemopreventive activity of statins. More specifically, anti-tumor effects exerted by statins have been shown to include: 1) inhibition of tumor growth, 2) induction of apoptosis, 3) reduce invasiveness and metastasis, and 4) effects on angiogenesis.

Ad 1. Lovastatin, mevastatin, and simvastatin, but not pravastatin, reduced tumor growth of human melanoma cell lines HT144, M14, and SK-MEL-28 *in vitro* with IC_{50} values between 0.8 and 2.1 μM. [65]

Ad 2. Jani *et al.* observed induction of apoptosis by lovastatin in murine B16F10 melanoma cells through a geranylation-specific mechanism [66]; Additionally, increased apoptosis, in a dose-dependent manner, was observed in human M14 cells after 72-h incubations (4-8 μM) of lovastatin, mevastatin, and simvastatin. [65] In human A375 melanoma cells, Shellman *et al.* also showed induced apoptosis by lovastatin. [67] Interestingly, Shellman and colleagues also performed add back experiments showing that supplementation of GPP, but not FPP, blocked the apoptotic effect of lovastatin which indicates apoptosis must involve proteins dependent on geranylgeranylation. [67]

Ad 3. Atorvastatin (1-3 μM) reduced invasiveness of A375M, CHL, SK-MEL-28 and WM 166-4 melanoma cells in an experiment performed by Collisson and colleagues. *[68]* In this experiment, atorvastatin (4 dd 10 mg/kg orally also reduced metastasis of A375M melanocytes in severe combined immunodeficient (SCID) mice. [68] Likewise, Jani *et al.* showed reduced metastasis by lovastatin and simvastatin in murine B16F10 melanoma cells. [66] Experiments reported by Glynn *et al.* also showed decreased invasiveness by lovastatin, mevastatin, and simvastatin on HT144, M14, and SK-MEL-28 cells. [65]

Ad 4. Lovastatin (2-12.5 μM) exhibited a concentration-dependent pro-angiogenic influence on A375M and G361 cells in an angiogenesis model with a co-culture of HUVEC cells (human umbilical vein endothelial cells) and human diploid fibroblasts (HDF). [69] However, in nonmelanoma cells, some studies with low-dosed statins have suggested increased angiogenesis. [59]

Some statin-mediated effects appear to be completely independent of HMG-CoA reductase and cholesterol lowering. E.g., some experiments with statins in the closed ring form, which do not inhibit HMG-CoA reductase, do show *in vitro* anticancer effects. [70] Further investigations on these cholesterol-independent pathways are needed.

Examples of the cholesterol-independent pathways that have been suggested are:

- binding to the leukocyte function antigen-1 (LFA1) which has an important role in leukocyte migration and T-cell activation. [71]
- inhibition of the proteasome [70;72;73] which could for instance account for effects on the cyclin-dependent kinase inhibitors (CDKIs) p21 and p27 [74], and increased fibrinolytic activity [75].
- altered membrane receptor function due to changes in membrane fluidity caused by cholesterol depletion. For example, melanocortin receptor (MC1R) [76] or

insulin-like growth factor receptor function [77-79], both of which are involved in melanocyte and melanoma growth.

In addition, some investigators suggest direct toxic effects of cholesterol lowering are involved. [80] Malignant cells metabolize cholesterol differently and, therefore, may be more sensitive. However, the evidence for this hypothesis is (very) limited.

Although *in vitro* and animal experiments in general show promising results, some critical issues should be mentioned. E.g., pravastatin, the only hydrophilic statin, does not exhibit clear chemopreventive effects in most experiments. Moreover, most studies have used statins at serum concentrations and dosages that exceed doses applied for the treatment of hypercholesterolemia. Lovastatin dosed at ~1 mg/kg/day, for example, yields steady-state serum concentrations of 0.15–0.3 μM. [81] Often tumor cell lines were only sensitive to lovastatin at higher concentrations, e.g. 1.0-12.5 μM. [65;67;69]

Interestingly, some agents may have synergistic chemopreventive action together with statins. For example, d-γ-tocotrienol (5 μM) together with lovastatin (1 μM) totally blocked cell growth, whereas lovastatin (12%) and d-γ-tocotrienol (8%) individually showed only limited growth inhibition in these concentrations. [82] Other agents that have been suggested in combination with statins are NSAIDs, bisphosphonates, GGTIs, phosphoinositide 3-kinase (PI3K) inhibitors, CDKI, MEK inhibitors, and tyrosine kinase inhibitors. [59]

Evidence for efficacy in humans

Originally, RCTs testing statins for cardiovascular disease were the first to report on a possible decreased cancer incidence with statin use. [56] Ironically, concerns about increased cancer incidence with low cholesterol led to inclusion of cancer as a secondary safety outcome in these trials. Since then, a large number of meta-analyses and observational studies investigating statin use and cancer incidence were performed.

Additionally, two abstracts appeared on a preliminary case control study comparing the use of statins among 74 melanoma cases and age, gender and race-matched controls. Preliminary results in this study were promising (OR = 0.55, $p = 0.11$). [83;84] However, to the best of our knowledge, the results of the final analysis have not been published.

Shortly after these reports, two large population-based studies reported decreased incidences of cancer. [85;86] Our group performed a large observational study (3129 statin users & 16976 nnon-users) in which statin use was associated with a 20%

decrease in cancer incidence (OR = 0.80, 95% CI = 0.66-0.96). The association was more pronounced with prolonged use (statin use ≥ 4 yrs, OR = 0.64, 95% CI = 0.44-0.93). [85] Subsequently, Poynter and colleagues reported, among 1953 patients with colorectal cancer and 2015 controls, a significantly reduced risk of colorectal cancer (OR = 0.50, 95% CI = 0.40-0.63) with the use of statins (≥ 5 years versus nonusers). [86] However, since then, research has shown conflicting, and generally disappointing results for statin use as a general cancer chemopreventive agents. [87-89] Moreover, some meta-analyses suggest differences in the associations between statin use and incident cases of different cancer types. [89]

Dellavalle *et al.* performed a formal Cochrane review on specifically incident melanomas as a secondary outcome of RCTs with primary cardiovascular outcomes. In this Cochrane review, 6 statin RCTs providing data on incident melanomas were included. Overall, 59 melanomas occurred among the participants randomized to statin treatment and 67 incident melanomas occurred in the placebo groups. The resulting odds ratio was 0.90 (95% CI = 0.56-1.44) indicating no statistically significant difference. However, due to the low numbers of incident melanomas, a (clinically relevant) association cannot be excluded. More importantly, three of the included RCTs studied pravastatin which may have, as *in vitro* studies have suggested, diminished chemopreventive activity. Interestingly, a subgroup analysis by type of statin showed a reduced melanoma incidence for lovastatin (OR = 0.52, 95% CI = 0.27-0.99). This analysis is, however, importantly limited by the fact that there was only one trial with lovastatin. The authors' final conclusions were "… does not exclude the possibility that these drugs (i.e., statins and fibrates) prevent melanoma …". [90]

Additional RCTs have been published since the Cochrane review. In a meta-analysis published in The Lancet, the Cholesterol Treatment Trialists' (CTT) Collaborators included 14 RCTs of statins and found no evidence for a decreased cancer incidence (RR = 1.00, 95% CI = 0.95-1.06). In a sub analysis among the trials for which melanoma incidence was available, there was also no statistically significant change in melanoma incidence (RR = 1.03, 95% CI = 0.71-1.50). [88] Another six similar meta-analyses have reported on melanoma incidence with estimates for melanoma incidence ranging from 0.84 to 1.5. [87;89;91-93] However, they mainly included the same RCTs.

Table 2 presents an overview of RCTs in cardiovascular disease comparing statins with placebo, no treatment or usual care and from which melanoma incidence was reported.

These clinical trials, however, have several disadvantages which include small numbers of incident melanomas, relatively short follow-up for melanoma incidence (ranging from 3 to 6 years) and, generally, of being a retrospective reviews of cardiovascular

trials in which the design was not adapted for the analysis for melanoma incidence. For instance, they would not be stratified for factors, we would recognize now as critical to melanoma development, such as the family history of melanoma, skin type, presence or absence of clinically atypical nevi et cetera. Therefore, retrospective analyses on these trials will always be of limited value.

The number of epidemiological studies reporting on the potential association between incident melanomas and statin use is very limited. Kaye and Jick reported a case-control study on cancer and statin use that performed in the GPRD (General Practitioners' Research Database) in the UK. In a sub analysis within this study, they observed a relative risk of 2.5 (95% CI = 0.78-7.3) among 79 incident melanoma cases between 1990 and 2002 and up to five controls matched on year of birth, sex, general practice, year of entry into the GPRD, and index date. The follow-up in this study ranged between 3 and 13.7 years with a median of 6.4 years. [94] However, the number of melanoma cases in this study was relatively small as reflected in the wide confidence interval.

In a larger case-control study, we also reported on statin use and melanoma incidence. In this study, we used data from the Dutch national pathological database and from PHARMO, a pharmacy database covering ~25% of the Netherlands. Among 1,318 melanoma cases (primary diagnosis 1991-2004) and 6,786 controls matched on gender, date of birth and geographic region, we could not validate an association between statin use (≥½ y) and melanoma incidence (OR = 0.98, 95% CI = 0.78-1.2). However, the Breslow's depth of the melanomas was reduced among statin users (–19%, 95% CI = –33% to –2.3%). In a pre-specified stratified analysis across gender, we observed that the difference was nonsignificant among women (–4.8%, 95% CI = –29.6% to 28.8%), and more pronounced in men only (–27.8%, 95% CI = –43.7% to –7.4%). The lack of an association on melanoma incidence in our study could be due to the relative short follow-up which was, by design, was 3 years for all individuals. {Koomen, 2007 1003 /id}

Noteworthy, in the PRIME study, a prospective cohort study, Gardette *et al.* recently observed a reduced cancer mortality, although statistically non-significant, among dyslipidemic men using statins as compared to untreated dyslipidemic men (OR = 0.41, 95% CI = 0.19-1.06). [96]

These observational studies, however, have the disadvantage of being non-randomized and observational for which (residual) confounding cannot be excluded. Moreover, risk factors critical to melanoma development, such as the family history of melanoma, skin type, presence or absence of clinically atypical nevi et cetera, will often not be available for adjustment in the analyses. If so, confounding may have resulted.

In summary, results of secondary analyses of cardiovascular trials and of observational research on the potential relation between statin use and incident melanomas are conflicting. Both these RCTs as well as the epidemiological studies have some important limitations such as potential residual confounding, and small numbers of incident melanomas and thus limited power. Therefore, efficacy of statins in melanoma chemoprevention can neither be validated nor excluded.

Safety, Tolerability & Compliance

In cancer chemoprevention literature, the excellent safety profile of statins in cardiovascular disease has often been pointed out. [12-14;97] Indeed, statins have relatively mild side effects in the doses used to prevent cardiovascular event. The most prominent side effects of statins are the so-called statin-related myopathy (i.e., muscle pain and weakness), elevated creatinine kinase (CK) levels and as a rare but life-threatening side effect, rhabdomyolysis. In RCTs the incidence of myopathy was 1.5-5%, whereas estimates in observational research indicated 5-10%. [98] In spite of the fact that the majority of side effects are mild, persistence to statins in the use for cardiovascular disease is poor with only ~25% of patients still compliant 5 years after starting statin therapy. [99] To ensure compliance and persistence, an excellent tolerability is needed.

In cancer chemoprevention, higher day doses may be required. In such high doses, the tolerability of statins has been proven to be limited due to dose-dependent side effects such as myopathy. In phase I /II trials for cancer treatment significant responses were only achieved with >25 mg/kg/day doses leading to dose-limiting toxicities (DLTs) including myalgia, muscle weakness, elevated CK activity, anorexia, ulcerative lesions, rhabdomyolysis, nausea, diarrhea, and fatigue. With very high statin doses, cardiomyopathy may even be a side effect. [100] In the trials mentioned, among others cycled dosing with 3-4 week intervals was introduced to prevent DLTs. [81;101] For melanoma chemoprevention, it remains uncertain which doses are required. However, since cell lines studies often indicate cytostatic rather than cytotoxic effects at achievable *in vivo* statin concentrations, continuous dosing is likely to be required. [102]

Numerous risk factors for statin-related myopathy have been described. [98] Among these risk factors is using high statin doses which, as mentioned before, may be required for chemopreventive effects. Some of the risk factors may be circumventable, such as excessive physical activity, perioperative period and concomitant use of drugs or grapefruit juice which precipitate drug interactions associated with elevated serum statin levels. For atorvastatin, lovastatin, cerivastatin or simvastatin, these are CYP3A4 inhibitors and for fluvastatin these would be CYP2C9 inhibitors. [98] Avoiding the risk

factor, temporary cessation of statin therapy or drug alternatives for the inhibitors can be options in these cases. Non-preventable risk factors, such as advanced age, female gender, (relative) renal insufficiency, hypothyroidism, alcoholism or (family) history of myopathy or CK elevation [98], should be considered as special subgroups in formal risk-benefit analyses. Some of the non-preventable risk factors might be considered contraindications for statin therapy, e.g., (relative) renal insufficiency.

The causal mechanism of statin-related myopathy is not entirely unraveled. Among the proposed mechanism is depletion of ubiquinone (also referred to as coenzyme Q10). Ubiquinone, a side-product in the mevalonate pathway, is widely used as a non-drug 'over the counter' (OTC) anti-aging agent, but studies on its long-term safety are sparse. Concomitant use of ubiquinone may, however, prove to be a good candidate to increase statins' tolerability. Indeed, Thibault and colleagues have used adding Q10 to lovastatin therapy for doses 30 mg/kg/day as a strategy to prevent statin-related myopathy and increase tolerability. From these preliminary data, this strategy seems to be promising. [81]

Further research is needed to explore the precise mechanisms involved in statin-related myopathy and, after required statin doses have been established, to determine the long-term safety of this chemopreventive strategy.

In summary, long-term safety data for low dose statins is excellent, but may be less favorable for higher doses that are likely to be required for chemoprevention of melanoma. Development of a chemopreventive strategy including risk factors for statin-related myopathy and preventive measures may ameliorate the risk-benefit ratio.

Conclusion Statins

Statins inhibit HMG-CoA reductase leading to inhibition of isoprenylation of several proteins involved in melanoma development and progression, such as ras, rhoA and rhoC, and which are dependent on this posttranslational prenylation. HMG-CoA independent pathways may, however, also be involved. Experiments have shown anti-tumor effects of statins to include: 1) inhibition of tumor growth, 2) induction of apoptosis, 3) reduce invasiveness and metastasis, and 4) effects on angiogenesis. These *in vitro* and animal experiments show promising results. However, concentrations and dosages used in these experiments often exceed doses applied for the treatment of hypercholesterolemia. Additionally, chemopreventive activity may depend on which statin is used (e.g., lovastatin > pravastatin).

Up to now, the results of secondary analyses on cardiovascular trials and observational have been conflicting. Both study types have some important limitations, such as

such as lack of power, relatively short follow-up, low doses and imperfections in study designs. Thus, the promising results observed in preclinical experiments can neither be validated nor excluded.

Although, long-term safety data for low dose statins are excellent, they may be less favorable for higher doses that are likely to be required for melanoma chemoprevention. Development of a chemopreventive strategy including risk factors for statin-related myopathy and possible preventive measures, such as adding ubiquitinone to statin therapy, may ameliorate the risk-benefit ratio. First, however, efficacy in humans should be sufficiently proven.

Further studies on the involved pathways and possible cross links with other pathways, cholesterol-independent pathways, dependence of efficacy on melanoma mutational status, required dosages, possible differential effects between statins, and the temporal and dose-response cause effect relationships are required.

Fibrates

Fibrates are used as lipid-lowering therapy to prevent cardiovascular events. This drug class consists of bezafibrate, clofibrate, ciprofibrate, etofibrate, fenofibrate, gemfibrozil, simfibrate, and ronifibrate. The hypothesized mechanism by which fibrates alter lipid metabolism is thought to be peroxisome proliferators activated receptor-α (PPAR-α) agonism [80], which stimulates the oxidation of fatty acids.

Mechanism of action

The interest in a possible association between use of fibrates and cancer has been raised by three observations. First, ecological research showed an increased cancer incidence with low cholesterol. [57] Secondly, gemfibrozil promoted the development of liver cancer in rodents. [58] Thirdly, decreased cancer incidences have been reported in RCTs testing lipid-powering drugs for cardiovascular disease. [56]

The molecular mechanisms underlying potential chemopreventive properties of fibrates are not clearly defined. Several mechanisms have been hypothesized. For example, some authors believe that direct toxic effects of cholesterol lowering on melanoma cells may be responsible. In explanation, cholesterol lowering may have differential effects in malignant cells and normal cells because cancerous cells metabolize cholesterol differently. [80] The possible relationship between cholesterol and cancer are, however, poorly understood.

An alternative hypothesis concerns PPAR-α or PPAR-γ agonism by fibrates which is assumed to mediate growth inhibition and apoptosis. [103-105] Grabacka and colleagues demonstrated inhibition of migration by fenofibrate in a murine B16F10

and a human SkMell88 melanoma cell line. These effects were reversed by a PPAR inhibitor. The authors suggested PPAR-α is involved. However, in an *in vitro* study of Mössner *et al*. PPAR-γ specific agonists, such as rosiglitazone, inhibited cell proliferation in four melanoma cell lines dose-dependently, whereas a specific agonist of PPAR-α receptor had no such effect. [104] Therefore, some researchers believe PPAR-γ agonism is involved in the chemopreventive effects of fibrates on melanoma. To test the hypothesis that PPAR-γ is important for the risk of melanoma development, Mössner and colleagues also investigated the possibility that variations in the gene encoding PPAR-γ influence melanoma risk. In two independent case-control studies with in total 832 melanoma cases and 790 controls, they studied two gene variants (P12A[rs1801282] and C161T [rs3856806]). In one study, cases, compared to controls, were more likely to be a homozygous carrier of a *T allele of the C161T polymorphism in exon 6 of PPAR-γ (6.0 versus 2.0%; p <0.01). After adjusting for melanoma risk factors, such as skin type and nevus count, the association was still significant (OR = 5.2, 95% CI = 1.7-16.0). In the second case-control study, however, this finding could not be replicated. They finally concluded that the investigated PPAR-γ polymorphisms are not likely to constitute a significant risk factor for melanoma risk among German Caucasians. [106] These conflicting results, however, warrant further study.

Alongside with growth inhibition and apoptosis, fibrates may also have antimetastatic effects. Grabacka *et al.* showed that hamsters with allograft melanoma cells and treated with oral fenofibrate developed significantly fewer metastatic lung foci compared to controls. [107]

Evidence for efficacy in humans

In the Cochrane review by Dellavalle and colleagues, seven fibrate trials provided data on incident melanomas. In five of these RCTs, incident melanomas were diagnosed. Although there was an overall 42% reduction in melanoma incidence with use of fibric-acid derivatives (OR = 0.58, 95% CI = 0.19-1.82), this reduction was not statistically significant. Subgroup analyses by gender, trial funding, or type of fibrate, failed to show statistically significant differences in melanoma outcomes. [90] The value of these subgroup analyses is, however, limited due to small numbers.

In a meta-analysis that also included RCTs with a shorter duration (≥ ½ year in stead of ≥ 4 years), Freeman *et al.*, reported an overall odds ratio of 0.45 (95% CI = 0.20-1.01). [92] Most of the included trials, however, were also included in the Cochrane review. Additionally, for these clinical trials several disadvantages apply which were mentioned earlier (see statins – efficacy in humans).

To our knowledge, since the Cochrane review, no additional cardiovascular RCTs

studying fibrates have been published that reported the number of incident melanomas.

Some observational studies have focused on fibric-acid derivatives and cancer incidence. For instance, Poynter *et al.* published a case-control study among 1953 cases with colorectal cancer and 2015 controls. However, in this study, cases did not use fibrates more often than controls (OR = 1.08, 95% CI = 0.59-2.01). [86]

Epidemiological studies on fibrates and, specifically, melanoma incidence are thus far not available. Some epidemiological studies on statins and cancer or melanoma did, however, include a drug group of 'other lipid-lowering drugs' but this also includes bile acid-binding resins and nicotinic acid and its derivatives. {Graaf, 2004 1398 /id;Koomen, 2007 1003 /id} Moreover, recently Gardette and colleagues demonstrated in the PRIME study that cancer mortality among dyslipidemic men using fibrates is about half the cancer mortality among untreated dyslipidemic men (OR = 0.52, 95% CI = 0.28-0.97). [96]

In conclusion, although secondary analyses of cardiovascular trials with fibric-acid derivatives in two available meta-analyses have been promising, data from observational research or new clinical trials are largely lacking. The lack of such new subsequent studies is likely to be a reflection of the diminished interest in fibrates as lipid-lowering therapy.

Safety, Tolerability & Compliance

Over the last four decades, both clinical experience and large long-term RCTs in the cardiovascular setting have provided safety data on gemfibrozil, fenofibrate, bezafibrate, and ciprofibrate. Side effects related to fibric-acid derivatives include abdominal pain, dyspepsia, myopathy, myalgia, elevated CK levels, rhabdomyolysis, reversible increases in serum creatinine and urea, and cholelithiasis. Venous thrombosis, pulmonary emboli, and increases in homocysteine levels (clinical relevance uncertain) have also been reported. [108]

Myopathy, myalgia, elevated CK levels, and rhabdomyolysis are consistently reported with the use of fibric-acid derivatives, both in monotherapy as well as in combination with statins. Although rare, these side effects, especially rhabdomyolysis, are among the most serious safety risks of fibrate exposure. Both rhabdomyolysis and other muscle symptoms occur more frequently with gemfibrozil (~3.7 per 10,000 person years, 95% CI = 0.8-11) than with fenofibrate (~0 per 10,000 person years, 95% CI = 0-15). The mechanism of fibrate-related myotoxicity is not entirely unraveled, but the risk seems to be increased for patients with diabetes, renal failure, advanced age, hypothyroidism, and most importantly with concomitant use of statins. [108] Notorious

is the concomitant use of gemfibrozil with cerivastatin or fluvastatin. Gemfibrozil precipitates a drug-drug interaction leading to increased exposure of these statins metabolized via CYP2C8/9, which in turn has been shown to be related to an incidence rate of rhabdomyolysis of ~1,000 per 10,000 person years. [108] Due to reports of rhabdomyolysis, with concomitant use of gemfibrozil, cerivastatin was withdrawn from the market in 2001.

Increases in serum creatinine levels have been observed with fenofibrate, bezafibrate, ciprofibrate, and, less commonly, gemfibrozil. Both an increased production of creatinine as well as a reversible decrease in glomerular filtration rate (GFR) has been postulated as the molecular mechanism behind this side effect. [108] Several studies, however, did not show decreased renal function nor an increased incidence of renal failure. Moreover, in patients without impaired renal function, creatinine elevations are reversible upon discontinuation of the fibrate. In patients with preexistent renal dysfunction, however, fibrates should be used cautiously in adjusted doses. [108;109]

Fibrates appear to be lithogenic meaning that they increase the cholesterol saturation in the bile and may cause gallbladder disease. Risk factors for coronary artery disease are, however, also risk factors for gallbladder disease. Epidemiologic studies comparing the incidence of gallbladder disease with and without fibrate therapy are, therefore, likely to overestimate the incidence of this side effect. Nevertheless, this side effect has been validated with trial data [108] and should be considered a relatively rare but potentially serious side effect.

In the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial, use of fenofibrate, compared to placebo, seemed to be associated with slight increases in the incidence of deep venous thrombosis (1.4 versus 1.0%), and pulmonary emboli (1.1 versus 0.7%). [109] Whether these findings indicate true side effects or if they are artifacts due to multiple simultaneous comparisons in this dataset remains under debate. [108;109]

A number of potential health benefits related to use of fibrates has been demonstrated or suggested. For example, clofibrate has been shown to reduce myocardial infarctions, for example in the Coronary Drug Project and a WHO trial. In this latter study, however, clofibrate, compared to placebo, was reported to be associated with a significant increase in overall mortality. Subsequent analyses have demonstrated that the increase was artificially caused by the study design which resulted in a biased follow-up of the participants randomized to clofibrate. [109] Nevertheless, analyses of cardiovascular, cancer-related and overall mortality within the target population should be part of any chemoprevention trials since these would be essential to assess the overall risk-benefit balance.

An additional potential health benefit was observed in the FIELD trial. Among diabetes patients, less progression of albuminuria was observed with fenofibrate use. [110] Within the Diabetes Atherosclerosis Intervention Study (DAIS) a reduction in proteinuria in the fenofibrate group was reported as well. [109]
Overall, the safety profile of fibrates is good if used for lipid-lowering as an alternative for, or additional to, statins. However, the required doses of fibrates as a melanoma chemo-preventive drug are unclear and long-term data on overall mortality rates and rare side effects are limited. These data would be essential for formal risk-benefit ratio analyses.

Conclusion Fibrates

Despite the promising results in two meta-analyses, the evidence for efficacy of fibrates in melanoma or cancer chemoprevention is inconclusive. Additionally, a valid molecular mechanism for the antineoplastic effects of fibric-acid derivatives has not been sufficiently described so far. Thus, further research on the molecular mechanisms behind and required dosing for the potential chemopreventive effects of fibrates on melanoma is warranted and the efficacy of fibrates in melanoma chemoprevention cannot be validated yet. Subsequently, long-term safety and mortality data would be required to assess the risk-benefit balance for melanoma chemopreventive strategies which include the use of fibrates.

Retinoids (Vitamin A and derivatives)

The group of the so-called retinoids includes vitamin A and its derivatives. Analogs are either naturally occurring or synthetically derived. First generation retinoids include vitamin A (all-*trans* retinol), tretinoin (all-*trans* retinoic acid), and isotretinoin (9-*cis* retinoic acid). Acitretin and etretinate belong to the 2nd generation retinoids, whereas adapalene, bexarotene, and tazarotene are examples of 3rd generation retinoids.
Retinoids are in use as acne treatment or anti-aging agent, but may also be used for several other indications, such as acute promyelogenous leukemia (APL). [80;111] Natural retinoids are also present in dietary sources, and are involved in several physiological processes among which vision, embryonic development, and regulation of growth and cell differentiation. [112]

Mechanism of action

Retinoids are thought to exert most of their effects by binding to retinoid acid receptors (RAR) and retinoid X receptors (RXR) in the cellular nuclei leading to altered gene transcription. [112;113] Different genes encode the α, β, and γ receptors which in

turn have two (RAR-α, RAR-γ) or four (RAR-β) splice variants. Tretinoin binds and activates only the RAR receptors, whereas isotretinoin is both a RAR and RXR agonist. [112] Rexinoids, 3rd generation retinoids, selectively bind to RXR which is hypothesized to be especially involved in proapoptotic effects. One of these agents, bexarotene, has been approved by the FDA for cutaneous T-cell lymphoma. [13] Because melanoma is known to be relatively resistant to apoptosis, retinoids and rexinoids, in particular, may also be interesting candidates for melanoma chemoprevention.

Chemopreventive effects exerted by retinoids/rexinoids may include: 1) inhibition of tumor growth, 2) promotes cell differentiation, 3) induction of apoptosis, 4) proangiogenetic effects, and 5) reduced invasiveness and metastasis.

Ad 1. Tretinoin markedly reduced cell growth of B16 murine melanoma cells at a concentration of 10^{-7} M. [114] Additionally, mice treated with vitamin A before being inoculated with murine melanoma cells had significantly decreased tumor growth compared with controls. [115] Moreover, CD437, a synthetic RAR-γ selective retinoid, inhibited the cell growth *in vitro* of three human melanoma cell lines (MeWo, SK-Mel23, and MV3) in a concentration-dependent matter (IC_{50} value: 5×10^{-6} M), whereas tretinoin did not. In the same study, CD437 was shown to decrease tumor volume in a xenograft MeWo mouse model. [116]

Ad 2. Retinoids have also been shown to promote cell differentiation of the mouse B16 melanoma cell line. [111]

Ad 3. CD437 was observed to induce apoptosis in MeWo melanoma cells *in vitro* after 72 h incubation at a concentration of 5×10^{-6} M. [116] Likewise, in another study, CD437 also promoted marked apoptosis in A375 melanoma cells at this concentration. [117]

Ad 4. Tosetti *et al.* postulated additional antiangiogenic effects of retinoids since tretinoin has shown antiangiogenic effects in several systems. [118] although antiangiogenesis was demonstrated in other tumor types, it has not been demonstrated (yet) for melanoma.

Ad 5. In an experiment by Edward and colleagues, pretreatment with 10^{-6} M tretinoin of metastatic B16 melanoma cells resulted in a significant inhibition of lung colonization after injection of 10^5 cells into the tail vein of mice. [119]

Although RAR and RXR receptors are generally thought to be involved in these chemopreventive effects, the exact mechanisms remain unclear. Moreover, studies with synthetic retinoids have revealed that apoptosis and growth inhibition mediated by these agents are likely to be independent of this retinoid signaling pathway. [120;121] These RAR/RXR independent pathways are supported by several observations:

- apoptosis could be induced in tretinoin-resistant cells.

- retinoid receptor antagonists failed to inhibit apoptosis induced by synthetic retinoids.
- retinoid related molecules that do not bind to retinoid receptors can be effective inducers of apoptosis. [121]

Alternative mechanisms that may be involved are inhibition of mitogen-induced c-*fos* expression [114], NF-κB activation mediated by retinoid acid inducible gene I through a CARD-containing adaptor protein VISA [117], and enhanced production of reactive oxygen species (ROS) dependent on Rac activity [122]. Examples of additional hypothesized signaling pathways include increased expression of p16, p21, p27, p53, and bax, decreased expression of Id1 protein, and down-regulation of mitogen-activated protein kinase and bcl-2. [80]

Overall, *in vitro* studies of murine and melanoma cell lines have produced some evidence for chemopreventive effect of retinoids and rexinoids on melanoma. However, the evidence as yet is not well enough established and the involved mechanisms are not distinctly defined.

Evidence for efficacy in humans

Anticancer effects of retinoids in certain types of human cancers are well-established. For instance, tretinoin (Vesanoid®) is used in the treatment of APL and has been approved by the FDA for this indication. In addition, high-dose isotretinoin has been successfully used in the chemoprevention of nonmelanoma skin cancer (NMSC) in patients with xeroderma pigmentosum. It reduced the incidence of NMSC by 63%. [123] The evidence for a role of retinoids in melanoma chemoprevention is, however, preliminary. Studies on the dietary intake of vitamin A have shown promising results. In a case control study among 542 melanoma cases and 538 controls, Naldi *et al*. reported an OR of 0.57 (95% CI = 0.39-0.83) for the highest quartile of retinol intake versus the lowest quartile. [124] Similarly, Feskanich and colleagues, in a cohort study among 162,000 Caucasian US women, observed a relative risk ratio for incident melanoma of 0.39 (95% CI = 0.22-0.71) for consumption of ≥1800 mcg/day of retinol as compared to <400 mcg/day (*p*-linear trend = 0.01). [125] Strong correlation between different food items and food groups as well as between diet and other health behaviors, however, dramatically complicate the interpretation of such nutritional and observational studies.

To our knowledge, there are no studies evaluating the effect of retinoids on melanoma incidence in humans. Despite this lack of definite data, a number of studies have evaluated the effect of topically or orally applied retinoids on surrogate markers lesions of melanoma, dysplastic or atypical nevi. Originally, Meyskens and colleagues

performed two case series with topical tretinoin and oral isotretinoin, respectively, for patients with dysplastic nevi. Only 3 and 8 patients, respectively, completed the study. Importantly, these studies did not include a control treatment. [126;127]

Edwards and Jaffe reported a preliminary randomized double-blind trial in which they randomized 21 patients with multiple large dysplastic nevi to either 0.05% tretinoin or placebo solution, both topically. Of the 8 patients randomized to tretinoin, 3 discontinued the study. Two of these patients discontinued due to local irritation. Seven of the 15 dysplastic nevi that were treated with tretinoin had completely disappeared or had reverted to normal, benign nevocellular nevi. [128] However, the small number of patients and the large proportion of drop-outs in the tretinoin group preclude definite conclusions. [128]

Halpern *et al.*, in a more recent trial, studied the effect of topical treatment with once daily 0.05% tretinoin or, if tolerated, twice daily 0.1% tretinoin for 6 months versus no treatment. An effect was observed on transformation of clinical appearance (including color, size, and border irregularities), and likewise, a statistically significant was shown on histological change toward benignity (for cellularity, cellular atypia, and proliferative cellular nuclear antigen). [129] Correspondingly, Stam-Postuma and colleagues evaluated topical treatment for 4 months with either 0.1% topical tretinoin, 0.1% tretinoin plus 1% hydrocortisone, or placebo cream. In their study, topical tretinoin 0.1% showed only clinical improvement with no improvement in the degree of atypia, possibly due to the limited number of biopsies. {Stam-Posthuma, 1998 1407 /id}

Due to the lack of validation of the predictive value of dysplastic nevi as a predictor of future incident invasive melanomas, the interpretation of these surrogate marker studies remains uncertain. As an additional limitation, these studies used different definitions for 'dysplastic nevi'. Noteworthy, toxicity has been substantial in these studies as indicated by the large proportion of drop outs and the high rate of patients experiencing side effects. Interestingly, some authors reported reappearance of a dysplastic nevus 1 year after cessation of topical tretinoin therapy. (128 and Stam-Postuma *et al.*, verbal communication)

Safety, Tolerability & Compliance

Retinoids' side effects include skin irritation following topical treatment and cheilitis (lip inflammation), xerosis, ocular effects, hepatotoxicity, hair loss, teratogenicity, bone toxicity, and serum lipid abnormalities following oral treatment. [80] Dose-dependent mucocutaneous irritation affects nearly all patients and is often the dose limiting side effect [113], but it is, in many patients, a temporary side effect [129].

From a doctor's point a view topical treatment may be preferred since it involves less

(serious) side effects. However, the use of topical retinoids in skin cancer chemoprevention trials, for example for patients with dysplastic nevi or in transplant patients, has been restricted by the irritation they cause. New, less irritating, formulations could be of interest. However, adherence to the application regimen with topical treatment may prove to be too big a hurdle for the use of topical retinoids in melanoma chemoprevention. Systemic retinoid therapy on the other hand has been associated with substantial toxicity [80] and thus may also lead to relatively rates of discontinuation.
Another concern, is the teratogenicity of retinoids. For example, isotretinoin exposure during pregnancy may cause craniofacial, cardiac, thymic and central nervous system (CNS) defects in about 30% of the developing fetuses. [131] Among children born without anatomical defects, an increased incidence of developmental delays and other CNS effects has been observed. Preventing fetal exposures has proven to be a difficult task requiring comprehensive risk management programmes. [131] After discontinuation of retinoid treatment pregnancy should be avoided until the drug is essentially cleared from the body. For some retinoids, such as etretinate and acitretin, this period is up to 2 years. This feature excludes its use as a chemopreventive agent among women of childbearing age. Retinoids should therefore only be considered for high risk target populations that would exclude women under the age of 45.

Conclusion Retinoids

Although retinoids have been considered a candidate for melanoma chemoprevention over the last decades, data on the efficacy in humans are still largely lacking. Evidence from experimental research is also inconclusive. Moreover, teratogenicity and limited tolerability lead to concerns whether retinoids as a monotherapy could be suitable as a melanoma chemopreventive strategy. Research should, therefore, focus on possible synergistic combinations with other chemopreventive agents.

Imiquimod and analogs

Imiquimod is prescribed and approved by the FDA for the treatment of external genital and perianal warts (caused by human papilloma virus), multiple actinic keratoses and superficial basal cell carcinomas. [13] It is an immune modifier that stimulates the immune system through Toll-like receptors, particularly TLR-7. [12] Imiquimod has been shown to induce apoptosis and, therefore, has also generated interest as a topically applied potential chemoprevention agent. [132]

Mechanism of action

The pivotal mechanism of action of imiquimod is stimulation toll-like receptors (mainly

TRL7) on dendritic cells, B cells and plasmacytoid cells which triggers a T helper cell type 1 (Th1) immune response and induces transcription of Th1 cytokines, such as interferon-α (IFN-α), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-12. [13;132-134] In this way, imiquimod activates mature dendritic cells after binding to TRL7 and activation signals will be sent to the T cells with the aid of co-stimulatory molecules such as CD40, CD80 and CD86. [132] Consequently, the Th1 immune response results in the activation of naïve T cells to transform into antigen-specific T cells directed against antigens expressed on condylomata, basal cell carcinomas and other potentially immunogenic skin lesions. [132]

Until recently, experiments with imiquimod did not focus on possible chemopreventive effects towards cutaneous melanoma. However, recently, some preliminary evidence was generated by Schön and colleagues. They performed experiments to test for effects of imiquimod and resimiquimod on apoptosis and also possible direct toxic effects. No direct toxic effects were observed on four different melanoma cell lines (Mel-HO, Mel-2A, A375, and MeWo) and normal human melanocytes (established from five different donors). Thus, they did not observe direct cytotoxicity. However, marked concentration-dependent pro-apoptotic effects on the Mel-HO and A375 melanoma cell lines were demonstrated with imiquimod concentrations ranging from 5 to 50 μg/ml. Normal melanocytes, Mel-2A or MeWo melanoma cells showed markedly weaker, if detectable at all, induction of apoptosis with imiquimod. In contrast, resiquimod, an analog of imiquimod, did not induce apoptosis in either of the cell lines studied. [133]

Evidence for efficacy in humans

Cancer chemopreventive effects of imiquimod have been observed in several settings, mainly involving (precursor lesions) of skin cancer. For example, phase II RCTs in which patients with actinic keratoses (AK), a premalignant condition that may progress to squamous cell carcinoma (SCC), were treated with 5% imiquimod three times per week topically, have shown statistically significant improvement in clinical and histological appearance, and the average number of AK. [132] Additionally, open label phase II studies have also demonstrated beneficial effects on superficial and nodular basal cell carcinoma (BCC). Similarly, preliminary studies have suggested regression after local application of 5% imiquimod cream for additional precursor lesions, such as Bowen's disease (SCC *in situ*), and vaginal intraepithelial neoplasia (VIN). [132] The evidence for melanoma chemoprevention specifically, however, is scarce.

In two case reports, regression of lentigo maligna (LM, melanoma *in situ*) lesions that could not be excised were observed. [132;135] Moreover, in a small case series of five

patients, Wolf *et al.* observed complete clearance of LM lesions after 13 weeks of application each night of 5% imiquimod cream. [136] We believe these results, although positive, should not be considered true melanoma chemoprevention because if left untreated not all LM lesions will progress to invasive lentigo maligna melanoma (LMM) and the latent period is estimated to be 10-50 years. {Stevenson, 2005 1510 /id} Likewise, in a case of disseminated cutaneous metastatic melanoma, local control of tumor growth has been observed after treatment with imiquimod three times per week for 18 weeks. [138] Although this may indicate that imiquimod could be beneficial for cutaneous metastatic melanoma if radiotherapy or surgery is impossible [138], if these results predict chemopreventive activity is uncertain.

No human studies, to our knowledge, have evaluated the effect of imiquimod on melanoma incidence. Thus, imiquimod has not yet been studied for true primary melanoma chemoprevention.

Nevertheless, human data on the effects of topical imiquimod on atypical nevi, surrogate markers lesions of melanoma are available. Somani and colleagues, in a small case series of three patients, evaluated the effect of imiquimod applied five nights per week for 12 weeks on a selected clinical atypical nevus. Imiquimod treatment failed to cause lesional resolution in these patients. [134] Likewise, Dusza *et al.* have studied topical imiquimod in a pilot study among 10 patients with atypical nevi and at least 8 large nevi (≥ 5 mm) on the trunk. Standardized photographs were compared at baseline and 4 weeks after completion of 16 weeks of imiquimod treatment (5% cream applied 3 times per week). In addition, histological assessment was performed of each patient's 4 largest study nevi. Size and morphology showed no obvious changes, but 4 of 14 treated nevi and 0 of 14 untreated nevi showed histological changes suggestive of partial regression ($p = 0.03$). [139]

Investigators of the University of Arizona are currently testing an analog of imiquimod among patients with dysplastic nevi. [12] This study may be an important step forward in unraveling the chemopreventive potential of imiquimod and its analogs.

In summary, some, but not all, of these preliminary studies have shown promising results. More importantly, definite data on melanoma incidence or validated precursors are lacking.

Safety, Tolerability & Compliance

In general, the side effects of topical imiquimod are mild to moderate. Side effects include local skin reactions (LSR), nausea, vomiting, headache, muscle weakness, fever, flu-like symptoms and fungal infection. [80]

LSR are most frequent, dose and frequency dependent and usually subside after a

resting period. Severe LSR usually are the DLT and some studies have reported that 16% of patients (4/25) required 4-week rest periods after a four-week treatment period with 5% imiquimod cream three times weekly. [132]
Although LSR are not considered to be severe medical conditions, they may have important implications for adherence in long-term therapy that would be required for melanoma chemoprevention.
Systemic side effects are rarely reported [132], but presumably are more likely to occur if large areas of the body would be treated or with application on areas with thin skin such as the face.
Since imiquimod treatment is often restricted to a duration of 6-16 weeks [132] , the long-term safety data required to evaluate the risk benefit ratio for melanoma chemo-prevention are lacking.

Conclusion Imiquimod and analogs

Imiquimod, and possibly some of its analogs, can be considered candidates for melanoma chemoprevention. Thus far, however, data from *in vitro* and *in vivo* experiments as well as human efficacy data are scarce and inconclusive. Additionally, long-term safety data are lacking.

Acetaminophen

Acetaminophen is a frequently used analgesic and antipyretic drug that, in most countries, is available both as an OTC drug as well as on prescription. Acetaminophen is also referred to as paracetamol and has been demonstrated to be a selective COX-3 inhibitor. [140] Its anti-inflammatory action is relatively weak and therefore it is not considered to be a NSAID.

Mechanism of action

Experimental studies on acetaminophen's effects on melanoma murine models or cell lines are very limited. Recently, however, Vad and colleagues have reported on two such studies. They tested an acetaminophen concentration of 100 μM which showed considerable toxicity towards B16F0 and B16F10 murine melanoma cells and SK-MEL-28, MeWo, and SK-MEL-5 human melanoma cell lines, resulting in a loss of cell viability of 40 ± 3, 45 ± 7, 66 ± 8, and 60 ± 5%, respectively. No significant toxicity was observed in three nonmelanoma cell lines (BJ, Saos-2, PC-3). Thus, selective toxicity towards melanoma cells with an IC_{50} of ~100 μM was observed. Adding glutathione (GSH) prevented toxicity in SK-MEL-28 melanoma cells, whereas 1-bromoheptane, a GSH depleting agent, increased acetaminophen induced toxicity. Additionally,

acetaminophen led to ROS formation and mitochondrial toxicity in these cells. The authors suggest that tyrosinase plays a role in acetaminophen's toxicity and that acetaminophen is a tyrosinase substrate. [141]
In a second study, Vad *et al.* studied the *in vivo* efficacy and toxicity of acetaminophen in a B16F0 skin melanoma tumor model in mice. At acetaminophen doses of 60, 80, 100, and 300 mg/kg/day, from day 7 until 13 post melanoma cell inoculation, tumor growth inhibition by 7 ± 14, 30 ± 17, 45 ± 11 and 57 ± 3%, respectively, was demonstrated. If acetaminophen was dosed from day 1 through day 13, the inhibition was similar. [142]
Overall, these two studies show promising, but limited, evidence for chemopreventive activity of acetaminophen against melanoma.

Evidence for efficacy in humans

Human data on the effect of acetaminophen on melanoma are very limited as well. Interestingly, Wolchok *et al.* observed two partial responses in a phase I dose-escalation study among 27 patients with stage III/IV melanoma. In this study, patients received acetaminophen doses every 3 weeks (10, 15 or 20 g/m^2) combined with carmustine (BCNU, 10 to 150 mg/m^2), every other cycle. To prevent acetaminophen toxicity, 6-8 hours after acetaminophen infusion had stopped, N-acetylcysteine (NAC) was infused (loading dose of 140 mg/kg in 1 h with subsequently 17.5 mg/kg/h for at least 19 h or until acetaminophen levels had dropped below 20 mg/L). [143] Obviously, however, these results may simply reflect effect of carmustine and may not predict any chemopreventive potential.
Some epidemiological studies investigating NSAIDs and melanoma incidence have used acetaminophen as a comparison drug. For instance, Harris and colleagues reported that they did not observe an association between acetaminophen and the risk of malignant melanoma. In their case control study, among 110 women with melanoma and 609 controls, they observed an OR of 0.95 (95% CI = 0.45-1.98). [42]
Asgari and colleagues, in a large cohort study, also included exposure to acetaminophen in their cohort study in which they investigated the association between melanoma incidence and NSAID exposure. However, they did not report findings on the association between use of acetaminophen and incident melanoma. [47]
Friis *et al.* have also investigated the association between acetaminophen use and cancer (among which melanoma). In contrast with the studies previously mentioned, their interest was raised by concern about the carcinogenic potential of acetaminophen. This concern originates from the fact that phenacetin, the precursor of acetaminophen, was withdrawn from the market due to an established link with urinary tract tumors.
The standardized incidence rate (SIR) observed by Friis *et al.* in the total cohort of

acetaminophen users was 0.9 (95% CI = 0.6-1.2). After excluding patients with prescriptions of aspirin and other NSAIDs, the SIR was 0.6 (95% CI = 0.2-1.3). Thus, an association cannot be excluded nor confirmed based on these data. [144]

Safety, Tolerability & Compliance

In normal doses, acetaminophen only rarely causes side effects. However, when liver enzymes catalyzing the normal conjugation reactions are saturated, acetaminophen will be metabolized by mixed function oxidases. As a result, N-acetyl-*p*-benzoquinone-imine, a toxic metabolite, is formed which is inactivated by conjugation with GSH. If GSH is depleted, toxic effects on the liver and also in the kidney will occur. [145]

Side effects of acetaminophen are dermatologic and allergic reactions, such as urticarial rash or exanthema, hypothermia, and renal failure after chronic exposure. Among patients with Glucose-6-Phosphate Dehydrogenase (G-6-PD) deficiency, acetaminophen may cause anemia, hemolysis and methemoglobinemia. [145]

In doses just above the normal therapeutic doses, however, acetaminophen may cause liver failure. Patients with special risk factors, such as preexistent liver failure, exposure to CYP2E1 inducers, such as carbamazepine, isoniazide or barbiturates, or chronic alcohol exposure, have an increased risk of liver failure if exposed to acetaminophen overdose. Single acetaminophen overdose can be relatively safely treated with NAC infusion. Chronic acetaminophen overdose, however, cannot and often leads to the need for liver transplantation. [145] Therefore, if future experiments would demonstrate that high doses of acetaminophen are required for melanoma chemoprevention, safety aspects are likely to preclude its use as such.

Conclusion Acetaminophen

Preliminary promising results have been generated for acetaminophen in human melanoma cells, a murine melanoma model and in a phase I study treating phase III/IV melanoma patients (combined with carmustine). The first few epidemiological studies, however, have been disappointing. Acetaminophen doses in these studies may have been too low. In general, evidence for acetaminophen as a potential chemopreventive drug is inconclusive and very preliminary.

Dehydroepiandrosterone

Dehydroepiandroterone (DHEA) is a physiologic steroid that is produced in response to adrenocorticotropin (ACTH) stimulation by the adrenal gland. [146] Physiologically, DHEA is predominantly present as dehydroepiandrosterone sulfate (DHEAS), and is a precursor of androgens (e.g., testosterone) and estrogens [147], but other physiologic

roles of DHEA and dehydroepiandrosterone sulfate (DHEAS) have remained unclear. In many counties, DHEA is marketed as a dietary supplement and, therefore, are available in OTC formulations which do not require approval of the regulatory authorities, such as the FDA and European Medicines Agency (EMEA). Beneficial effects of DHEA have been claimed for numerous indications. For most of these, however, evidence is preliminary, if not lacking at all. One of the claims is chemopreventive potential toward cutaneous melanoma. [148]

Mechanism of action

A small number of experiments have investigated the effects of DHEA on melanoma. Richardson *et al.*, in an attempt to investigate why women have a survival benefit in metastatic melanoma, have performed *in vitro* experiments with DHEA. At a concentration of 1nM DHEA, they observed significantly enhanced invasion of A375 melanoma cells. In contrast, *in vitro* experiments by Kawai and colleagues, showed DHEA dose-dependently inhibited the growth of B16 mouse melanoma cells and enhanced melanin production, which may indicate induction of differentiation. [149] In conclusion, there is hardly any experimental evidence to support claims of chemopreventive activity of DHEA towards melanoma.

Evidence for efficacy in humans

To the best of our knowledge, only a single study investigated the association between DHEA and incident melanoma in humans. In a nested case-control study, the mean serum DHEA and DHEAS levels of 23 melanoma cases and 43 controls (matched for age, sex and race) were compared. No statistically significant differences in de DHEA(S) levels were detected between cases and controls. [148]

Safety, Tolerability & Compliance

In physiological doses DHEA is considered to be safe. However, good quality long-term safety data for higher doses are lacking.

Conclusion Dehydroepiandrosterone

Both experimental and human data on the chemopreventive potential of DHEA(S) have been disappointing. However, the number of studies that have been reported is small. Nevertheless, DHEA does not seem to be a good candidate as a melanoma chemopreventive drug.

Discussion

Initially, our literature search resulted in a large number of references. However, most of these had to be excluded and about 75% of the finally included references did not emerge from the systematic literature search. We believe this is a reflection of the fact that 'chemoprevention' is not defined as a MESH term. Research would certainly benefit from such a MESH term.

Although there was a large number of preclinical studies available for some candidate chemopreventive drugs, the interpretation remains troublesome. Particularly, preclinical *in vitro* and animal models usually have not been validated. Similarly, biomarkers and precursor lesions have also not been validated. Moreover, different definitions for precursor lesions, such as atypical / dysplastic, have been used in the present literature.

Additionally, experimental research usually includes one or two agents of a larger drug class. Some drug classes, such as NSAIDs, may, however, be chemically rather diverse. We believe experimental research should include at least one example of each chemical subclass. In explanation, what may be interpreted as lack of effectiveness of a complete drug class, could very well be a result of differential effects of different subclasses or even of individual agents. The same problem may arise in observational research. For example, the disappointing results for statins in observational research do not exclude differential effectiveness for lovastatin. Freeman and colleagues calculated that based upon the lovastatin subgroup analysis (which included only one trial), 244 people would need to be treated for 5 years to prevent one case of melanoma. Similar effectiveness (which cannot be assumed *a priori*) in a high risk population would decrease this number needed to treat and may even result in a realistic chemopreventive strategy.

Since the temporal dose-response and cause-effect relationships between the duration and dose of chemopreventive drugs and incident invasive melanoma are unknown, it is not clear which study design is to be preferred. Duration of drug use and also follow-up in many studies may have been too short and daily doses may not have been high enough.

For chemopreventive drugs to move forward from *in vitro* research, animal experiments and observational studies towards RCTs and ultimately clinical practice, overall acceptable risk-benefit ratio for the target population is to be expected. To achieve this, after efficacy has been proven, a *sine qua non* in this issue, full risk-benefit analyses should be performed to show the overall health impact for subpopulations at high risk of developing (a second) melanoma. Such risk-benefit analyses should take into account all important health

outcomes (Fig. 2). For example a risk-benefit analysis of aspirin should not only include cancer reductions in melanoma, but also in colorectal, esophagus, stomach, lung, breast, and ovarian cancer, as well as benefits on other health aspects, such as reductions of myocardial infarction, pulmonary embolism, and occlusive cerebrovascular events. In addition, risks of long-term aspirin treatment should include all important drug related adverse events, such as GI bleeds, ulcers, perforation, and haemorrhagic stroke. [55] However, the balance between health benefits and risks is complicated by several issues, such as the lack of clear-cut definitions for the target population to be treated, but also by age. Specifically, with increasing age not only do the absolute risks of cardiovascular events and GI bleeds increase, but simultaneously melanoma risks are changing. Lack of evidence on the temporal and dose-response cause-effect relationships even further complicate these issues since the expected prevalence of adverse effects depends on required dose and duration. Consequently, the influence of different chemopreventive strategies, varying in drug dose, duration, definition of the target population in order to include individuals at highest risk of cancer development and excluding individuals at highest risk of developing adverse events, with or without additional interventions to prevent adverse effects, and the age-specific changes in the risk–benefit ratio should be investigated. Recently, an international expert group, however, concluded that "gaps in our understanding of appropriate dose, duration, and age of use, do not support a formal risk–benefit analysis". [55]

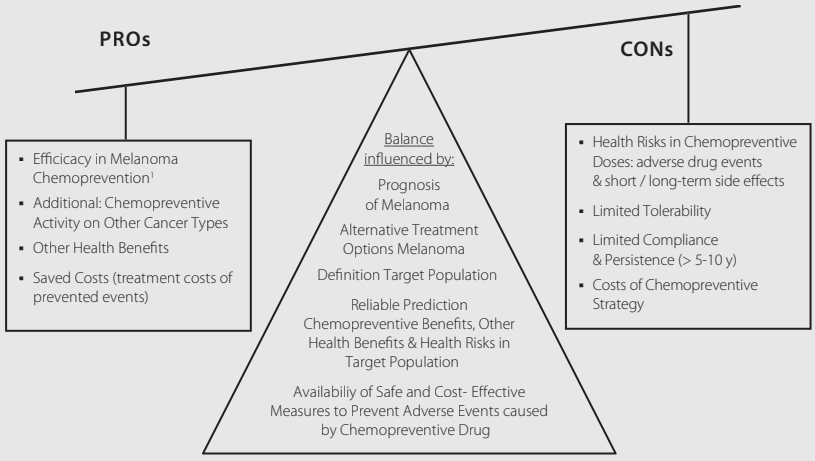
Nevertheless, among high risk (sub)populations, melanoma chemoprevention may prove to be an innovative approach additional to sun protection measures to control the increasing burden of melanoma in the future.

Conclusion

Considerable preclinical evidence of efficacy as a melanoma chemopreventive drug exists for aspirin, NSAIDs and statins. Data on clinical efficacy and long-term safety with doses required for melanoma chemoprevention, however, are still sparse. Validated preclinical models are urgently needed to move melanoma chemoprevention forward. In future research, special attention should be paid to explore possible differential effects within a drug class, temporal dose-response relationships, and to possible synergistic or antagonistic effects. Research should also focus on how to define the target populations.

Chemoprevention may prove to be an innovative approach additional to sun protection measures to control the increasing burden of melanoma among high risk

Figure 2 Risk-Benefit Balance of Melanoma Chemoprevention Strategies



[1] prevented or delayed incident melanomas including possible shifts in prognostic factors (e.g., distribution of Breslow depth, in situ/invasive, % ulcerated etc.) & inhibited progression of incident melanomas such as reduced tumor growth, invasiveness, metastasis.

individuals. Lack of definite data on efficacy in humans and profound long-term safety data in the required doses, however, preclude the use of chemopreventive drugs for melanoma in current practice. Success factors for melanoma chemoprevention to be useful in patient practice will likely be:

- Little-to-no toxicity, including mild but inconvenient side effects to not only ensure safety, but also tolerability and adherence
- A sufficiently motivated target population, e.g. patients with previous melanoma (or other types of cancer) or premalignant lesions would be more likely to be motivated to use a chemopreventive drug for at least 5 to 10 years.
- A clear-cut definition of the high risk subpopulations at whom chemoprevention should target based upon validated prediction models, mutational status and, if possible, validated early biomarkers of invasive melanoma risk
- A clear-cut definition of contraindications and predictors for individuals prone for the adverse events the chemopreventive drug may cause in order to withhold the drug from these individuals or to present additional preventive measure to them.

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