

Salicylic acid (SA), CAS no. 69-72-7

Synonyms: Salicylate, 2-hydroxybenzoic acid, keralyt, acetylsalicylic acid, methyl ester

Salicylic acid (Figure 1) is a Beta hydroxyl acid obtained from the bark and wintergreen leaves of the white willow and encompass bacteriostatic, fungicidal and keratolytic effects. Salicylic acid (SA) is a colorless, crystalline organic carboxylic acid. The substance is used in many skin-care products for the treatment of acne, psoriasis, callouses, keratosis pilaris and warts and is produced and/or imported to EU in 10 000 - 100 000 tpa.

For the studies in this summary, different substance modifications of the substance have been used as a substitute, e.g. Acetylsalicylic acid (Aspirin), sodium salicylate and methyl salicylate.

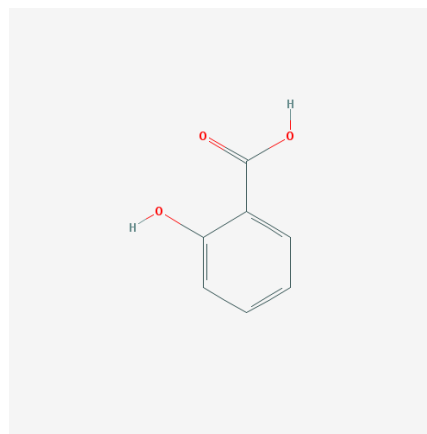


Figure 1: 2D structure from PubChem

Acetylsalicylic acid and methyl salicylate are the results of esterification of salicylic acid and hydrolysis of methyl salicylate produces sodium salicylate. The compounds are closely related and the following statement from WHO has been used as guidance for this summary: “All salicylic acid derivatives except combinations with corticosteroids (M01B) or opioids (N02AJ) are classified in N02BA - Salicylic acid and derivatives, as it is difficult to differentiate between the use of salicylates in rheumatic conditions and other therapeutic uses of salicylates.” (WHO 2017).

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Mazaud-Guittot et al. (2013)

Summary: The study aimed to investigate the effect of mild analgesics on testis morphology and endocrine function. Three different drugs were used (Paracetamol, Aspirin and Indomethacin) but only data concerning Aspirin will be included in this summary. Human fetal testis (n=62) from gestation week (GW) 7-12 were cultured with exposure to Aspirin at 10^{-4} to 10^{-7} M. Testicular hormone levels of testosterone and Insulin-like factor 3 were measured by Radioimmuno Assay (RIA) and Anti-Müllerian hormone (AMH), prostaglandin D₂ and E₂ (PGD₂/PGE₂) was assayed by ELISA. Testicular cells (germ and Sertoli cells) were counted using the semiautomatic counting CAST grid image analysis software and mesenchymal cells of the interstitial tissue were identified by COUP-TF II staining. For assessment of nuclear receptor-mediated agonist or antagonist activities of Aspirin a reporter cell line expressing androgen, estrogen and peroxisome proliferator-activated γ -receptors was used. The study results showed no change in testis morphology, cell count or number of interstitial

cells. Regarding testosterone production, Aspirin showed a significant dose-response relationship increasing the level in the youngest fetal testis (8-9.86 GW) but not in the older (10-12 GW) and when all ages were pooled, no significant effect was observed. The AMH production was strongly stimulated by Aspirin and the PGE₂ was significantly inhibited. No significant effect was reported on the remaining parameters.

Study quality and assessment: The study is well-described but no CAS number (no.) and purity was reported for the substance used. The study is assessed to be of high quality and provides strong evidence for an endocrine disrupting (ED) mode of action (MoA) from Aspirin exposure.

Albert et al. (2013)

Summary: This study investigates the endocrine disrupting properties of mild analgesics in the adult human testis. Three types of analgesics were used in parallel studies but this summary will only cover the Aspirin part. Tissue used for the study came from prostate cancer patients who had not received any hormone therapy and NCI-H295R steroid producing human cell line. Only testis displaying spermatogenesis (assessed by transillumination) was used. Orchiectomy was performed and testis was transported on ice and processed as followed. The testicular tissue were fragmented and cultured by the procedure of Testis Explants Assay (TEXAS) and the media contained 10⁻⁵ and 10⁻⁴ M of Aspirin (purity > 99%). To determine the levels of hormones in the cultured media, Radioimmuno Assay was applied for the measurement of testosterone and insulin-like growth factor 3 (INSL3), Enzyme-Linked immunosorbent Assay was conducted to measure Inhibin B and for estimation of prostaglandins (PGD₂ and PGE₂) Enzyme Immunosorbent assay was applied. To evaluate gross morphology the testis explants were fixed in Bouin fixative, stained and examined under a light microscope. No change in the gross morphology of the testis was found. Measurements described in the following text include values after 24 h and 48 h of culturing. Testicular testosterone production was decreased but did not reach statistical significance. For the NCI-H295R cell line, exposure to 10⁻⁵ and 10⁻⁴ M Aspirin significantly reduced testosterone level. A reduction was also seen in the levels of INSL3, PGD₂, PGE₂ and Inhibin B production by the adult human testes.

Study quality and assessment: The study is well described and thorough although CAS no. of the substance used could not be found. It is assessed to be of high quality and since data only showed a significant value in NCI-H295R cell line the study provides moderate evidence for ED MoA by direct exposure to Aspirin.

Abend et al. (1991)

Summary: The aim of this study was to examine the feedback mechanism from T3 / T4 on TSH by looking at the role of type II 5'Deiodinase (5'D-II). The study was carried out in 3 parts of which only one part is relevant to this summary. A small *in vitro* investigation was conducted to look at the activity of 5'D-II and it was carried out using 100 and 200 pM [¹²⁵I]-T₄ as substrates. To investigate if sodium salicylate could be a direct inhibitor of 5'D-II, an assay was done with aliquots of normal pituitary homogenates for activity in the presence of 0, 2 and 10 mM sodium salicylates. The results from the study showed that the administrated dose of Salicylate did not inhibit the 5'D-II enzyme activity directly.

Study quality and assessment: The study has some shortcomings – the number of pituitaries used is not given and the CAS no. and purity of the substance used could not be found. The study is assessed to be of medium quality and it did not provide any evidence for an ED MoA on the activity of 5'D-II.

Larsen, P.R. (1972)

Summary: This study was conducted to clarify the effect of SA on the protein binding of the two thyroid hormones (T_3 / T_4) in human serum. The investigation was made up from several smaller studies.

1- The effect of addition of sodium salicylate on the ultrafiltrable fraction of T_4 (UFT₄) and T_3 (UFT₃) *in vitro*: Sodium salicylate was added in increasing amounts to pooled human serum. The concentrations of salicylate used were within the therapeutic (20-30 mg/100 ml) or toxic (60mg/100 ml) range (with humans as a reference for therapeutic and toxic values). A progressive increase in both of UFT₃ and UFT₄ was seen after sodium salicylate exposure.

2- The effect of dilution of human serum containing salicylate on UFT₃ and UFT₄: Two samples of human sera, one control and one salicylate-enriched were progressively diluted and a simultaneously determination of UFT₃ and UFT₄ was conducted. The result revealed a progressive decrease in the effect of salicylate when diluted and for UFT₃ it was significant at 1:2 dilutions.

3- Comparison of the effect of sodium salicylate and barbital on the binding of T_3 and T_4 in human serum and to human serum albumin: Diluted serum or human serum albumin was used in this study and a final concentration of sodium salicylate was established at 15 mg/ml. The result showed an increase of UFT₃ and UFT₄ for both Salicylate and barbital.

4- Effects of sodium salicylate on T_3 / Thyroxin-binding-prealbumine (TBPA): For this study the ultrafiltration system and simultaneous labeling with T_3 -¹³¹I and T_4 -¹²⁵I was used in the presence of 1.3×10^{-6} M TBPA and the result showed that addition of salicylate (15 mg/100 ml) increased both the UFT₃ and the UFT₄.

5- Effects of sodium salicylate on T_3 and T_4 binding to Thyroxine-binding-globulin (TBG): The binding was examined in an assay system described in an earlier study. The result showed that both T_3 and T_4 were displaced from TBG (thyroxine-binding-globulin) by sodium salicylate.

Study quality and assessment: The text is written with addition of several references to earlier studies regarding descriptions of methods used and in the section with results it is a challenge to distinguish result from the study conducted from results obtained from other experiments. No report of CAS no. and purity of the substance used could be found. The study quality is assessed to be medium but the evidence for a thyroid disrupting MoA of SA *in vitro* is strong.

Hansen and Mogensen (1964)

Summary: This study investigated the effect of sodium salicylate on the uptake of ¹³¹I-labelled 1-triiodothyronine by human erythrocytes. For this part of the study, human venous blood was obtained from donor blood and 4 portions of erythrocytes (2 x washed + 2 x unwashed) were prepared for use in a scintillation counter. To all samples ¹³¹I-labelled 1-triiodothyronine was added in the process (concentrations kept below 0,1 µg/ml) and 25 % W/v salicylic acid solution were added in proximately calculated amounts. The result from the study showed that by adding sodium salicylate to

the blood samples an increase in the T₃ uptake was seen in erythrocytes. An additional study was performed where erythrocytes were washed six times and then suspended in physiological saline solution (instead of plasma). No increase in the T₃ uptake was observed under those conditions. According to the authors the effects observed are a result of the influence of salicylate on plasma proteins and not a direct effect on the erythrocytes.

Study quality and assessment: The quality of the study is assessed to be medium. No information on CAS no and purity of the substance used was given and there is no report of any control group. The study provides no evidence for a thyroid disrupting MoA of sodium salicylate through binding of T₃ to erythrocytes *in vitro*.

Wolff et al. (1961)

Summary: The aim of the study was to investigate T₄ displacement from serum proteins in human serum after addition of natrium salicylate. The study consists of 3 experimental parts where only two of them are relevant to this assay. Pooled normal human serum (one batch) obtained from Baxter Laboratory was used in all studies and butanol solutions of thyroxine were added. For the first experimental part natrium salicylate ($3 \cdot 10^{-3}$ M) was added to serum before measurement by reverse flow electrophoresis. For the second part natrium salicylate ($3 \cdot 10^{-3}$ M) was added to the (NH₄)₂CO₃ buffer (pH was adjusted after addition). The displacement was measured at 4 different T₄-concentrations 0.15 µg T₄/ml (0.19 µM), 0.28 µg T₄/ml (0.36 µM), 1.54 µg T₄/ml (2.0 µM) and 2.08 µg T₄/ml (2.7 µM). The result from the first study showed that natrium salicylate tended to displace T₄ from Thyroxine-binding pre-albumin (TBPA) onto thyroxine binding globulin (TBG). However, the result was more reproducible in the second part where natrium salicylate was added to the buffer. In this part it was possible to measure a significant displacement of T₄ from TBPA to TBG at low T₄-concentrations and when the amounts of T₄ was higher, TBG was saturated and then T₄ was displaced and bound to albumin.

Study quality and assessment: The study is well-described but regarding the substance used it is only mentioned that it is of reagent grade but no CAS no. was given. Overall the study is assessed to be of high quality and it provides strong evidence for a thyroid disrupting MoA of natrium salicylate.

4.10.3.3 *In vivo* effects with regard to an endocrine mode of action

Kristensen et al. (2012)

Summary: This study aimed at investigating effects of mild analgesics (paracetamol, indomethacin and Aspirin) on *ex vivo* rat fetal testis development. Only results relevant to Aspirin will be included in this summary. To examine the effects of the mild analgesic, Sprague-Dawley rat testis (gestation day 14.5) was used in a 3 day *ex vivo* organotypic culture system with exposure to Aspirin at doses of 0, 1, 10 and 100 μM . Testosterone, Insulin-like factor 3 (Insl3) and prostaglandin D₂ (PGD₂) concentrations were determined in the medium at 24, 48 and 72 h by Coat-A-Count Total Testosterone Kit (testosterone), Enzyme-linked immune sorbent assay (Insl3) and Prostaglandin D₂-Mox EIA Kit (PGD₂). Leydig cells were prepared and counted by systematic random sampling in a CAST-grid stereotactic system with a reference to an earlier study. For evaluation of gonocyte apoptosis in the testis, a TUNEL assay with the in Situ Cell Death Detection Kit was conducted and finally gross morphology of the seminiferous tubules was examined. The result from the study showed significantly decreased testosterone levels at all Aspirin concentrations, with 20 – 75 % compared to control and for PGD₂ Aspirin led to a modest decrease in the production at all time-points. No significant data was obtained for gross morphology, leydig cells number and rate of gonocyte apoptosis.

Study quality and assessment: The study is well described and thorough all though there was no report of CAS number. and purity of the substance used. The study is assessed to be of high quality and it provides a strong evidence for an anti-androgenic ED MoA after Aspirin exposure.

Kristensen et al. (2011)

Summary: The study consisted of 3 parts (*in vivo*, *ex vivo* and epidemiology) and the *in vivo* part will be described here. This study had a focus on intrauterine exposure to mild analgesics as a risk factor for development of male reproductive disorders in rats. Pregnant rat dams were exposed to acetylsalicylic acid at doses of 150, 200 and 250 mg/kg/day from GD 13-21 with cesarean section conducted on GD 21. For dose-response analysis, anogenital distance (AGD) in male fetuses was analyzed by the calculated ADG index (AGDi) divided by the cube root of the body. Analysis of testosterone and the production by testes were performed by a method with a reference to an earlier study. The results showed reduced AGD compared to control but due to fetal growth retardation AGD was undetectable in a number of fetuses and statistical results are not presented. A significant reduction of testosterone by testes was measured.

Study quality and assessment: The study is well described although no CAS number and purity of the substance used could be found but overall the quality is assessed to be high. In the article it is noted that no signs of general toxicity were observed (no liver toxicity, normal maternal body weight gain, litter size, live fetuses, resorption, implantation and sex ratio when compared with controls). Thus, the findings regarding testosterone and AGD provide strong evidence of an anti-androgenic MoA after Aspirin exposure.

Kristensen et al. (2011)

Summary: The study consisted of 3 parts (*in vivo*, *ex vivo* and epidemiology) and the *ex vivo* part will be described here. This study had a focus on intrauterine exposure to mild analgesics as a risk factor for development of male reproductive disorders in rats. Testes from male rat fetuses (n=8) obtained by caesarean section on GD 14.5 were incubated with two testes in each experiment, for 3 days in a media with or without Aspirin at concentrations of 1 μ M and 10 μ M. The media concentration of prostaglandin D₂ (PGD₂) and testosterone were measured by Prostaglandin D₂-Mox EIA kit after 24, 48 and 72 h. The result from the study show a dose dependent reduction in testosterone and PGD₂ with a significant result for testosterone at 10 μ M Aspirin at all time points and a significant result for PGD₂ at 48 and 72 h.

Study quality and assessment: The study is well-described although no information on CAS number and purity of the substance used could be found. The quality is assessed to be high and it provides strong evidence for an anti-androgenic MoA.

Gupta et al. (2003)

Summary: The aim of this study was to compare the developmental toxicity of Aspirin (CAS 50-78-2) in rats using selected dosing paradigms. To allow a direct comparison between the responses of Sprague-Dawley (SD) rats (in this study) and Wistar rats (from Kimmel et al.1971), the study design and dose levels were based on the work of Kimmel et al (1971). The study was conducted in two parts with a single dose study and a multiple dose study and in both cases, timed-mated SD rats were assigned. In the first study (single dose) groups of 7 rats were orally exposed (by gavage) to acetylsalicylic acid (ASA) on gestation day 9 (0, 250, 500 or 625 mg/kg), 10 (0, 500, 625 or 750 mg/kg) or 11 (0, 500, 750, 1000 mg/kg). In the second study (multiple doses) groups of 20 rats were orally (by gavage) treated with ASA from gestation day 6 to 17 at concentrations of 0, 50, 125 or 250 mg/kg. On gestation day 21 all rats were killed, and fetuses were examined and following parameters were noted: numbers of corpora lutea, implantation sites, late and early resorptions, viable and dead fetuses, individual fetus weight, placenta weight and finally all fetuses were examined for external and visceral anomalies and developmental variations with focus on ventricular septal defects (VSD) and midline defects (MD). The results from the study showed a high concordance between Wistar and SD rats regarding developmental anomalies with the exception to hydrocephalus in Wistar rats and the VSD in the SD rats. Whether ASA was administrated as a single dose or during the organogenesis (GD 6-17), the malformations were similar. All registrations of malformations are presented in a table and only in the high dose-group are they statistically significant. Hypoplastic testes were seen in 2 out of 137 fetuses and only in the highest dose group and along with ectopic adrenals, ablepharia was only detectable in the multiple dose study and not in the single dose study.

This paper is included in the REACH registration dossier for Salicylic acid on developmental toxicity / Teratogenicity

Study quality and assessment: The study is well-written and thoroughly described and contains details on both animal housing conditions and CAS number of the substance used and it is assessed to be of high quality. The results provide weak evidence for ED-related adverse effects after exposure to Aspirin

Conte et al. (1999)

Summary: The aim of this study was to examine the effect of Aspirin on plasma testosterone, pregnenolone, progesterone, 17OH-progesterone, androstenedione, dehydroepiandrosterone and 17 β -estradiol in response to human chorionic gonadotropin (hCG). Healthy men (n=8) age 20-30 years was examined in a placebo-controlled, single-blinded study and to test the efficacy of Aspirin as a prostaglandin-blocker an additional study was conducted where seminal prostaglandin E₂ (PGE₂) were determined at the same doses and times used in the experimental protocol. All subjects received the same four treatments, separated by an interval of at least 1 month. 1) Placebo Aspirin + hCG, 2) Aspirin + hCG, and for control 3) Aspirin + placebo hCG, 4) placebo Aspirin + placebo hCG. Administration of 1 tablet (800 mg) Aspirin or placebo Aspirin was done orally two times a day for 7 days (i.e. 2 days before and 5 days after administration of hCG or placebo hCG). For the experiment 5000 IU hCG was injected intramuscular (IM) and blood samples were collected after 2, 24, 48, 72 and 96 h and in the same period, all participants were asked to collect semen specimens (by masturbation) for PGE₂ measurements. Parameters measured by radioimmune assay were plasma testosterone, pregnenolone, progesterone, 17OH-progesterone, androstenedione, dehydroepiandrosterone, 17 β -estradiol and PGE₂. The study result showed that Aspirin significantly lowered the seminal level of PGE₂ and significantly reduced the response of testosterone, 17 OH-progesterone, androstenedione and dehydroepiandrosterone to hCG stimulation (assessed by the mean integrated area under the curve). No change was seen with regard to 17 β -estradiol, pregnenolone and progesterone when hCG was administrated.

Study quality and assessment: The study is well-described and thorough with the exception that CAS no and purity of the substance used was not given. The study is assessed to be of high quality and because of the significant results it provides strong evidence that the androgen response to hCG is inhibited by Aspirin treatment.

Davis et al. (1996)

Summary: This study has a two-fold purpose. One part investigates the effect on maternal reproduction in rats after oral exposure to salicylic acid. The other part looks at the structure-toxicity relationship between acetylsalicylic acid (ASA) and salicylic acid (SA) with respect to the effects measured in part one. Sprague-Dawley virgin female rats (n=105) at the age of 63 days was mated and the presence of a copulating plug marked gestation day 0. Randomly assigned animals were divided into dose groups receiving SA at the level of 0 mg/kg/day, 20 mg/kg/day, 80 mg/kg/day, and 200 mg/kg/day. A single group received a dose of ASA at 260 mg/kg/day. All groups were exposed by oral gavage on day 15-21 during gestation and administration of the compound was conducted twice a day (one half morning / one half 6-8 h after). Parameters recorded from the dams were body weight (measured on day: 0, 6 and 15-21), duration of gestation, labor time (time between the first and last born) and gross examination of uterus and ovaries (post-mortem). Registrations from pups included examination for external abnormalities, number of viable/non-viable, sex determination and weight. The results of the study showed that the groups exposed to SA 200 mg/kg/day and ASA 260 mg/kg/day had a delay in the onset of labor, an increase in labor time and a significantly increase in maternal perinatal mortality. Regarding the treatment-associated fetotoxicity only the group exposed to ASA 260 mg/kg/day showed a significant increase in stillborn pups and peripartum death. Additionally, ASA and SA were well-tolerated in all treatment groups No substantial potency difference between the salicylate congeners, ASA and SA could be established only the differences in toxicity profile were evident.

Study quality and assessment: The study is well-described but no information about CAS no. and purity could be found regarding sodium salicylate and acetylsalicylic acid. Overall the quality of the study is assessed to be high and from the result found it provides a moderate evidence of adverse effect on maternal reproduction and fetotoxicity including prolongation of labor and gestation after SA exposure.

Abend et al. (1991)

Summary: The aim of this study was to examine the feedback mechanism from T₃/T₄ on TSH by looking at the role of type II 5'Deiodinase (5'D-II). The study was carried out in 3 parts of which only one part is relevant to this summary. Three groups of male Sprague-Dawley rats were exposed to 1) Intraperitoneal injection of 40 nM NaOH vehicle (control), 2) Intraperitoneal injection of 2 µmol/100g 3-methyl-4',6-dihydroxy-3',5'-dibromo-flavone (EMD 21388) and 3) 30 mg/100 g sodium salicylate administered by oral gavage. All rats were sacrificed 1 h after exposure by decapitation and trunk blood and pituitaries was collected. From serum, values of thyroid-stimulating hormone (TSH), total T₄, total T₃ and free T₄ was measured by Radioimmunoassay (RIA) and the level of salicylates was determined by a modification of Trinder's method with a reference to an earlier paper. The results from the study showed that administration of salicylates significantly decreased the serum total T₄ concentration.

Study quality and assessment: Although sodium salicylate was not the main focus of the study, the findings are relevant to this summary because a significant decrease in the serum total T₄ concentration was observed (value of free T₄ is not given for salicylate). In general the article has a number of shortcomings for instance, the age and number of animals used is not given in the text, only body weight is mentioned and no information on CAS number and purity of the chemical used could be found. Overall the study is assessed to be of medium quality and it provides moderate evidence for a thyroid disrupting MoA after exposure to sodium salicylic acid.

Overman and White (1983)

Summary: The aim of this study was to compare the teratogenic effect of methyl salicylate in hamsters after oral and topical exposure. Virgin female hamsters were individually mated and the pregnant rats were grouped into either an oral or topical treatment group. For Oral exposure (by intubation) two dose levels were established at 0 mg/100 g bw (control) and 175 mg/100 g bw. For the topical application four groups were established at levels of 0 mg/100 g bw (control, shaved and treated with saline solution and washed after 2 h.), 0 mg(control, anesthetized with Nembutal and shaved), 350 mg/100 g bw (applied to a clipped area and washed after 2 h), 525 mg/100 g bw (applied to a clipped area and washed after 2 h). All exposures to methyl salicylate were conducted once at 7 days and 9 hours into the pregnancy and at day 9-12 all animals were sacrificed. To monitor if the topical and oral doses were received by the animals, blood samples were obtained on a regular basis by heart puncture under light ether anesthesia and centrifuged for spectrophotometrically reading and to a 50 µl blood sample, 1 ml of modified Trinder reagent was added which precipitates protein and gives a color if salicylate is present. To determine if salicylate also reached the embryos an assay was done with a pooled sample of fetuses (oral treatment group) and compared to maternal salicylate levels from blood samples taken at the same time (assayed by the same procedure). Morphological examinations of the embryos were done on day 9 of gestation. The study result showed similar embryo malformations in both treatment groups and included both the cranium and spina where

failure of closure involved the midbrain region. The monitoring result showed a higher plasma concentration and a faster peak of salicylate after oral exposure than after topical treatment and regarding the comparison of salicylate levels in fetus and mother, it showed that salicylate was reaching the fetus but in lower concentration.

Study quality and assessment: The study is well-described but more information on housing conditions, CSA no. and purity of the substance used would have been preferred. The number of maternal animals used and the size of each litter are not given in the text or any table but the number of litters can be found in table 1. No statistics were conducted. Overall the study is assessed to be of medium quality. The study provides moderate evidence for developmental adverse effects on skeletal malformations after oral and topical exposure to methyl salicylate and no evidence for ED related adverse effects.

Didolkar et al. (1980)

Summary: This study examines the effect of Aspirin (acetylsalicylic acid) on spermatogenesis in rats. The study examined two age-groups of male albino rats (n=12), Norwegian strain. Group one: Immature male rats, age 21-24 days and Group two: Adult male rats of proven fertility - both groups received doses of Aspirin at 0 mg/100 g bw (control) and 5 mg/100 g bw by oral administration once daily for 30 days. All animals were killed 12-13 h after the last exposure and testis was excised, blotted and weighed. Samples from testis were collected for histology, assays for hyaluronidase, β -glucuronidase and sorbitol dehydrogenase were conducted and testicular nucleic acids were extracted and estimated. The result from the study shows that Aspirin caused a significant ($P < 0.05$) decrease in testicular weight in the group of immature rats. A decrease in the activity of testicular enzymes was observed for hyaluronidase and sorbitol dehydrogenase in both groups. Regarding spermatogenesis, for both groups, Aspirin caused an impairment of the later stages by a decrease in numbers of spermatids and morphologically it was registered that the nuclei of the pachytene spermatocytes were increased (statistically insignificant).

Study quality and assessment:

The study is well-described but assessed to be of medium quality due to the lack of information regarding CAS no. and purity of the substance used, and there is no information on general toxicity in the animals. The study provides strong evidence for adverse effects on spermatogenesis after Aspirin exposure.

Balasubramanian and Ramakrishnan (1979)

Summary: The aim of the study was to investigate the effect of acetylsalicylic acid (Aspirin) individually and in combination of prostaglandins (PGs) on carbohydrate and thyroid metabolism in rats. An *in vivo* and an *in vitro* study were performed, but the *in vitro* part and parts of the *in vivo* study focused on glucose and glycogen and will not be included here. The following groups were established: Control (*ad libitum* feeding), control (*pair-fed*), Aspirin 30 mg/day for 6 weeks (Chronic), Aspirin 60 mg as single dose (acute group), PGF_{2 α} 100 μ g intraperitoneal, PGE₂ 100 μ g intraperitoneal (IP), Aspirin (60 mg, single dose) + PGF_{2 α} , Aspirin (60 mg, single dose) + PGE₂ and a withdrawal group with 2 exposure-free weeks following exposure to 30 mg/day for 6 weeks. Aspirin was administered through the diet and the exposure to radioactive iodine (Na¹³¹I) and (U-14C) glucose (5 μ Ci) was administered by intraperitoneal injection. Parameters measured for the *in vivo*

part included %uptake of injected radioactive iodine in thyroid and serum protein-bound iodine. The result from the study showed a decrease in percentage uptake of injected Na¹³¹I and plasma protein-bound iodine (PBI) by the thyroid gland in the groups exposed to Aspirin (acute and chronic) and Aspirin + PGs but no effect on the parameters was seen in the PGF_{2α} and PGE₂ groups. In the group where Aspirin was withdrawn from the diet the two parameters were restored to normal levels.

Study quality and assessment: The description of the study has a number of shortcomings starting with the section of materials and methods where information regarding treatment, size of the groups, housing conditions and procedures for preparation and administrations of PG, radioactive iodine and IP injected glucose is referred to earlier studies. No report of CAS no. and purity could be found for the substance used. Based on the lack of information the study is assessed to be of low quality but it provides moderate evidence for a thyroid disrupting MoA of Aspirin

Beall and Klein (1977)

Summary: This study was designed to determine if maternal food restriction would enhance the teratogenic effects of salicylic acid. Charles River, CD rats were mated and day 0 of pregnancy was determined by sperm in vagina. From a group of 49 pregnant rats four groups of similar size were established and received I) Food *Ad Libitum* (control), II) Food *Ad Libitum* + 250 mg/kg acetylsalicylic acid adm. orally by gavage (0.5 ml/100 g bw) suspended with vehicle (2,5% aqueous Tween 80) from day 7-10 of pregnancy, III) Restricted food (6 g/day) from day 6-15 after mating along with vehicle (control) and IV) Restricted food + 250 mg/kg acetylsalicylic acid administration orally by gavage (0.5 ml/100 g bw) from day 7-10 of pregnancy. Food consumption was measured on a daily basis, all rats were weighed every 3 days and on day 21 after mating all rats were killed. Parameters investigated included offspring abnormalities, number of live pups, sex, bw of the pups, litter size and resorption sites. For examination of skeletal defects two-thirds of the pups from each litter were fixed and stained with alizarin red and for soft tissue examination the rest were fixed in Bouin's solution and examined by serial slicing. The result from the study showed statistical significant values for increase in resorption sites for group IV, reduced mean body weight of pups in group II and increase in developmental defects (rib abnormalities, craniorachischisis and umbilical hernia, eye defects) in both Aspirin-treated groups. The combination of food restriction and exposure to Aspirin increased the incident of abnormalities from 24.4 % in group II (=32 pups) to 66.3 % (=59 pups) in group IV.

Study quality and assessment: The study is well-described but more information on housing conditions and the age of the rats would have been preferred and there was no report of CAS no. and purity of the substance used. The study is assessed to be of medium quality and it provides a moderate evidence for developmental adverse skeletal and soft tissue effects induced by Aspirin.

Wilson et al. (1977)

Summary: This study investigates the embryo toxicity and comparative distribution of acetylsalicylic acid in pregnant rats and rhesus monkeys. For all animals, gestation day 0 was assigned when sperm was found in vaginal lavage. First, two studies on embryotoxicity were performed. In the rat study, a weight adjusted volume of acetylsalicylic acid (suspended in 0.3% aqueous solution of carboxymethyl-cellulose) was administrated orally (by gavage) twice a day on gestation day 9-12 at doses of 0 (control), 100, 150, 175 and 200 mg/kg (2-8 litters/dose gr.). Embryo removal was

conducted at 1, 2, 4, 8 or 17 h after last exposure on GD12 or they were allowed to continue their pregnancy and removed on GD20. Blood samples for preparation of plasma were taken by cardiac puncture under light ether anesthesia at 1, 2, 4, 8 and 17 h after exposure. For the pregnant monkeys (n=8) acetylsalicylic acid was administered orally (by gavage) twice a day on gestation day 23-32 at doses of 100 and 150 mg/kg (no report of a control group). Blood sample for serum preparation was taken by venipuncture at day 4, 5 or 10 at 1, 2, 4, 8 and 17 h after gavage. Hysterectomy was performed at same intervals after the last gavage. For both groups of animals, parameters measured were plasma binding, and concentration of salicylic acid in plasma, placenta and embryonic tissues and finally all fetuses were examined for external, visceral and skeletal abnormalities. Monkey embryos were additionally examined for heart beat. To compare the distribution of acetylsalicylic acid in rats and monkeys, an additional rat study was performed and similarly to the monkey study 100 mg/kg was chosen as the low dose and 150 mg/kg was set as the high dose in this rat study. The study result from the rat part showed a significant effect on intrauterine death, growth and malformation (cardiac, brain and skeletal) at doses of 150 and 200 mg/kg. Maternal plasma concentration was significantly higher in non-pregnant than pregnant rats at 100 mg (2 and 17 h after dosing) and 150 mg/kg (after 2 h) and the teratogenic (maternal) dose of 150 mg/kg resulted in an embryo concentration greater than 60 µg/g maintained for minimum 16 h/day. In monkeys both exposure doses (100 and 150 mg/kg) resulted in a slight increase in intrauterine death and transitory growth retardation. Maternal plasma concentration was not consistent but showed a significant difference between non-pregnant and pregnant animals at 100 mg/kg with a significant higher concentration in non-pregnant at 2, 4 and 8 h after dosage. The mean embryo concentration never exceeded 36 µg/g in monkeys. When the two groups of animals were compared, the highest value of unbound salicylate was found in rat plasma.

Study quality and assessment: Overall the study is well described although information on CAS number, and purity of substance used was not available and more information on housing conditions would have been preferred. The study is assessed to be of medium quality and since the exposure doses were well tolerated by the maternal animals at levels below 200 mg/kg the study provides high evidence for adverse effects on embryonic development, growth and survival in rats and moderate evidence for adverse effects on embryonic growth and survival in monkeys.

Tuchmann-Duplessis et al. (1975)

Summary: The aim of the study is to look at the effects of prenatal administration of acetylsalicylic acid (ASA) in rats. Two groups of pregnant rats (COBS CD Charles River) were established and randomly divided in two dose groups (n=16/group), 0 mg/kg/day and 200 mg/kg/day. ASA, suspended in 1% tragacanth gum, was administered by gastric intubation twice a day starting on day 15 until the end of pregnancy. Registrations were made on gestation length, parturition time and effects on offspring (stillborn and death). The results revealed a statistical significant difference between the two groups with a prolongation of pregnancy in treated dams. The parturition time was also prolonged and in the treated group 2 out of 16 dams died due to an extended period of contractions. No significant observations were registered regarding effect on offspring survival. The dams tolerated the treatment well with no mortality or change in behavior during exposure.

Study quality and assessment:

The study is well-described although the purity of the tested substance is not reported and more information on housing conditions would have been preferred. Overall the study is assessed to be of medium quality and it provides strong evidence for adverse effects on gestation length and parturition.

Larsen, P.R. (1972)

Summary: This study was conducted to clarify the effect of SA on the protein binding of the two thyroid hormones triiodothyronine (T₃) and thyroxine (T₄). An *in vivo* and an *in vitro* experiment was conducted. In the *in vivo* part of the study Aspirin was administered to humans (n=2) for a period of 8-10 days in quantities sufficient to obtain a serum salicylate level of 20-25 mg/100 ml. Three baseline determinations were obtained during a 6 day control period prior to the study. During the period of treatment samples of serum were collected every other day and the free T₃ and free T₄ was estimated by ultrafiltration (UF). The results from the study showed an immediate and persistent increase in the UFT₃ and UFT₄ in both humans.

Study quality and assessment: The study is not described in a structured way and it contains several references to earlier studies for description of methods used. There was no report of batch number of the Aspirin used. The *in vivo* part is assessed to be of medium quality. Although only two subjects were assigned to the study, it provides moderate evidence for thyroid ED MoA after Aspirin exposure.

Collins et al. (1971)

Summary: The aim of this study was to investigate the effect of methyl salicylate on rat reproduction. The study included a main part and a supplemental study but due to a mixed-compound exposure in the supplemental study, the result was found to be non-relevant and only the main part will be included in this summary. Osborne-Mendel rats were divided in groups of 20 pair (litter mated) and fed methyl salicylate through the diet for 100 days prior to mating at the levels of 0, 500, 1500, 3000 and 5000 ppm. Two litters, F_{1a} and F_{1b} were produced by F₀ and on day 4 all litters were reduced to include maximum 10 pups per litter. At weaning F_{1b} were pair-housed and mated (20 pairs per group). Same procedure was followed for the following generations. Parameters investigated were fertility index (number of litters cast/number of females exposed to mating), Litter size, number of live born, viability index (number of live-born/total number born), surviving from day 0-4, survival index (number alive at day 4/number born alive), number of progeny weaned at day 21, weight of weanlings and abnormalities by external examination. Autopsy and histopathological examination (liver and kidney) was only performed on the weanlings from the third generation. The results from the study revealed significant findings at dose levels of 3000 and 5000 ppm regarding a decrease in the average litter size, number of live-born progeny, number of survivors to day 4 and number of survivors to day 21. The decrease in number of live-born appeared to be dose-related. At the lower dose levels only a non-significant decrease was observed.

This paper is included in the REACH registration dossier for salicylic acid on toxicity to reproduction.

Study quality and assessment: The study has a number of shortcomings. For example, the details on diet preparations, the description of the 3 generation study and the reason for the choice of concentrations refers to earlier studies and is only roughly described in the text. No report of CAS number and purity of the substance used could be found. Overall the study is assessed to be of medium quality and it provides moderate evidence for reproductive adverse effect after exposure to methyl salicylate during pregnancy.

Hansen and Mogensen (1964)

Summary: This study investigated the effect of sodium salicylate on the uptake of ¹³¹I-labelled 1-triiodothyronine by human erythrocytes. Human patients (n=9) were given 1 g sodium salicylate three times a day for 4 days and serum concentrations of salicylic acid were measured. From a 20 ml venous blood sample, 4 portions of erythrocytes (2 x washed + 2 x unwashed) were prepared for use in a scintillation counter. To all samples ¹³¹I-labelled 1-triiodothyronine was added in the process (concentrations kept below 0.1 µg/ml). The study result showed an increase of triiodothyronine (T₃) uptake in erythrocytes of patients given salicylate.

Study quality and assessment: The the study is assessed to be of medium quality. No information on CAS number and purity of the substance used was given and there is no report of any control group. The evidence for a thyroid disrupting MoA is moderate.

Warkany and Takacs (1959)

Summary: The aim of the study was to conduct an experimental production of congenital malformations in rats by salicylate exposure. The study was composed of two experimental parts. In the first part 116 female rats were mated and the presence of sperm in vagina marked the first day of pregnancy. One single dose of 0.1-0.5 cc (cubic centimeter) methyl salicylate was administrated subcutaneously on gestation day (GD) 9, 10 or 11. In the second study 43 pregnant rats received a single dose of 60-180 mg sodium salicylate also administrated subcutaneously on gestation day 9, 10 or 11. For control group, 105 females were used. The results obtained in the first part (methyl salicylate) showed a weight reduction in most of the pregnant females of 15-25 g after treatment, 26 of the 116 dosed animals died and 47 had fetal resorptions. The last 43 pregnant rats were sacrificed on gestation day 21 and 298 progeny were obtained. At examination 45 of the offspring were externally visible malformations and in the remaining 253 pups that appeared externally normal, 75 showed skeletal abnormalities. In the second study (sodium salicylate) 6 of the 43 rats died (all dosed more than 120 mg) and 24 had fetal resorptions. The remaining 13 were sacrificed on GD 21 and 100 progeny were obtained. At examination 15 were externally abnormal and in the group of 85 that was externally normal, 11 had skeletal abnormalities. In both groups malformations that were encountered were eye defects, fascial clefts, hydrocephaly, exencephaly, gastroschisis and irregularities of the vertebrae and ribs. In the control group of 105 female rats, 59 produced a litter and 484 progeny were obtained. No externally malformations were observed with the exception of 3 which had “wavy ribs”

Study quality and assessment:

The study has a number of shortcomings starting with no information on CAS number or purity of the two substances used. More information on housing condition, and the allocation of rats into dose groups would have been preferred and from the information given in the article it seems that the doses given is administrated with no consideration to body weight. Moreover, all progeny were pooled for

each group and litter effects were not taken into account. All data is only described in the text and not presented in any tables and no statistical analysis was conducted. The study quality is assessed to be low and due to the number of dead dams and resorptions it provides a weak evidence for adverse effect on fetal development.

Epidemiology study

Kristensen et al. (2011)

Summary: This study consisted of 3 parts (*in vivo*, *ex vivo* and epidemiology) and the epidemiology part will be described here. The focus of the study was to evaluate maternal use of mild analgesics during pregnancy in relation to congenital cryptorchidism in humans. A prospective birth cohort study was conducted in collaboration between the university hospital of Copenhagen (Rigshospitalet and Hvidovre Hospital) and the Turku University Central Hospital in Finland with the use of a self-administrated questionnaire (assessing the use of mild analgesic by indication, name, dosage, and gestational week of administration), completed by 2297 women from both countries and a computer-assisted interview over the telephone (addressing the use of analgesic), where 491 of the Danish mothers participated. Following criteria were established to obtain a genetically well-defined population: both the parents and grandparents of the unborn child should have been born and raised in Finland or Denmark with a maximum residence abroad of 10 years for the grandparents and father and 3 years for the mother. A total of 2521 mothers entered the Danish part of the study and 1071 boys were examined, of those 5 were excluded as dependent cases and 26 excluded due to missing data. From Finland a total of 2728 mothers participated were 1499 boys were examined and from that group 25 were excluded as dependent cases along with 4 due to missing data. The assessment of the testicular position in the newborns was performed by trained pediatricians. In the Danish part, findings in the self-administrated questionnaire indicated that many mothers strongly under-reported their use of analgesic unless they were specifically asked and for that reason only the results from the computer-assisted telephone interview were taken into account. The data from that part, showed that the use of mild analgesic was dose-dependently associated with congenital cryptorchidism and especially the use during the second trimester increased the risk - for acetylsalicylic acid data was reported to be significant. In the finish cohort the same association could not be identified, only a trend was seen in the second trimester.

Study quality and assessment: The study is well-described and is assessed to be of high quality. The study provides strong evidence of adverse effects of acetylsalicylic acid on male sexual development leading to congenital chryptorchidism.

Other studies

In addition to the above described studies a number of experiments have been conducted where the results are not directly related to an endocrine disrupting (ED) action but a possible contribution cannot be excluded.

Schardein et al (1969) presented a study with focus on male/female fertility (rats), the teratogenic potential (rats and rabbits) and the effect of treatment in the perinatal and postnatal period (rats). It should be noted that treated animals in all groups showed moderate to severe reduction of weight gain. Skeletal malformations, reduced litter size and reduced viability of the pups were noted and for the dams treated with aspirin in large doses (> 210 mg/kg) all pregnancies resulted in resorption of all fetuses.

Cappon et al. (2003) conducted a study with focus on comparing the developmental toxicity of Aspirin (acetyl salicylic acid) in rabbits when it was administered throughout organogenesis or during sensitive windows of development. A repeated dose study was conducted on GD 7-19 with doses of 125, 250 and 350 mg/kg and a single dose study was conducted on day 9, 10 or 11 with dose levels of 500, 750 or 1000 mg/kg. On GD 29 cesarean sections were performed and an examination of fetuses was done with focus on external, visceral and skeletal development but the results from the study showed no malformations associated with the exposure to Aspirin.

Erikson (1970) investigated the role of dosage and the frequency of administering on the prenatal effect in rats produced by salicylate. Late pregnancy effect in the fetuses included superficial liver and gastric hemorrhage and vessel abnormalities and increased death.

REACH Registration Dossier

Toxicity to reproduction :

Outline:

Following studies and reviews from “Toxicity to reproduction” in the REACH registration dossier has already been included here (*Collins et al.1971 and Chapin and Sloane 1997*) and summaries of their data can be found under the section for *in vivo* studies or reviews. The applicant also reported data from several additional studies and study reports that were not possible to retrieve in a search conducted up to the 15/11-2017. Based on information available in the REACH registration Dossier a short summary of all the additional studies has been added below.

Summary:

The studies did not add significant value or new information to the ED MoA or endocrine-related adverse effects on salicylic acid. In general the studies show a dose-related decrease in the average litter size and pup weight, effects on offspring viability and some studies investigated the effect on male/female reproduction but without any significant findings.

Study quality and assessment:

The quality of the unavailable studies cannot be assessed based on summaries available on REACH registration dossier. They all report to have a minimum of 20 animals/ dose group and a few is performed under GLP and follow a guideline. In the experiments that observe some changes, half of them report that the findings were significant so in general the studies are assessed to provide a moderate evidence for adverse effect on reproduction.

Developmental toxicity / Teratogenicity :

Outline:

Following studies and reviews from “Developmental toxicity / Teratogenicity” in the REACH registration dossier has already been included here (*Cappon et al.2003, Gupta et al.2003, Eriksson et al.1971 and Shardein et al.1969*) and summaries of their data can be found under the section of *in vivo* and *in vitro* studies. The applicant also reported data from several additional studies and study reports where some of them were available but did not provide any relevant data on ED endpoints and others were not possible to retrieve in a search conducted up to the 15/11-2017. Based on information available in the REACH registration Dossier a short summary of all the additional studies has been added below.

Summary:

Overall the studies did not add significant value or new information to ED MoA or endocrine-related adverse effects on salicylic acid. Most of the studies were conducted according to OECD guideline 414 and the study design of the remaining studies did not appear to follow a guideline. In general the studies provided information on decreased litter size and neonatal body weight, increased labor time, increased resorptions, skeletal abnormalities (vertebral and costal bones), hydrocephalus, delayed ossification, internal abnormalities (kidney), subcutaneous, liver and gastric hemorrhage and a reduction in fetal liver glycogen. A few studies presented new data showing that increasing dietary zinc reduced the teratogenic effects of salicylate, but in patterns different to strain (Wistar or Sprague-

Dawley) and from that suggesting that marginal zinc deficiency in certain pregnant women might increase the possibility of salicylate teratogenesis (Hackman et al.1984) but it was also concluded that Zinc deficiency did not cause any lesions detectable in fetal kidneys (Gossrau et al.1988). The new results presented do not contribute with results that are relevant for the ED MoA of Salicylic acid.

Study quality and assessment:

The quality of the studies cannot be assessed based on summaries available on REACH registration dossier but the largest part of the studies contained 15 animals or more per dose. Overall the additional part of the studies does not provide information relevant to the ED effect of Salicylic acid.

Other studies:

Outline:

A large part of the studies presented under “Other studies” in the REACH registration dossier were not possible to retrieve in a search conducted until the 15th of November 2017 and studies with endpoints that were considered to be of non-endocrine disruptive character has not been included here (abnormality of tail, facial malformations, decreased heart beat and focus regarding mouse palatal)

Repeated dose toxicity – Dermal + Oral:

Outline:

Overall the studies under “Repeated dose toxicity” in the REACH registration dossier, presents data that show an effect on the kidneys, liver, skin (dermal lesions) and bones (density and lesions). Data did not contribute with results that are relevant for the ED effect of Salicylic acid.

Reviews

Reviews on SA have been evaluated in the search for additional relevant studies not found in the literature search (Kristensen et al. 2016; Lapczynski et al. 2007; Belsito et al. 2007; SCCNFP 2002; Chapin and Sloane 1997). One of the reviews is included in the REACH registration dossier for Salicylic acid on toxicity to reproduction (Chapin and Sloane 1997).

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

The androgenic activity is also investigated *in vitro* and *ex vivo* and the studies showed decreased testosterone production after acetylsalicylic acid exposure in all cases except one. The three studies showing a decrease includes investigations in H295 cells (Albert et al.2013) and *ex vivo* rat fetal testicular tissue (Kristensen et al. 2012 and Kristensen et al. 2011). However, *ex vivo* studies in human testis did not show clear reductions in testosterone production after acetylsalicylic acid exposure. One study showed a reduction in testosterone levels in adult testis but it did not reach statistical significance (Albert et al.2013) and another study found results showing that Aspirin stimulated testosterone production in testis from gestation week 8-9 but no effects were seen in older fetal testis (Mazaud-Guittot et al. 2013). It is unclear whether the differences in effects on *ex vivo* testosterone levels are due to species differences or if there is a difference in either the methods used or the sensitivity of the testis at different time-points. Taken together, both the *in vitro* and the *ex vivo* data provide moderate evidence of an anti-androgenic mode of action of acetylsalicylic acid. The human studies available show that Aspirin significantly inhibit the androgen response to hCG stimulation in humans (Conte et al. 1991) and a significant decrease in testicular weight together with a decrease in the activity of testicular enzymes and an impairment of the later stages in the spermatogenesis were found by (Didolkar et al.1980). The adverse effects observed in human testis are likely related to the anti-androgenic MoA of acetylsalicylic acid. When all results are taken into consideration the data provide moderate evidence for an anti-androgenic ED MoA and adverse effects after exposure to acetylsalicylic acid.

The ED MoA of Salicylic acid has been investigated in several *in vivo*, *ex vivo* and *in vitro* studies (Table 1). Starting with the results from *in vitro* studies concerning the thyroid function, some of the studies found that exposure to salicylates increased the free fraction of T₃ and T₄ (Larsen et al.1972). Going further into details, other studies found that salicylate affects the binding capacity between T₄ and TBPA (Wolff et al.1961) and that T₄ then is displaced to TBG (Wolff et al.1961). A similar action of salicylate on the binding of T₃ to TBPA and TBG was shown (Larsen et al. 1972) and it appears that this leads to an increased binding to erythrocytes *in vivo* (Hansen and Mogensen, 1964). Available human studies provide results that back up the *in vitro* findings. A human study showed an increase in free T₃ and T₄ after Aspirin exposure and this was in line with their *in vitro* results (Larsen et al.1972). Furthermore, studies showed a decrease in total serum T₄ concentration (Abend et al. 1991) and increased uptake of T₃ by erythrocytes after salicylate treatment in humans (Hansen and Mogensen, 1964). Together, both the *in vitro* and the human studies provide strong evidence of a thyroid disrupting mode of action of salicylates and when all data are taken together, they provide strong evidence of a thyroid disrupting MoA of salicylates. No studies investigated endpoints relevant for evaluation of adverse effects related to thyroid disruption were found (Table 2).

In conclusion, salicylates meet the WHO definition of an endocrine disruptor with anti-androgenic ED MoA leading to adverse effects.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of Salicylic acid (SA).

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In Vitro</i>	<i>In Vivo</i>		
Albert et al 2013	The production of testosterone by human testis revealed a decreased level but did not reach statistical significance. In the NCI-H295R cell line, exposure to 10 ⁻⁵ and 10 ⁻⁴ M Aspirin significantly reduced testosterone production. A reduction was also seen in the levels of INSL3, PGD ₂ , PGE ₂ and Inhibin B production.		High	moderate
Mazaud-Guittot et al. 2013		Aspirin showed a significant dose-response relationship by increasing the level of testosterone in the youngest fetal testis (8-9.86 GW). Anti-Müllerian Hormone production was strongly stimulated. PGE ₂ was significantly inhibited	High	Strong
Kristensen et al. 2012		Decreased testosterone levels in rat fetal testis were found at all Aspirin concentrations. For PGD ₂ , Aspirin led to a modest decrease in the production at all time-points	High	Strong
Kristensen et al. 2011		The results showed reduced AGD compared to control but due to fetal growth retardation AGD was undetectable in a number of fetuses and statistical data are not presented. A significant reduction of testosterone was measured.	High	Strong
Kristensen et al. 2011		Dose dependent reduction in testosterone and PGD ₂ production in rat fetal testis with a significant result for testosterone at 10 µM Aspirin at all time points and a significant result for PGD ₂ at 48 and 72 h.	High	Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In Vitro</i>	<i>In Vivo</i>		
Conte et al. 1999		Aspirin significantly lowered the seminal level of PGE ₂ and significantly inhibited the androgen response of testosterone, 17 OH-progesterone, androstenedione and dehydroepiandrosterone to hCG stimulation in humans.	High	Strong
Abend et al 1991	The result did not provide evidence for the administrated doses of salicylates to directly inhibit enzyme activity.		Medium	none
Abend et al 1991		Administration of salicylates significantly decreased the serum total T ₄ concentration.	Medium	Moderate
Balasubramanian and Ramakrishnan 1979		Decreased percentage uptake of injected Na ¹³¹ I and plasma PBI by the thyroid gland in the groups exposed to Aspirin (acute and chronic) and Aspirin + PGs.	Low	Moderate
Larsen et al. 1972	Five smaller studies all confirmed the endpoint that addition of salicylate to human sera caused an increased in free T ₃ and T ₄	Increase in free T ₃ and T ₄ after administration of Aspirin to humans (n=2)	medium	In vitro – Strong In vivo – Moderate
Hansen og mogensen 1964	Increased uptake of ¹³¹ I-labelled 1-triiodothyronine by human erythrocytes after addition of sodium salicylate to human donor blood.	Increased uptake of ¹³¹ I-labelled 1-triiodothyronine by human erythrocytes in blood from humans exposed to sodium salicylate	Medium	Moderate
Wolff et al.1961	At low T ₄ concentrations in human serum most of the T ₄ was displaced from TBPA onto TBG by addition of natrium salicylate. In the situation where T ₄ was present in higher amounts TBG became saturated and T ₄ was further displaced to albumin.		High	Strong

Insulin-like growth factor 3 (INSL3), Prostaglandin D₂ (PGD₂), Prostaglandin E₂ (PGE₂), anogenital distance (AGD), hCG, protein-bound iodine (PBI), triiodothyronine (T₃), thyroxine (T₄), thyroxine-binding pre-albumin (TBPA), thyroxine binding globulin (TBG).

Table 2. Overview of potential endocrine-related adverse effects of Salicylic acid (SA):

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Gupta et al. 2003	Rats	The results from the study showed a high concordance between Wistar and SD rats regarding developmental anomalies with the exception to hydrocephalus in Wistar rats and the VSD in the SD rats. Whether acetylsalicylic acid was administered as a single dose or during the organogenesis (GD 6-17), the malformations were similar. Hypo-plastic testes were seen in 2 out of 137 fetuses and only in the highest dose group	High	Weak
Davis et al. 1996	Rats n=105	The results of the study showed that the groups exposed to 200 mg/kg/day SA and 260 mg/kg/day ASA had a delay in the onset of labor, an increase in labor time and a significantly increase in maternal perinatal mortality. Regarding the treatment-associated fetotoxicity only the group exposed to ASA 260 mg/kg/day showed a significant increase in the number of stillborn pups and peripartum death.	High	Moderate
Overman and white 1983	Hamsters	Failure of closure of the neural tube resulting in cranium bifidum and/or spina bifida	Medium	None
Beall and Klein (1977)	Rats n=49	Increase in resorption sites for group IV, reduced mean body weight of pups in group II and increase in developmental defects (rib abnormalities, craniorachischisis and umbilical hernia, eye defects) in both Aspirin-treated groups. The combination of food restriction and exposure to Aspirin increased the incident of abnormalities from 24.4% in group II (=32 pups) to 66,3% (=59 pups) in group IV.	Medium	Moderate
Wilson et al. 1977	Rats (n= 4-8) Monkeys (n=8)	The study result from the rat part showed a significant effect on intrauterine death, growth and malformation (cardiac, brain and skeletal) at doses of 150 and 200 mg/kg and for the monkey part the results showed that both exposure doses (100 and 150 mg/kg) resulted in a slight increase in intrauterine death and transitory growth retardation.	High	Rat study : Strong Monkey study: Moderate

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Tuchmann-Duplessis et al. (1975)	Rats n=32	The results revealed a statistical significant difference in the two groups with a prolongation of pregnancy for the treated dams. The parturition time was also observed to be prolonged and in the treated group 2/16 dams died due to extended period of contractions.	Medium	Strong
Collins et al. 1971	Rats 3.generations	Significant findings was observed at dose levels of 3000 and 5000 ppm regarding a decrease in the average number of litter size, number of live-born progeny, number of survivors to day 4 and number of survivors to day 21. The decrease in number of live-born appeared to be dose-related. At the lower dose levels only a non-significant decrease were observed.	Medium	Moderate
Didolkar et al. 1980	Rats n= 24	The result from the study shows that Aspirin caused a significant decrease in testicular weight in the group of immature rats. A decrease in the activity of testicular enzymes was observed for hyaluronidase and sorbitol dehydrogenase in both groups. Regarding spermatogenesis, for both groups, Aspirin caused an impairment of the later stages	Medium	Strong
Warkany and Takacs 1959	Rats n=159 pregnant rats	Abnormalities (external or skeletal) were observed in 146 out of 398 progeny. Out of 159 maternal rats, 32 died and 71 resorbed their fetuses after exposure	low	weak

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