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Development of Adverse Outcome Pathways relevant for the identification of substances having endocrine disruptors properties

Barbara Viviani[1], Elena Bernardini[3], Valentina Galbiati[1], Ambra Maddalon[1], Gloria Melzi[1], Miriam Midali[1], Melania Serafini, [1] Emanuela Corsini[1], Roberto Cosimo Melcangi[1], Eugenio Scanziani[2]

[1]Università degli Studi di Milano (UMIL), Dipartimento di Scienze Farmacologiche e Biomolecolari - DiSFeB, Via Balzaretti 9, 20133 Milan, Italy

[2]Università degli Studi di Milano (UMIL), Dipartimento di Medicina Veterinarie e Scienze animali - Via Dell'Università, 6, 26900 LODI (LO)

[3]Università degli studi di Milano (UMIL), Biblioteca del Polo centrale di Medicina e Chirurgia - Via Della Commenda, 19, 20122 Milan, Italy

Abstract

This report is the outcome of an EFSA procurement (NP/EFSA/PREV/2020/01) which aims at contributing to the development of adverse outcome pathways (AOPs) to be integrated in a network addressing uterine adenocarcinoma in mammals. The outcome is intended to support the identification of substances with endocrine disruptor mode of action. For this specific purpose, an evidence-based approach methodology was adopted. Available evidence was systematically mapped from the literature to identify Molecular Initiating Events (MIEs) and Key Events (KEs) linked to adverse outcome (AO) "uterine endometrioid adenocarcinoma" independently from prototypical stressors, by means of: 1. *a priori* defined search strategies initially addressing the AO and biologically plausible MIEs, 2. application of machine learning technique (Topic modelling) that automatically analyzes text data to identify biologically plausible KERs, 3. systematic literature review and critical appraisal of prioritized evidence, taking into account human, *in vivo* and *in vitro* studies. Estradiol and tamoxifen, two recognized human risk factors for endometrioid adenocarcinoma, were used as tool chemical compounds to empirically support the response and temporal concordance of the identified key event relationships (KERs). All evidence has been then integrated by means of the AOP conceptual network. An evidence-based AOP starting from activation of uterine estrogen receptor-alfa leading to endometrial adenocarcinoma via epigenetic modulation was postulated.

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Key words: Uterine adenocarcinoma, endocrine, estrogen, topic modelling, evidence-based, critical appraisal

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Correspondence: pesticides.peerreview@efsa.europa.eu

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Amendment: the corrigenda concerns an editorial mistake (wrong affiliation of Elena Bernardini). To avoid confusion, the original version of the External Scientific Report has been removed from the EFSA Journal but is available on request.

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1. Introduction

1.1. Background and Terms of Reference as provided by EFSA

Hormone-related cancers, like breast, endometrium, ovary, prostate, testis and thyroid cancers, share commonalities in the mechanism of carcinogenesis. Endogenous and exogenous hormones drive cell proliferation, increasing the opportunity for the accumulation of random genetic errors. Progression to a malignant phenotype depends on a series of somatic mutations that occur during cell division. Therefore, endocrine disruptor (EDs) impacting hormonal homeostasis can promote target tissue cell proliferation in hormonal dependent organs, leading to neoplasms. One example is the uterine adenocarcinoma, a common human malignancy that can be induced experimentally in laboratory animals following treatment with EDs. However, a direct link between the occurrence of rodent uterine adenocarcinoma and an endocrine mode of action relevant to human is not immediate.

To identify a substance as an endocrine disruptor, the Mode of Action (MoA) framework should be followed. It means that an endocrine MoA, an adverse effect and a biological plausible link between the MoA and the effect need to be demonstrated.

An adverse outcome pathway (AOP) framework is a useful tool to link an adverse effect with a molecular initiating event. An Adverse Outcome Pathway (AOP) describes a logical sequence of causally linked events at different levels of biological organisation, which follows exposure to a chemical and leads to an adverse health effect in humans or wildlife. AOPs are agnostics and can therefore be applied to any chemical able to reach and activate the MIE. AOPs are the central element of a toxicological knowledge framework, promoted by member countries through OECD, built to support chemical risk assessment based on mechanistic reasoning. These AOPs are available in the AOP Wiki, an interactive and virtual encyclopaedia for AOP development. Following their development and review, the endorsed AOPs are published in the OECD Series on Adverse Outcome Pathways."[1] . An AOP is conceptualized as a single sequence of events proceeding from the molecular initiating event (MIE) to the adverse outcome (AO) via a series of intermediate key events (KE). An AOP network is defined as an assembly of two or more AOPs that share one or more KE(s) in common (User's handbook for developing and assessing AOPs, 2018).

In this context, EFSA launched a negotiated procedure to develop AOPs relevant for the identification of substances having ED properties leading to a uterine adenocarcinoma as AO, applying an evidence-based approach including a systematic retrieval, screening for relevance and appraisal of available evidence.

The contract was awarded by EFSA to: Università degli Studi di Milano

Contract title: "Development of Adverse Outcome Pathways relevant for the identification of substances having endocrine disruptors properties"

Contract number: *NP/EFSA/PREV/2020/01*

The specific objectives of the contract as defined by EFSA are as follows:

1. Perform a systematic review to acquire available information on toxicity pathways leading to uterine adenocarcinoma, (disruption of key events in the pathway during different life stages e.g. in utero, perinatal, puberty, adult, physiological reproductive senescence) including chemical substances interfering with the pathway
   a) Define the search string
   b) Provide eligibility criteria to be used to retrieve and screen the evidence and perform the retrieval and screening of the evidence (two reviewers working in parallel on the same paper/study should be forecast).

[1] https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcomepathways_2415170x

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   c) Identify a critical appraise tool (e.g. OHAT-NTP) to appraise the internal validity of the studies and adapt where needed. Perform the appraisal of the study (two reviewers working in parallel on the same paper/study should be forecast).
   d) Propose a data model to extract the data. Perform the data extraction according to the data model.
2. Based on the evidence identified and appraised, develop an evidence-based AOP/AOPs for uterine adenocarcinoma. AOPs are intended to support regulatory applications. The focus shall be on mammals, specifically rodent (rats and/or mice) and humans.
3. Identification of missing data. The focus shall be on those inconsistencies or gaps that would have a direct bearing or impact on the confidence in the key event relationship (KER) and its use as a basis for inference or extrapolation in a regulatory setting. Uncertainties that may be of academic interest but would have little impact on regulatory application don't need to be described but can be included as recommendation.
4. Submission of the AOP to the AOP Wiki. Since approval of AOPs on AOP-Wiki requires peer review by the OECD, the timing of this step is beyond the tenderer's control. The tenderer commits itself to follow the approval process and will inform the contracting authority about the publication of the AOPs on AOP-Wiki. The developed AOP(s) are also intended to be published in the OECD Series on AOPs in a later stage. The text of the AOP description, including the list of references, should therefore conform, to the extent possible, with the OECD Style Guide (https://www.oecd.org/about/publishing/OECD-Style-Guide-Third-Edition.pdf) (OECD, 2015).

1.2. Additional background and Interpretation of Terms of Reference

Regulatory framework

In 2002, the World Health Organisation (WHO) through its International Programme for Chemical Safety proposed a definition for endocrine disruptors (ED) (WHO/IPCS, 2002) and in 2009 a definition of adverse effects (WHO/IPCS, 2009). Upon the endorsement of those definitions by the European Food Safety Authority (EFSA, 2013), the Scientific Committee on Consumer Safety (2014) and the consensus of the scientific community, criteria for the determination of ED properties pursuant to Regulations (EU) No 528/2012[2] have been set as defined in Commission Delegated Regulation (EU) No 2017/2100[3] and Commission Regulation (EU) No 2018/605[4] (amending Annex II to Regulation (EC) 1107/2009[5]) for biocidal products and Plant Production Products). According to these criteria, a substance shall be considered as having ED properties if it accomplishes all the following conditions:

a) *it shows an adverse effect in [an intact organism or its progeny]/[non-target organisms], which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;*

b) it has an endocrine mode of action, i.e. it has the potential to alter the function(s) of the endocrine system;

c) the adverse effect is a consequence of the endocrine mode of action.

[2] Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products Text with EEA relevance. OJ L 167, 27.6.2012, p. 1–123. Available online: http:// data.europa.eu/eli/reg/2012/528/oj

[3] COMMISSION DELEGATED REGULATION (EU) 2017/2100 of 4 September 2017 setting out scientific criteria for the determination of endocrine-disrupting properties pursuant to Regulation (EU) No 528/2012 of the European Parliament and Council

[4] COMMISSION REGULATION (EU) 2018/605 of 19 April 2018 amending Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties OJ L 101, 20.4.2018, p. 33–36 Available online:http://data.europa.eu/eli/reg/2018/605/oj

[5] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ L 309, 24.11.2009, p. 1–50. Available online: http://data.europa.eu/eli/reg/2009/1107/oj

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Conclusions about whether a substance meets the ED criteria must be drawn, as requested by Regulations (EU) No 528/2012 and (EC) No 1107/2009.

Main features of the uterine adenocarcinoma

Uterine (endometrial) adenocarcinoma (UA), the most common uterine cancer, is a type of cancer that arises in the layer of cells that form the inner epithelial lining of the uterus (endometrium). Based on clinical, pathological and molecular features, uterine adenocarcinomas in humans have been classified into two broad categories: type I and type II (Sherman, 2000).

Type I adenocarcinoma (synonyms: type I carcinoma, endometrioid adenocarcinoma) is the most common type, accounting for approximately 80% of cases. It is often referred as endometrioid adenocarcinoma since it is a well differentiated (low grade) tumour characterized by a glandular growth pattern resembling normal endometrial epithelium. Clinically, is often characterized by a favourable prognosis. Type I adenocarcinoma is estrogen-dependent, estrogen-receptor-positive and arises in a background of endometrial hyperplasia. It is frequently associated with microsatellite instability accompanied by mutations in PTEN, K-RAS, and β-Catenin. It can be polypoid or infiltrative, the latter can spread transmurally through the uterine wall and to adjacent organs. Involvement of regional lymph nodes can occur and, in advanced stages, the tumour may metastasize to distant organs including lungs, liver, bones, and other organs (Robbins and Cotran, 2014).

Type II adenocarcinoma comprises about 10-20% of UA cases and has a non-endometrioid morphology. It is non-estrogen-dependent, estrogen-receptor-negative and has a serous, papillary, or clear cell morphology (Sherman, 2000). Clinically, it is characterized by an aggressive clinical course, and a propensity for early spread and poor prognosis (Bansal et al. 2009). It arises in a background of endometrial atrophy. Type II adenocarcinoma is associated with TP53 mutation, P16 inactivation, HER-2/neu overexpression, E-cadherin reduced expression. This classification that sub-divide uterine adenocarcinomas into types I and II is not completely accurate since a minority of endometrial cancers may exhibit shared characteristics (Bansal et al. 2009).

The dependence of Type I uterine adenocarcinoma on estrogens is supported by different epidemiologic observations:

- the raised incidence rate for uterine adenocarcinoma in the population in conjunction with widespread use of unopposed estrogen replacement therapy in menopause (Sherman 2000)
- the decreased incidence with the decline in the use of this hormone preparations (Sherman 2000)
- the increased risk of UA as a consequence of the use of oral contraceptives based on estrogens (Weiss et al. 1976; 1980)
- the 2-to 3 times increase in the relative risk of developing UA in patients under tamoxifen therapy, due to its agonistic action on estrogen receptors in endometrial tissue (Passarello et al. 2019).

Prolonged estrogenic stimulation of the endometrium is associated with atypical endometrial hyperplasia (Sherman 2000). Endometrial hyperplasia is characterized by an increased (pathological) proliferation of the endometrial epithelial cells compared with normal proliferative endometrium. It can be subdivided in two morphological categories: non-atypical hyperplasia and atypical hyperplasia, a pre-neoplastic lesion associated with an increased risk of developing type I uterine adenocarcinoma.

Further details are reported in EFSA PPR Panel et al. 2023.

Endocrine disruption and cancer

Hormones, by promoting cell proliferation, allow the accumulation of random genetic mutations due to DNA replication errors, which may give rise to a malignant phenotype (Henderson et al., 1982). Not only endogenous hormones, but also exogenous hormones and chemicals are held accountable for carcinogenesis (Diamanti-Kandarakis et al. 2009; Gore et al. 2015). Against this background, endocrine-disrupting chemicals (EDs) can pose a significant concern to the possible carcinogenic health threat on hormone-sensitive organs and tissues (Diamanti-Kandarakis et al. 2009; Gore et al. 2015). The mechanisms by which endocrine

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disruptors exert their effect can include those acting via nuclear receptors, non-nuclear steroid hormone receptors, non-steroid receptors, orphan receptors, enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that converge upon endocrine and reproductive systems (Diamanti-Kandarakis et al. 2009).

Epidemiological studies suggest a potential role for estrogenic EDs in increased risk of uterine endometrial adenocarcinoma, although the understanding of the direct role of EDs and the molecular mechanisms involved are still inconclusive (Gore et al. 2015).

The relevance of animal models in uterine adenocarcinoma

Additional information about the possible role of EDs on the onset of UA comes from animal studies. In particular, the rodent can be considered a biologically useful model for human UA. Although some differences in the gross anatomy of the female reproductive tract and cycle between humans and rodents exist, there are also common features that make animal models biologically relevant to be representative of pathways relevant for UA in humans (Table 1. Treuting et al., 2017; Koeble and Bimonte-Nelson 2016; vom Saal et al. 1994; Klauning et al. 2016).

Table 1. Main differences and similarities in female reproductive cycle physiology, aging and pathology between rodents and humans.

Female reproductive cycle	**Differences**	**Similarities**
Physiology (adults)	Cycle length shorter in rodents as compared to humans (4-5 days against 28 days, respectively) In rodents the cycle is organized through four different phases (metestrus, diestrus, proestrus/ovulation and estrus) rather than three as in humans (follicular, periovulatory/ovulation and luteal phase)	Hormones involved and their fluctuations are similar, e.g. the increase of 17β-estradiol levels preceding ovulation is followed by its decrease and concomitant progesterone rise
Aging	Sequence of states in the transition period to anestrous and consequent endocrine status in dependence on rats and mice strain	Progressive ovarian hormonal imbalance involving both 17β-estradiol and progesterone levels

Incidence of uterine adenocarcinomas in rodents is dependent on the strain. While rodent strains used in chronic toxicity and carcinogenic studies such as Sprague-Dawley rats and Wistar rat have a low incidence of uterine adenocarcinomas, other strains (Donryu, DA/Han, BDII/Han) have a high incidence of this type of tumour (Nagaoka et al. 1990; 1994; Kaspareit-Rittinghausen et al.1987). In Donryu rats there is a progression from endometrial hyperplasia with atypia at 8 months of age, with increasing incidence and severity degree over time to endometrial carcinoma at 15 months (Nagaoka et al. 1990; 1994). Interestingly, these sequential changes are associated to an increased estrogen-to-progesterone ratio, that is 7 times higher at 12 months of age (Nagaoka et al. 1990; 1994). Further details are reported in EFSA PPR Panel et al. 2023.

The relevance of the Adverse Outcome Pathways frame

Animal models provide an opportunity to gain a fundamental understanding of key elements underlying UA pathology. Nevertheless, factors like variable persistence of EDs in the body and environment, duration of exposure, variable latency between exposure and effects, multifactorial nature of carcinogenesis, differences in animal experimental designs makes difficult to infer causality in humans. In this context, uncertainty related to extrapolation from the animal model to humans for type I UA represents a critical issue, being uterine adenocarcinoma addressed as a regulatory endpoint.

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Understanding the underlying mechanisms of adverse effects and their integration in the framework of risk assessment may help to overcome this issue by establishing causality and addressing uncertainties allowing a more accurate prediction of biological responses leading to UA and the possible role of EDs.

To this purpose, the AOP framework (OECD, 2013) provides a powerful tool to assemble and organize all available data for endocrine activity and adversity arranging lines of evidence into a logical sequence of causally linked events, triggered by chemical exposure and leading to an adverse outcome (AO).

The project is part of a collaborative effort with EFSA to develop AOPs relevant for the identification of substances having endocrine disruptors properties (EFSA PPR Panel et al. 2023).

2. Data, Methodologies and Tools

2.1. Data

The initial postulated AOP and AOP network dealing with estrogenic activity leading to uterine adenocarcinoma developed by EFSA (Annex A, **Section 3.2**) created a conceptual map that was used as a starting point.

It should be noted that in the protocol (Annex A) the term used to refer to the initial AOP and AOP network developed as conceptual framework was "putative" instead of postulated. It was decided at a later stage to align the terminology to that of the PPR Panel Scientific opinion (EFSA PPR Panel et al. 2023)

Human observational data, *in vivo* and *in vitro* experimental data have been collected exploring peer reviewed literature, as described below.

2.2. Methodologies

To address the assigned tasks, a strategy was defined *a priori*, and reported in fig. 1.

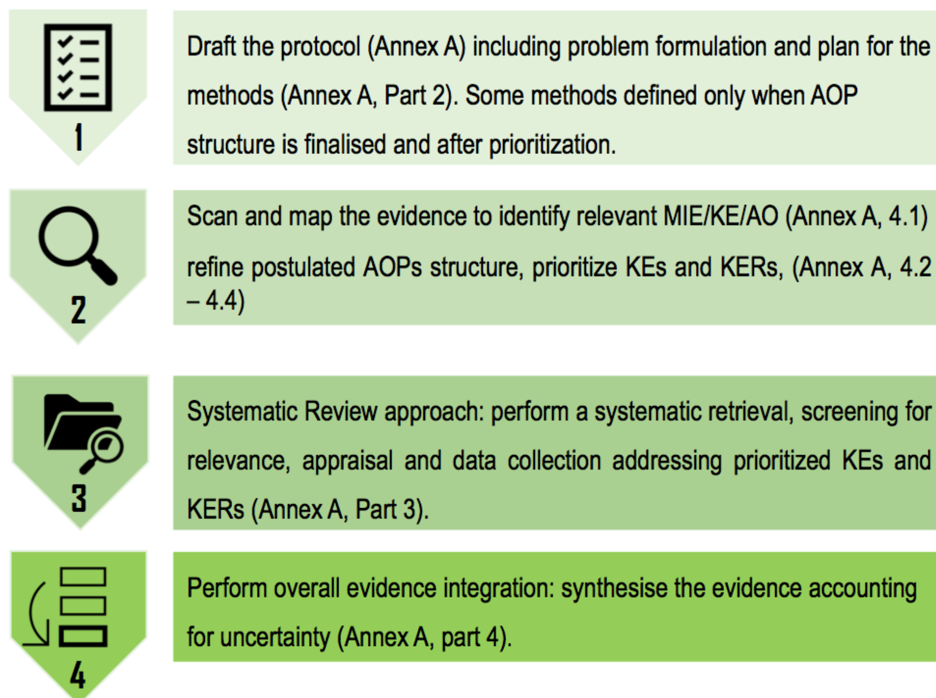

Figure 1. Summary of the strategy adopted to develop the project

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2.2.1. Step 1 – Protocol definition and problem formulation

A pre-defined protocol has been developed in collaboration with EFSA following the approach indicated in EFSA, 2020. The protocol delineates *a priori* each step of the project, and it is provided in Annex A.

As part of the protocol development, main assessment questions (Qs) and sub-questions (SQs) were identified (Annex A, Section 4.3) and refined as part of the systematic review process. Briefly, the assessment Qs and SQs are summarized below:

Q1. What is (are) the adverse outcome pathways (AOPs) relevant for the identification of substances having endocrine disruptive properties and leading to a uterine adenocarcinoma as adverse outcome (AO) based on the available evidence assessed in light of biological plausibility, essentiality and empirical evidence?

Q2. What is the overall certainty in the adverse outcome pathways (AOPs) relevant for the identification of substances having endocrine disruptive properties and leading to a uterine adenocarcinoma as adverse outcome (AO).

> SQ1. What is the level of certainty in the relationship between the MIE (upstream KEs) and the downstream KE(s)?
>
> SQ2. What is the level of certainty in the relationship between the upstream KE(s) and the AO(s)?

Given the novelty of the approach chosen (application of artificial intelligence and evidence mapping), it was difficult to anticipate the most appropriate methodology to adopt and deviations from the protocol were undertaken (see section 2.3 and Annex A).

2.2.2. Step 2 - Mapping the evidence to identify relevant MIEs/KEs/AO; refine postulated AOPs and prioritise KEs and KERs

Based on the initial AOP (Annex A, 3.2), the literature was **preliminarily explored** for the selected MIE "Estrogenic activity" and the AO "Uterine Adenocarcinoma" using broad search strings (Annex B) elaborated to retrieve the highest number of records in public peer-reviewed literature. The information collected has been **scanned and mapped** to address possible KEs and additional MIEs that could be part of the pathways from endocrine perturbation to the AO. To support this, a machine learning technique (I.e. Topic modelling) was used.

The outcome of this exercise was also used to **refine the postulated AOP and AOP network** (EFSA PPR Panel et al. 2023) and to **distinguish** MIEs and KEs whose contribution to the pathway is well established and those whose role is either unknown or less established. In accordance to the protocol (Annex A, 4.2 and 4.4), identified MIEs and KEs for which knowledge was not sufficiently well established were prioritized for subsequent activity.

The evidence mapping methodology is detailed in the below paragraphs, with emphasis to the machine learning technique – topic modelling as a tool to scan and map the evidence to draft and integrate the initial AOP structure.

2.2.2.1. Preliminary literature search

Bibliographic databases (Table 2) have been searched for relevant records using tailored search strings. The search strategy included *ad hoc* combinations of search terms with relevance to the field of "estrogenic activity", "sexual hormones receptors" and "uterine adenocarcinoma". Search terms has been defined by the project team on the base of experts' knowledge and analysis of key studies (i.e. reviews and a sample of relevant primary research studies), medical thesauri MeSH (Medical Subject Headings, for relevant and appropriate terms) and EMTREE (Embase index terms). Pubmed and Embase databases were searched for words in the title or abstract and by using the standardized indexing terms, assigned to each record; Scopus and Web of Science (Tab. 2) were searched only for words in the title or abstract.

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Strings, number of records obtained for each of the combinations of search strings together with the used Boolean operators, have been collected in **Annex B.**

Searches were limited to English language.

Table 2. Bibliographic databases searched for relevant studies

Source of information	Coverage date	Platform
PubMed	Inception–present	PubMed (NLM)
Embase	Inception-present	www.embase.com
Science Citation Index Expanded (SCI-EXPANDED)	1944–present	Web of Science (all database UNIMI subscription)
Book Citation Index– Science (BKCI-S)	2005–present	
Emerging Sources Citation Index (ESCI)	2015–present	
Conference Proceedings Citation Index- Science	1990-present	
BIOSIS Citation Index (BCI) --	2009-2014	
SciELO Citation Index	2002-present	
Derwent Innovation index	2009-2014	
KCI-Korean Journal Database	1980-present	
Russian Science Citation Index	2005-present	
Data Citation Index	2009-2014	
Zoological Record	2009-2014	
CABI	1973-present	
Current Contents	1998-present	
Scopus	Inception--present	www.scopus.com

Records were collected in Endnote and duplicates removed.

2.2.2.2. Topic selection process and evidence mapping

The collected records (corpus) have been scanned by Topic modelling (Rao, R. N. and Chakraborty, M 2021), a machine learning technique that clusters papers according to their semantic similarity and provides set of words (topic) occurring in the corpus using a probabilistic model.

All topics retrieved (**Annex C**) have been collected and organized in excel spreadsheets (fig. 2, **Annex D**) and screened against the general eligibility criteria reported in the protocol (Section 5.1, Annex A). Adjunctive

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specific criteria have been introduced to refine the assessment of topics obtained after the broad search strategy (Table 3).

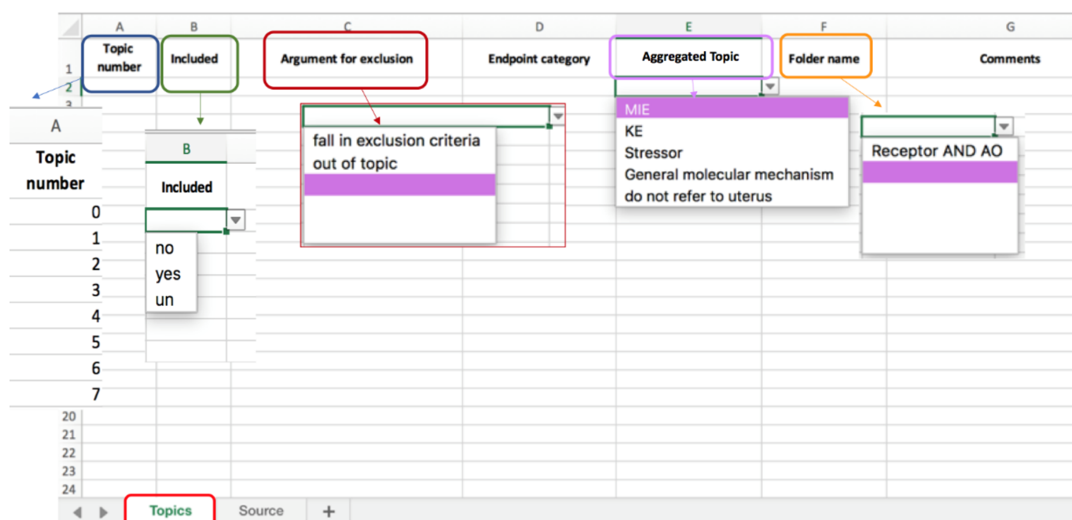

Figure 2. Topics tabular organization in an excel spreadsheet (Annex D).

Table 3. Specific criteria for selecting topics

IN	All topics addressing MIEs, KEs, generic mechanisms, physiology (e.g. estrous cyclicity, menopause) and pathology (e.g. obesity, polycystic ovary) related to UA All topics addressing the uterus and connected organs (e.g. ovary, adipose tissue, brain areas involved in the control of sexual behaviour)
OUT	All except above

These specific criteria have been added *a posteriori,* after a pilot analysis of a restricted number of topics, due to recognition that the planned eligibility criteria were not optimal for a broad literature search as that performed by topic modelling. This deviation to the protocol did not alter the scope of the analysis rather it was instrumental to better characterize MIEs, KEs and general mechanistic information associated with the concept of estrogenic activity leading to uterine adenocarcinoma.

Each topic has been screened against eligibility criteria (Tab. 3 above and Protocol, Section 5.1) by two independent reviewers, to minimise the risk of bias. All topics have been finally discussed against the selected criteria by the whole dedicated team (four reviewers) to be excluded or included.

The whole team clustered topics in relevant evidence streams (Fig. 2) based on the biological domain addressed as *per* expert knowledge in addition to the records collected in each corpus. The identified biological domains referred to each topic were reported in the "Endpoint category" column of the excel spreadsheet (Annex D).

2.2.2.3. Refinement of the structure of the postulated AOP and network

The result of the exercise previously discussed on the topics, their screening for relevance and their aggregation in evidence streams was used to identify the most plausible MIEs, KEs and KERs to be integrated in the postulated AOPs and network via expert judgment.

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According to this, the structure of the postulated AOPs and AOP network was fine-tuned and further developed in this contract or by EFSA ((EFSA PPR Panel et al. 2023).

2.2.2.4. Prioritization of KEs and KERs

According to the protocol (Annex A, 4.2 and 4.4), identified KEs and KERs for which knowledge was not sufficiently well established were prioritized based on two fundamental criteria:

- the biological plausibility of the KERs is not fully established in the scientific community (non-canonical)

- measurability of the KEs (if not measurable the KE is excluded by the AOP).

Prioritized KEs and KERs were developed in the context of this contract carrying out a systematic retrieval, screening for relevance, data extraction and appraisal of the evidence as reported in Phase 3 of the protocol (Annex A) and briefly summarized in the following sections. Otherwise, KEs/KERs considered well established were described and quantified based on expert knowledge, seminal papers and information retrieved from the systematic approach if any (Annex A 4.4). Further details are provided in the following sections

2.2.3. Step 3 – Systematic review and evidence integration

A systematic review approach and data collection for prioritized MIE, KEs and KERs was performed according to the protocol (Annex A, Phase 3) as synthetized in fig. 3. Key elements of the protocol phases in Fig 3 are described in the following sections.

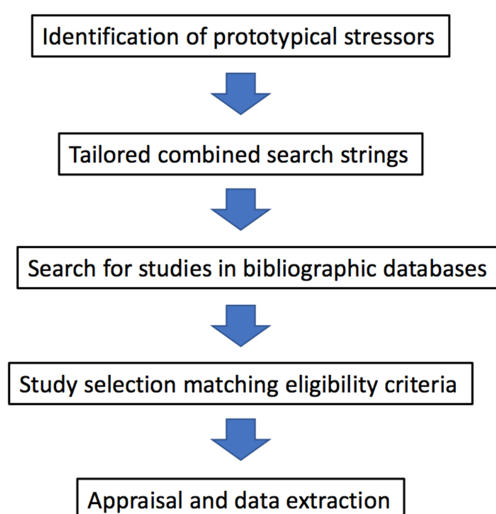

Figure 3. Systematic review approach and data collection for prioritized MIE, KEs and KERs

2.2.3.1. Systematic literature review

Chemical stressors used to define search boundaries

A stressor-based approach was adopted to support the assessment of the empirical evidence. Human exposure and evidence demonstrating a causal link to UA were considered determinant features to select **prototypical stressors** relevant for the characterization of the KERs. Estradiol and Tamoxifen were identified as suitable stressors since exposure to humans is documented and have been identified as critical risk factors for the occurrence of uterine adenocarcinoma in humans (Sherman et al. 2000; Weiss et al. 1976; 1980; Passarello et al. 2019).

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Strings and literature searches

Terms for the stressors were combined with relevant terms addressing the prioritized KEs and the AO (**Annex E**). Bibliographic databases searched are reported in table 2.

Pubmed and Embase databases were searched for the selected terms in the title or abstract and by using the standardized indexing terms, assigned to each record; Scopus and Web of Science (Tab.2 except Derwent Innovation index) were searched only for the terms selected in the title or abstract or keywords.

2.2.3.2. Evidence appraisal

Eligible studies (see eligibility criteria in the Protocol, Annex A, section 5.1) addressing prioritized KEs and KERs were evaluated for sources of uncertainty in the evidence and categorized for Risk of Bias (RoB)[6] using a tailored version of the OHAT-NTP RoB tool (NTP, 2019). This was adapted after a pilot phase to improve the performance of the assessors and to address human diagnostic research studies (DRS), not included in the OHAT-NTP RoB tool. DRS are designed to look for better ways to identify a particular disorder or condition and in human are often based on analysis of tissues obtained from groups characterized by a specific disease compared to tissues derived from people without the disease. Description of DRS and deviation from the original OAHT/NTP tool are reported in Annex A, section 5.5. DRS represent the only type of human studies retrieved according to the inclusion criteria.

The appraisal was performed for *in vitro*, *in vivo* and human studies (lines of evidence). Table 4 shows the different questions and domains appraised for each line of evidence (*in vitro*, *in vivo* and human studies) used to support the development of postulated AOPs.

Table 4- RoB assessment questions and domains for line of evidence

		LINES OF EVIDENCE		
		In vitro	**In vivo**	**Human Diagnostic Research Studies**
Selection bias				
Was administered dose or exposure level adequately randomised?		YES	KeyQ	/
Did selection of study participants result appropriate comparison groups?		/	/	KeyQ
Confounding bias				
Did the study design or analysis account for important confounding and modifyng variables?		/	/	YES
Performance bias				
Were experimental conditions identical across study groups?		YES	YES	/
Attrition/exclusion bias				
Were outcome data complete without attrition or exclusion from analysis?		/	YES	YES
Detection bias				
Can we be confident in the exposure characterisation?		KeyQ	KeyQ	/
Can we be confident in the outcome assessment?		KeyQ	KeyQ	KeyQ
Selective reporting bias				
Were all measured outcomes reported?		YES	YES	YES
Other bias				
Were there other potential threats to internal validity?	Cytotoxicity	KeyQ	/	/
	Replicates/ Repetitions	YES	/	
	Culture medium composition	YES	/	
	Toxicity	/	YES	

[6] Risk of bias: definitely low, probably low, probably high, definitely high

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During the RoB assessment the different lines of evidence for each prioritised KE were collated following a categorisation by endpoints as measured in the studies .

Endpoints measured in the studies has been classified as being of **low (Tier 1), moderate (Tier 2) or high (Tier 3) RoB**. These tiers were derived weighing the appraisal of the individual RoB domains where some were identified as key for the overall appraisal, through the use of an algorithm that combine the answers to the appraisal questions and allocate studies to RoB tiers.

To summarize the results and provide a visual overview of the general strength and weakness for all studies and endpoints included in the analysis, a heatmap reporting the scores obtained for each question designed by a different colour (e.g. definitely low: dark green, probably low: light green, probably high: light red, definitely high: red) has been prepared and presented in the result section.

Refinement of selection criteria based on appraisal training

From a preliminary evaluation of the literature retrieved for the human and *in vitro* lines of evidence, emerged the need to elaborate on the acceptability of the comparator used in the studies.

Background

Human line of evidence

Primary research of the human line of evidence was populated by studies designed to answer questions relevant for clinical assessment, (e.g. why tamoxifen is a risk factor for uterine adenocarcinoma? Does the drug under investigation impact on survival?).

The structure of these studies is based on the comparison between two populations, both with uterine adenocarcinoma and receiving a different drug treatment (e.g. with or without tamoxifen treatment)

In vitro line of evidence

Cell lines used as *in vitro* model of uterine adenocarcinoma are obtained by primary or metastatic tumours and all of them display a collection of different mutation and/or expression of key genes for Type I uterine adenocarcinoma like PTEN and alteration of the related pathway (PI3K/AKT) (see table below) (Van Nyen et al. 2018).

Cell Line	PTEN Deletion/ missense mutation	KRAS Missense mutation	TP53 Splice mutation/ Missense mutation/ Deletion	PI3K/Akt Pathways Alteration	Microsatellite Instability
AN3CA	√		√	√	High
ECC-1	√		√	√	High
HEC1A		√	√	√	High
HEC1B		√	√	√	Low
Ishikawa	√		√	√	High
MFE-280			√	√	Low
RL-95-2	√		√	√	High

Table 5. Endometrial cancer (EC) cell line information. Genomic alterations of the most commonly used type I EC cell lines. Adapted from Van Nyen et al. 2018.

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Definition of the comparator for AOP development

Analysis of the selected studies was finalized to identify AOP(s) relevant for the identification of substances with endocrine disruptive properties impacting on the normal uterus (endometrium) and leading to uterine adenocarcinoma as adverse outcome.

The normal endometrium consists of luminal columnar epithelium and tubular glands, composed of epithelial cells and stroma, this latter representing the supporting fibrovascular tissue (The Human Protein Atlas - endometrium; https://www.proteinatlas.org/). During the development of type I uterine adenocarcinoma the endometrium goes into morphological modifications, through the stages of non-atypical hyperplasia and atypical hyperplasia[7] (Robbins and Cotran, 2015). This transformation is under the control of prolonged estrogenic stimulation (Robbins and Cotran, 2015) and it is accompanied by modifications in molecular mechanisms and loss of homeostatic control resulting in the development of a neoplastic phenotype, i.e. uterine adenocarcinoma.

For this AOP a critical challenge was the identification of endpoints/molecular mechanisms (KEs) relevant for the transformation of normal endometrium into a neoplastic phenotype, following prolonged estrogenic stimulation. Consistently, the definition of the comparator for each line of evidence was defined as summarized in the table 6.

Table 6. Comparator definition for each line of evidence

Lines of evidence	Comparator
Human	healthy population
In vivo	animals not exposed to estradiol or tamoxifen
In vitro	non-cancer cell lines representative of uterine epithelium

All studies not accomplishing these criteria were tagged as "No-Compar" and excluded by appraisal and data extraction. The same apply for case-case studies and case report studies in human line of evidence.

Characterisation of the MIE and KEs: uncertainty identification

As explained in previous paragraphs, evidence for each line (i.e., *in vivo*, *in vitro* and human) was analysed for RoB and for unexplained inconsistencies between different studies. This exercise allowed to identify the source, the direction and the magnitude of any potential bias and provided an overall evaluation of the consistency, confidence and precision of the data set. The work has been preparatory to the KER certainty analysis included in the opinion EFSA PPR Panel et al. 2023.

Such an appraisal of the results, and related uncertainties identification, has been instrumental to draw conclusions on the biological plausibility of the link between UA and the endocrine activity for the postulated AOP.

The likelihood of an observed effect was assessed considering the validity (Tier) of the studies appraised for each line of evidence (body of evidence) and the combination of the outcome from the three lines of evidence per cluster and endpoint. The rationale underlying the ratings for each line of evidence is explained in detail in the result section (3.3.1.1 to 3.3.2.2). The overall likelihood of an observed effect (from the overall body of evidence level and based on RoB and unexplained inconsistencies following the principle of the NTP-OHAT) was defined as:

7 Endometrial hyperplasia is defined as an increase of endometrial glands relative to stroma, resulting in an increased gland-to-stroma ratio when compared with normal proliferative endometrium (Robbins and Cotran, 2015).

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- Very Likely: There is very high confidence in the body of evidence for an association between exposure to estradiol and tamoxifen and effect/s (e.g. there is much evidence showing consistent effect/s).
- Likely: There is high confidence in the body of evidence for an association between exposure estradiol and tamoxifen and effect/s (e.g. there is evidence showing consistent effects).
- As likely as not (ALAN): There is low confidence in the body of evidence for an association between exposure to estradiol and tamoxifen and effect/s (e.g. there is evidence showing inconsistent effects).
- Not Likely: There is very low confidence in the body of evidence for an association between exposure to estradiol and tamoxifen and effect/s (e.g. there is evidence showing consistent no effects).
- Inadequate evidence: There is insufficient evidence available to assess if the exposure to estradiol and tamoxifen is associated with and health effect/s or data are missing.

2.2.3.3. The inclusion of the evidence in the AOP framework considered the strengths and the weaknesses in a collection of human, animal and *in vitro* studies that constitute the body of evidence for a specific cluster.Data extraction

Data extraction was performed after the appraisal to focus the analysis on the more robust studies (Annex A, 4.4). The criteria adopted to retain the studies for data extraction, AOP development and quantification of the KERs were:

- *In vitro* or *in vivo* studies only: Tiers 1 and 2

- Human studies: all Tiers

- Studies including more than one line of evidence (e.g. *in vivo* and *in vitro* or human, *in vivo* and *in vitro*) addressing the same or related endpoints: all Tiers

The data model for extraction was tailored for each study type (i.e. human, *in vitro*, *in vivo*) and was provided (see **Annex F1, F2 and F3**).

2.2.4. Step 4. Adverse Outcome Pathway development

An AOP is conceptualized as a single sequence of events proceeding from the molecular initiating event (MIE) to the adverse outcome (AO) via a series of intermediate key events (KE). A KE represents a hallmark of this causal chain and has to be identified in biologically relevant models to be representative of a pathway relevant for a human disease. The core of an AOP design is the definition of the relationships sequentially linking the different KEs, that are the Key Event Relationships (KERs). KERs provide a quantitative description of the relationship between KEs and detailed mechanisms of biological processes based on all the available evidence, and therefore support the causal link and the biological plausibility between a pair of KEs towards the AO, according to a weight of evidence approach.

The AOP developed in the present report (Annex I) is based on data and information obtained for the three lines of evidence of prioritized KEs and KERs, integrated with KEs and KERs that were not prioritised according to the protocol (see Annex A, section 4.4), and as described in section 2.2.2.3 of the present report.

The AOP conceptual framework was developed in accordance to OECD guidance.[24,25]

2.2.4.1. Methods for quantification of KERs certainty

Quantification of the KERs is out of the scope of the present report and have been addressed in collaboration with EFSA PPR Panel and results are reported in EFSA PPR Panel et al 2023.

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2.3. Deviations from the protocol

During the assessment, deviations from the protocol were introduced a *posteriori* to (i) optimize the process and obtain relevant results from the available literature, (ii) overcome practical concerns (e.g. time limitation, rearrangement of the team). Overall, the adopted deviations have a low impact on the project as defined by the protocol.

Deviations have been tabulated and annotated in the protocol in details at the end of any relevant section (Annex A).

3. Results

3.1. Mapping the evidence – Topic modelling outcome

The search strategy adopted to retrieve the evidence for the postulated AOP and AOP network connecting "Estrogenic activity" (MIE) to "Uterine adenocarcinoma" (AO) included tailored combinations of search terms with relevance to the field of the MIE and AO (Section 2.2.3 and Annex B for details).

A total of 84.722 records were collected from the combination of "estrogenic activity" and "endometrioid adenocarcinoma" strings in the different databases after deduplication (Tab. 7).

Table 7. Total records retrieved from searched databases before and after deduplication (*24th April 2021*)

Database	**Number of records retrieved from the combination of "estrogenic activity" and "Endometrioid adenocarcinoma" strings**
Pubmed	11545
Embase	16803
Scopus	21206
Web of Science	54965
Total	105519
Total after deduplication	**84722**

A joint embedding the 84.722 documents and word vectors have been created to find dense clusters of documents (topics) and to identify the words that attracted the documents to the dense area (topic words). As a result, 401 topics were identified, associated to an equivalent number of corpora collecting all related records (Annex C). The outcome of the analysis of the retrieved topics is summarized in fig.4.

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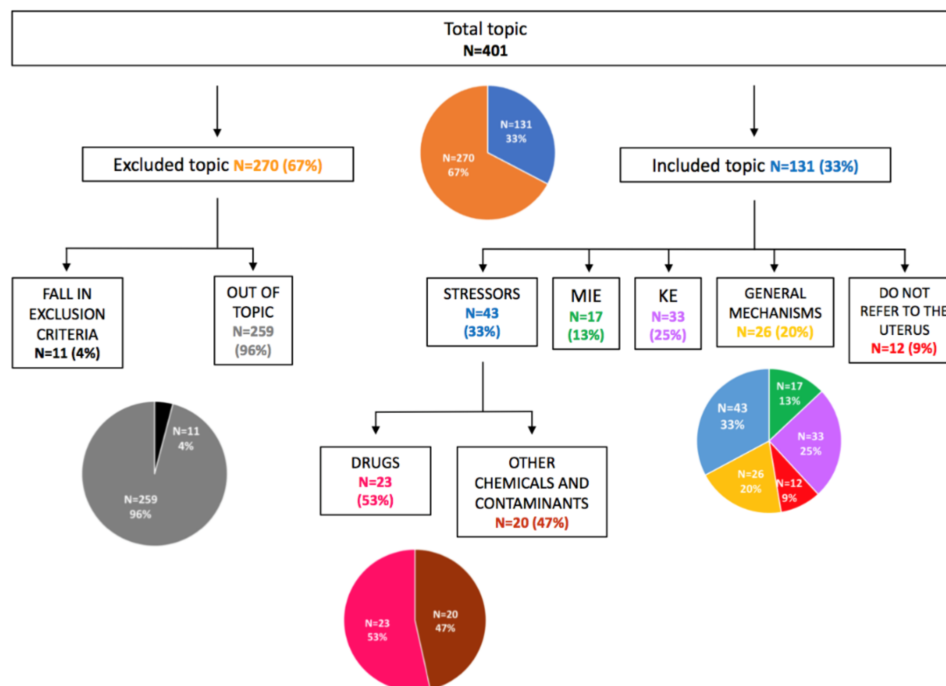

Figure 4. Overview of topic selection process and results. The list of topics for each area is given in Annex D.
N= number of topics, in brackets: percentage of total number of topics in each section

Two-hundred seventy topics were excluded because falling in the exclusion criteria (e.g. addressing uterine adenocarcinomas other than type I) or addressing subjects out of the scope of the report (Tag: out of topic, e.g. osteosarcoma or tumours other than uterine adenocarcinoma) (Fig. 4).

One-hundred thirty-one topics out of 401 resulted to be related to the assessment question and met the eligibility criteria (Fig. 4). Included topics were clustered together as "Stressors", "MIEs", "KEs", "General mechanisms" and "extra-uterine events" (do not refer to the uterus) (Fig. 4), as described in section 2.2.2.2.

Topics addressing potential **stressors** (n=43) are grouped in drugs (n=23) and other chemicals/environmental contaminants (n=20) connected with sexual hormones activity or ED activity (Fig. 4). The complete list of stressors and corresponding topics is reported in Annex D.

Topics collected under **"MIEs"** (n= 17)**, "KEs"** (n=33) **and "General mechanisms"** (n= 26) have been further organized in ten **subgroups** based on the biological domain (defined *as per* expert knowledge) and on the records collected in each corpus (Fig. 5 and Annex D, Endpoint category column).

Two **MIEs subgroups** have been identified:

- related to "Steroid hormone receptors" - mainly referring to estrogen (nuclear and membrane) and progesterone receptors (13% of in-uterus factors topics) together with coregulators (coactivators and corepressors) of nuclear receptors (11% of in-uterus factors topics), and their activation;

- related to "Estrogen Synthesis and metabolism" (11% of in-uterus factors topics), and associated specific enzymes (e.g. aromatase, steroid sulfatase/sulfotransferase, 17β- hydroxysteroid dehydrogenase isozymes) as well as principal pathways leading to the synthesis / metabolism of estrogens and sexual hormones.

The most represented **general mechanisms/KEs subgroups** are: "Proliferative control", collecting 28% of in-uterus factors topics, and "Epigenetic regulation", collecting 14% of in-uterus factors topics, followed by "Inflammation and immune response" (collecting 7% of in-uterus factors topics), "Gene expression" and "Processing regulating cell fate" (each collecting 6% of in-uterus factors topics), "proteolysis" (collecting 3% of in-uterus factors topics) and Gap junction (1% of in-uterus factors topics) (total=59).

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The minor number of **topics not strictly referring to the uterus** (n=12) tag human pathological conditions (i.e. obesity, n=1; anovulation, n=1; polycystic ovary, n=1) leading to unopposed estrogen exposure (Passarello et al. 2019) and physiological pathways (i.e. estrous cyclicity, n=1; neuronal control of sexual behavior, n=2; ovary and hormonal regulation, n=4; menopause, n=2) with a control on estrogen production. (Fig. 5).

The topic modelling outcome prompted to the distinction of topics into uterine and not referring (solely) to uterus as functional to the AOP network development (see Fig.5).

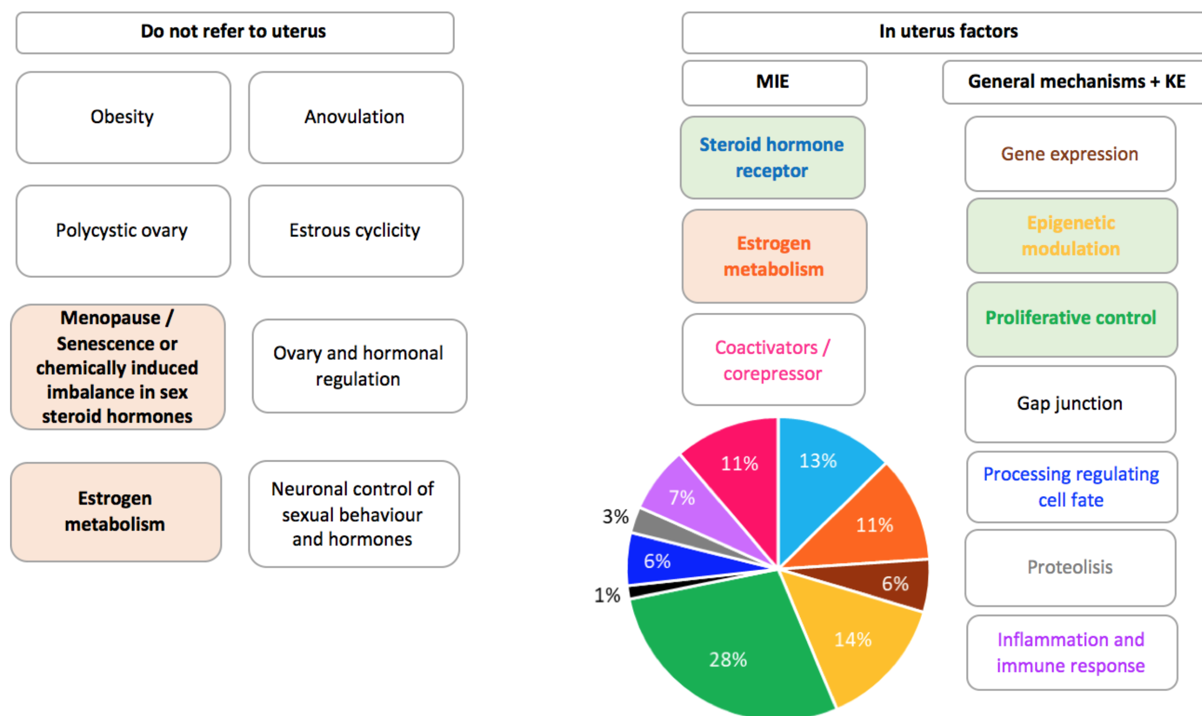

Figure 5. General pathways and effectors (MIEs and KEs) identified by topic modelling. Each box refers to the topics identified as part of the iterative process using topic modelling. The list of topics for each area is given in Annex D. Pie chart shows the % distribution of topics of in-uterus factors (MIE and General mechanisms + KEs; text in each box replicates the colour of the corresponding section in the pie). Coloured boxes represent those selected for developing the AOP network. Orange boxes represent topics developed by EFSA, Green boxes those in this report.

3.2. AOPs revision and KEs and KERs prioritization

Evidence clustered following the application of Topic Modelling was mapped, based on biological plausibility, against the initial postulated AOPs.

To frame an AOP network leading to UA, a selection of topics was identified (Fig 5). Topics related to extra-uterine events (Fig 5, orange boxes) were investigated by EFSA (EFSA PPR Panel et al. 2023) and are not discussed in the current report. Topics addressing intra-uterine events (Fig. 5, green boxes) were considered in the present report for the development of the designed postulated AOP "Estrogenic activity" leading to "Increased proliferation in estrogen dependent tissue/organ" and "Uterine adenocarcinoma".

Criteria to select the most impacting MIE/KEs in the AOP developed in this report were based on biological plausibility and expert knowledge; the number of topics addressing specific endpoints was also taken into account as a measure of interest of the scientific community and literature availability. Table 8 summarizes MIEs and KEs that were selected and those prioritised.

Table 8. Lists the selected MIE, KEs and those prioritized for appraisal

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Selected MIE/KE	Prioritised (Yes/No) /MIE or KE	Reason
Estrogen Receptor activation	No/MIE	well-established
Proliferation control	No/KE	well-established
Epigenetic modulation	Yes/KE	knowledge not well established
DNA repair	Yes/KE	knowledge not well established
Accumulation of mutation	Yes (only animal models)/KE	knowledge not well established in animal models

"Estrogen dominance" and "endometrial hyperplasia" (i.e. increased pathological proliferation of the endometrial epithelial cells compared with normally proliferative endometrium), are considered as well-established anchor points of a biologically plausible AOP for UA. Indeed, "Estrogen dominance" leads to hormonal dependent uterine adenocarcinoma, an adverse outcome that is preceded by increased proliferation in estrogen dependent tissues and organs (Sherman, 2000). As such, the **KE dealing with proliferation and cell cycle control** is the one where the highest number of topics were allocated.

In utero estrogen receptor (over)activation may summarize both the concept of "Estrogenic activity" as well as "Estrogen dominance" identified respectively as **MIE** and KE in the postulated AOP framing the initial conceptual map. As such, uterine estrogen receptors activation represents a nodal event where extra-uterine factors converge through the control of estrogen levels. Two main classes of estrogen receptors exist: the nuclear intracellular receptor family (ERα and ERβ) and G protein-coupled membrane receptors membrane, the most studied is GPR30. All were tagged by topic modelling with most topics addressing ERs (55%). ERs are considered the primary receptors activated by estrogens in the uterus (Walker et al. 2004) and their activation has been selected as MIE to be further developed.

Cancer is a disease characterized by widespread epigenetic alterations which may contribute to the malignant properties of cancer cells (Dawson et al. 2012). "**Epigenetic regulation", the second identified KE** (14% of in-uterus topics), is less established in UA pathogenesis. Evidence suggesting estradiol as a player in epigenetic mechanisms through the recruitment of ER has been identified in breast cancer (Kovacs et al. 2020). The observation that activated ER can attract co-regulatory proteins like histone acetyltransferase (Thomas & Gustafsson, 2011) or rules enzymes involved in epigenetic DNA modulation, supports the biological plausibility of the **KER "ER activation" leading to "Epigenetic modulation".**

Other KEs emerging from the analysis of the corpora associated to "Epigenetic modulation" were **"DNA-repair" and "Accumulation of mutation**". "Accumulation of mutation" addresses another established feature of human uterine adenocarcinoma. Sequencing of genomes of human Type I uterine adenocarcinoma allowed to identify common mutation which are considered as hallmark of this tumour type in humans. Mutations and genetic abnormalities that are considered prevalent in Type I uterine adenocarcinoma are in the PTEN (tumour suppressor gene) PIK3CA (oncogene), KRAS, ARID1A (regulator of chromatine structure), microsatellite instability. In particular, PTEN mutations have been identified in more than 20% of atypical hyperplasia, a frequent precursor of endometrial carcinoma (Mutter et al. 2000; Elleson et al. 2015), providing a link with proliferation control in the uterus. In general, knowledge of this KE is less consolidated in animal models, although knock-out mice for PTEN (PTEN +/-) represents a transgenic UA model. This KE/KER was then prioritized and considered for appraisal for the *in vivo* line of evidence.

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3.3. Systematic review and evidence integration for prioritised KE, MIE, KER

3.3.1. Systematic review and eligibility assessment results

The search strategy adopted to address the prioritized KEs (see Table 8) included tailored combinations of search terms with relevance to the field of **epigenetics (Epi), DNA repair (DNA-rep) and accumulation of mutations (Mut)** in the framework of uterine adenocarcinoma (endometrioid) (in line with the protocol, see Annex A). All Epi, DNA-rep and Mut terms have been combined with search string concerning Adverse Outcome (uterine adenocarcinoma) and stressors (1=tamoxifen; 2=estradiol). Strings and number of records obtained for each of the combinations of search strings, together with the used Boolean operators, have been collected in **Annex E.** Records from all databases were collected in Endnote and duplicates were removed. After importing record in DistillerSR (Annex A, section 5.3), a further removal of duplicates has been performed.

4569 unique references were identified, screened and included as eligible studies. Results of this process are summarised in the Prisma flow diagram reported in fig.6. As part of the title and abstract screening phase the records were clustered in three lines of evidence (Table 9).

Table 9. Line of evidence and associated records type

Line of evidence		Type of record
1	Human	Clinical, observational and diagnostic research studies
2	*In vivo*	Experimental studies on animal models
3	*In vitro*	Experimental studies on in vitro test system

Title and abstract screening for relevance and eligibility left 1012 full text assessed for eligibility. 480 were classified for human, 516 for *In vivo* and 711 for *In vitro*. For Human 229 records were excluded and 231 were included. For *in vivo* 303 records were excluded and 182 were included. For *in vitro* 225 records were excluded and 452 were included (Fig.6). Included records were clustered in "Reviews" and "Primary research studies", the latter being relevant as source of data to support the empirical evidence of the developed AOP (Fig. 6).

Full list of references included and excluded and reasons for exclusion are described in Fig.6.

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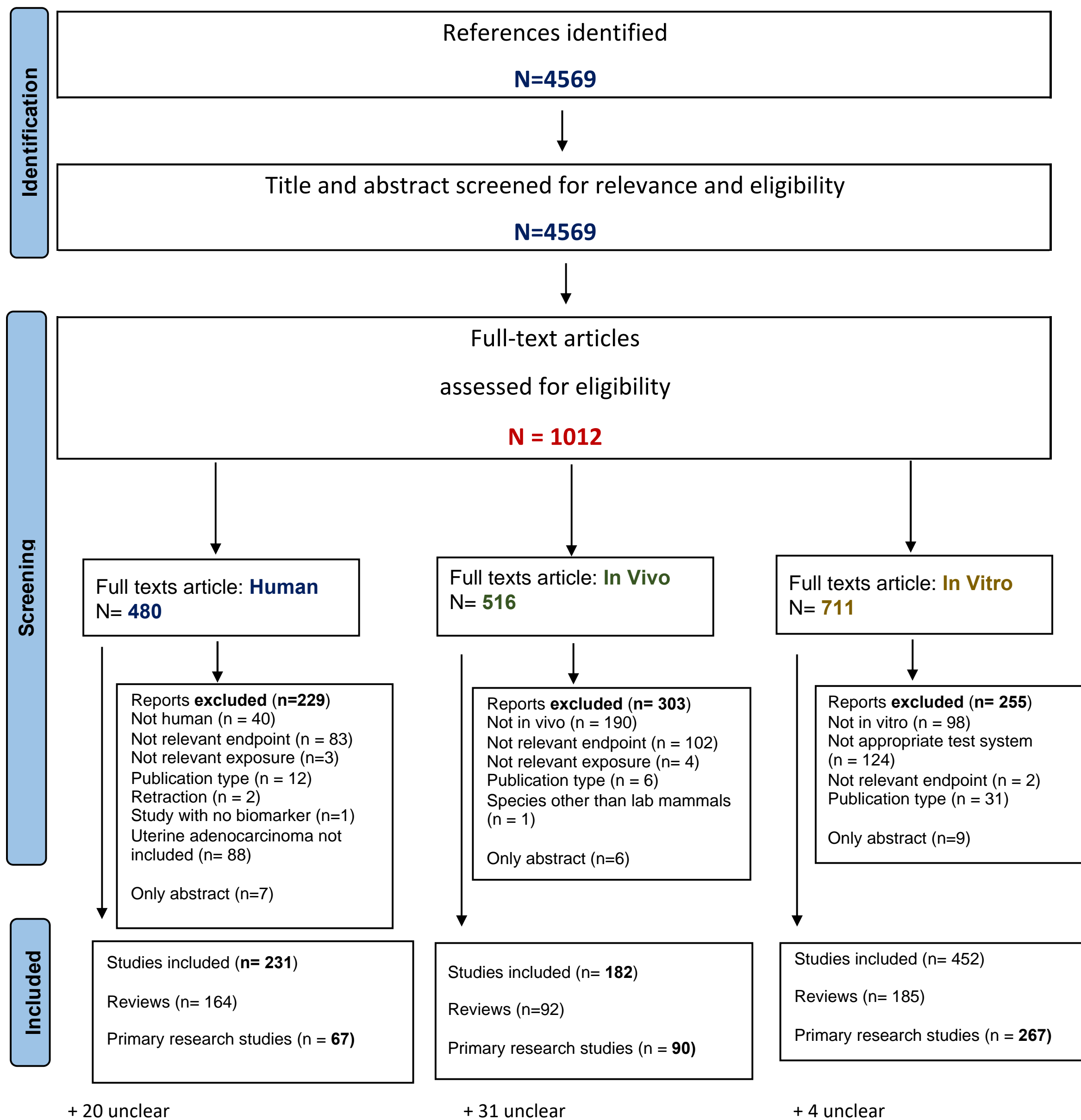

Fig. 6

PRISMA flow chart of the literature search process, including screening for relevance

Records addressing prioritized KEs/KERs were selected from "Primary research studies" for the three lines of evidence and included for appraisal based on the presence of an appropriate comparator (Fig. 7).

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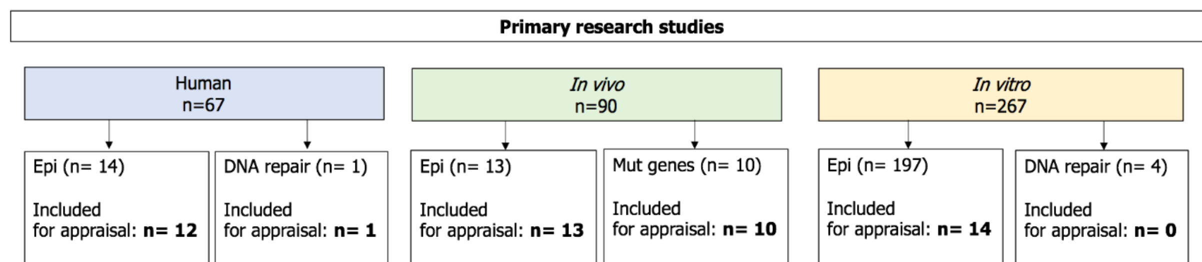

Fig. 7 Flow chart of studies addressing prioritized KEs/KERs, including screening for relevance. Epi: epigenetic modulation, Mut genes: mutated genes.

"Epigenetic modulation" indicates the processes that involve changes in gene expression without modifying the DNA sequence (Sharma et al. 2010). The main processes are DNA methylation, histone modification, alterations of factors involved in nucleosomes assembly and remodelling, and gene regulation by non-coding RNAs (Rodríguez-Paredes & Esteller, 2011). Studies describing both epigenetic mechanisms and modulated gene expression were then collected in the "Epigenetic modulation" cluster. This approach was adopted to optimize *a priori* the collection of a wide spectrum of information possibly connected through a common epigenetic mechanism but distributed between different records and different lines of evidence.

The drastic reduction in the number of *in vitro* records to be considered for appraisal was due to the lack of an appropriate comparator (cell models that recapitulate normal endometrial epithelial cells; see 2.2.3.2).

During the RoB assessment (see 2.2.3.2) the different lines of evidence on the prioritised KEs were collated following a categorisation by endpoint (see box 1) according to the study design.

Box 1. Evidence for prioritised KEs

KE prioritised	Endpoints	Line of evidence: RefID
Epigenetic modulation	Acetylation	Hu: Ref ID 296, 426 In Vivo: Ref ID 296, 494 In Vitro: Ref ID 296
	Gene expression	Hu: Ref ID 47, 426, 499, 596, 1066, 1418 In Vivo: Ref ID 572, 1068 In Vitro: Ref ID 47, 326, 426, 453, 499, 596, 667, 1066, 1418
	Methylation	Hu: Ref ID 33, 47, 426, 1066, 453, 918 In Vivo: Ref ID 47, 317, 918, 1042,1066 In Vitro: Ref ID 47, 337, 453, 918, 1066
	miRNA	Hu: Ref ID 405, 669, 1418 In Vivo: Ref ID 307, 326, 1062, 1600 In Vitro: Ref ID 326, 405, 667, 669, 1418
	Lnc_RNA	Hu: Ref ID 669 In Vivo: Ref ID 307, 1062 In Vitro: Ref ID 669
Mutated genes	Hyperplasia	In Vivo: Ref ID 239, 954, 3792, 4234

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	Uterine weight	In Vivo: Ref ID 2743, 4611
	Adenocarcinoma	In Vivo: Ref ID 4260
	Mutations	In Vivo: Ref ID 2172, 2175, 2743, 3515

3.3.2. Critical appraisal results

The outcome of the RoB appraisal is presented in full in Annex G1.1 for human, Annex G.1.2 for *in vivo* and Annex G.1.3 *in vitro* lines of evidence and is summarised in a graphical way in tables 10, 11, 12 (heatmaps). The appraisal for each study has been performed at endpoint levels because, for the same study, the design and conduct may affect the RoB differently depending on the endpoint measured.

The outcome is firstly summarised below per line of evidence (human, in vivo, in vitro).

The outcome is then presented per endpoints, including all lines of evidence (human, *in vivo* and *in vitro*); this accommodates also the fact that most of the studies address more than one line of evidence.

3.3.2.1. Critical appraisal results - summary by line of evidence

Human studies (Hu)

In the RoB analysis conducted using Distiller (Annex A, section 5.4), three articles and relative endpoints were judged in Tier 1 (Ref. ID# 426, 699, 1066). Nine records and relative endpoints were in Tier 3, (Ref. ID# 296, 499, 596, 33, 47). The main shortcomings of these type of research studies lies in the paucity of details describing the characteristics of the population involved, e.g. age, ethnicity and possible confounders (Q1 and Q2) (Annex G.1.1).

Table 10 Records and specific endpoints appraised in Tier 1 (low RoB) and 3 (high RoB) (see Protocol)

Prioritized KE	RefID	Specific endpoints		Appraisal questions					Tier
				Q1	Q2	Q3	Q4	Q5	
Epigenetic modulation	296	Acetylation	Modulator (ACLY mRNA)	NR	NR	DLRoB	DLRoB	DLRoB	3
			Modulator (ACLY mRNA)	NR	NR	DLRoB	DLRoB	DLRoB	3
			Modulator (ACLY, protein)	NR	NR	DLRoB	PHRoB	DLRoB	3
			Global H3 acetylation	DLRoB	DLRoB	DLRoB	PHRoB	DLRoB	3
	426		HDACs expression	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	1
	1066	Gene expression	PAX2 expression	DLRoB	NR	DLRoB	DLRoB	DLRoB	1
	47		TFF3 expression	NR	NR	DLRoB	DLRoB	DLRoB	3
	499		HOXB13 (transcription factor)	NR	NR	DLRoB	DLRoB	DLRoB	3
	596		Wnt	NR	NR	NR	PHRoB	DLRoB	3
	1418		Kruppel factor 9	PHRoB	NR	PLRoB	PHRoB	DLRoB	3
	426	Methylation	DNMT1 (Methyltransferase)	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	1
	1066		Hypomethylation, PAX2 promoter methylation	DLRoB	NR	DLRoB	DLRoB	DLRoB	1
	33		Global 5-me-cytosine	PHRoB	NR	DLRoB	DLRoB	DLRoB	3
	453		HOPX CpG methylation and expression	NR	NR	PLRoB	DLRoB	DLRoB	3
	918		RIZ1 (Methyltrasferase)	NR	NR	DLRoB	DLRoB	DLRoB	3
			RIZ1/ER ratio	NR	NR	DLRoB	DLRoB	DLRoB	3
	405	miRNA	miR181a, miR98	PLRoB	PHRoB	PHRoB	PLRoB	DLRoB	3
	1418		miR200c	PHRoB	NR	PLRoB	PHRoB	DLRoB	3
	669		miR646	PLRoB	NR	DLRoB	DLRoB	DLRoB	1
	669	Lnc_RNA	HOTAIR	PLRoB	NR	DLRoB	DLRoB	DLRoB	1

In light green are evidenced the endpoints in Tier 1. DLRoB: Definitely Low RoB, PLRoB: Probably Low RoB, PHRoB: Probably High RoB, DHRoB: Definitely High RoB, NR: not reported. Appraisal questions are reported in Annex A.

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In Vivo studies

In the RoB analysis conducted using Distiller, five articles were judged in Tier 1 (Ref. ID# 296, 326, 918, 1042). The rest being in Tier 3, due to several shortcomings such as lack of information on chemical source and purity, number of animals, blind, etc.

Table 11. Records and specific endpoints appraised in Tier 1 (low RoB) and 3 (high RoB) (see Annex A)

Prioritized Kes	RefID	Specific endpoints		Appraisal questions							Tier
				Q1	Q2	Q3	Q4	Q5	Q6	Q7	
Epigenetic modulation	296	Acetylation	tumor growth	DLRoB	DLRoB	PLRoB	DLRoB	DLRoB	PLRoB	NR	1
	494		E2, uterusproliferation and morphogenetic reactions	PHRoB	DLRoB	PLRoB	PLRoB	PHRoB	DLRoB	PLRoB	3
	47	Methylation	Hypomethylation, tumor growth	PLRoB	PLRoB	PHRoB	PLRoB	PHRoB	PLRoB	PLRoB	3
	1042		Demethylase expression (KDM4)	PLRoB	PLRoB	PLRoB	PLRoB	PLRoB	PLRoB	PLRoB	1
	1066		Hypomethylation, tumor growth	NR	PHRoB	PHRoB	NR	DLRoB	DLRoB	PLRoB	3
	317		Histone methyltransferase expression	PHRoB	PLRoB	PHRoB	PHRoB	PLRoB	PLRoB	PLRoB	3
	918		tumor growth	DLRoB	DLRoB	PLRoB	PLRoB	DLRoB	DLRoB	PLRoB	1
	1062	miRNA, LncRNA	LncRNA UcA1, miRNA-144	PHRoB	PLRoB	PLRoB	PLRoB	PHRoB	PLRoB	PLRoB	3
	1600		miRNA-451	NR	PLRoB	PLRoB	NR	PHRoB	DLRoB	DLRoB	3
	326		miR-203	DLRoB	DLRoB	PLRoB	DLRoB	DLRoB	DLRoB	DLRoB	1
	307		LncRNAs and mRNA	PHRoB	PHRoB	PLRoB	DLRoB	PLRoB	DLRoB	DLRoB	3
	572	Gene expression	Sox4	PHRoB	DLRoB	DLRoB	DLRoB	PHRoB	DLRoB	PLRoB	3
	1068		c-fos, c-june	PHRoB	PLRoB	PHRoB	PLRoB	PHRoB	PLRoB	PLRoB	3
Mutated genes	239	Hyperplasia	Erα splicing, hyperplasia E2	NR	PHRoB	PLRoB	PLRoB	PLRoB	PLRoB	PLRoB	3
	239		Erα splicing, hyperplasia TAM	PLRoB	PHRoB	PLRoB	NR	PLRoB	PLRoB	PLRoB	3
	954		IRS-1 null mutant mice, hyperplasia	PHRoB	DLRoB	PLRoB	PHRoB	PLRoB	PLRoB	PLRoB	3
	3792		PTEN+/- , simple hyperplasia	NR	PLRoB	PLRoB	NR	DLRoB	DLRoB	PLRoB	3
	3792		PTEN+/- , complex hyperplasia	NR	PLRoB	DLRoB	PLRoB	DLRoB	DLRoB	PLRoB	3
	4234		Wdr13 KO mice, hyperplasia	NR	DLRoB	PLRoB	NR	NR	PLRoB	PLRoB	3
	4611	Uterine weight	APC KO mice, uterine weight	NR	PHRoB	PHRoB	PLRoB	PLRoB	PLRoB	PLRoB	3
	2743		PTEN+/- uterine weight	NR	PHRoB	NR	DLRoB	DHRoB	PLRoB	PLRoB	3
	4260	Adenocarcinoma	Cre-loxP PTEN inactivation, adenocarcinoma	NR	DLRoB	PLRoB	NR	PLRoB	PLRoB	PLRoB	3
	2172	Mutations	mutant frequencies in lacl transgene	NR	PLRoB	DLRoB	DLRoB	PLRoB	DLRoB	PHRoB	3
	2175		TAM DNA adducts	NR	PLRoB	PLRoB	DLRoB	PLRoB	DHRoB	PHRoB	3
	2743		p53 and ras gene mutations	NR	PHRoB	NR	DLRoB	DHRoB	PLRoB	PLRoB	3
	3515		PTE KO, stroma Erα expression	NR	PLRoB	PLRoB	PLRoB	PLRoB	PLRoB	PLRoB	3

In light green are evidenced the endpoints in Tier 1. DLRoB: Definitely Low RoB, PLRoB: Probably Low RoB, PHRoB: Probably High RoB, DHRoB: Definitely High RoB, NR: not reported. Appraisal questions are reported in Annex A.

In Vitro studies

Tier 3 score in *in vitro* studies is mainly due to methodology not appropriately reported an/or lack of a sufficient number of replications (Q4), lack of information on chemical source and purity (Q3), in few cases lack of cytotoxicity studies (Q6).

Table 12 Records and specific endpoints appraised in Tier 1(low RoB) and 3 (high RoB) (see Annex A)

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Prioritized KE	RefID	Specific endpoints		Appraisal questions Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Tier
Epigenetic modulation	296	Acetylation	Acetylation, H3K27ac	PLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DHRoB	PLRoB	1
			Acetylation, H3	PLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	PLRoB	PLRoB	1
	47	Gene Expression	TFF3, Protein expression, 4 human cell lines (hypomethylated)	PLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	1
			TFF3, promoter activity, 4 human cell lines (hypomethylated)	PLRoB	DLRoB	PHRoB	DLRoB	DLRoB	PLRoB	DLRoB	DLRoB	3
			TFF3, TAM induced expression	PLRoB	DLRoB	PHRoB	DLRoB	DLRoB	PLRoB	DLRoB	DLRoB	3
	426		Kruppel factors	DLRoB	DLRoB	PLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	1
	453		HOPX, 3 human cell lines	DLRoB	DLRoB	DLRoB	PLRoB	DLRoB	DLRoB	PHRoB	PLRoB	1
	499		HOXB13	DLRoB	PLRoB	NR	DHRoB	DLRoB	NR	NR	DLRoB	3
	596		Wnts, 4 human cell lines	DLRoB	DLRoB	DLRoB	DHRoB	DHRoB	PLRoB	DHRoB	DLRoB	3
			Wnts, human non cancer cell line	DLRoB	DLRoB	DLRoB	DHRoB	DHRoB	PLRoB	DHRoB	DLRoB	3
	667		Cyclin, mouse non cancer cells	DLRoB	DLRoB	PLRoB	NR	DLRoB	DLRoB	DLRoB	DLRoB	3
			Cyclin, 2 human cell line	DLRoB	DLRoB	PLRoB	NR	DLRoB	DLRoB	DLRoB	DLRoB	3
	1418		Gene expression, miR200c gain of function	DLRoB	DLRoB	PLRoB	PLRoB	DLRoB	PLRoB	DLRoB	PLRoB	1
	1066		PAX2 expression, human normal endometrial cells (hypomethylated)	NR	DLRoB	PLRoB	DLRoB	DLRoB	DLRoB	PLRoB	DLRoB	1
			PAX2 expression, human Type I EC cells (hypomethylated)	NR	DLRoB	PLRoB	DLRoB	DLRoB	DLRoB	PLRoB	DLRoB	1
	326		miRNA,miR203 downregulated genes	DLRoB	PLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	PLRoB	1
	47	Methylation	hypomethylation, TFF3 promoter	PLRoB	DLRoB	PHRoB	DLRoB	DLRoB	PLRoB	DLRoB	DLRoB	3
	337		Demethylase expression, KDM1A, 4 different human cell lines	DLRoB	NR	DLRoB	NR	DLRoB	DLRoB	DLRoB	DLRoB	3
			KDM1A, E2 regulation, 2 different human cell lines	DLRoB	PLRoB	DLRoB	PLRoB	DLRoB	DLRoB	DLRoB	DHRoB	1
	453		HOPX promoter, 2 human different cell lines	DLRoB	DLRoB	DLRoB	PLRoB	DLRoB	PLRoB	PHRoB	DLRoB	1
	918		E2 induced RIZ1 expression, 2 different human cell lines	DLRoB	DLRoB	NR	PLRoB	DLRoB	PLRoB	PLRoB	DLRoB	3
			RIZ1 methyltransferase expression AN3CA cell line	NR	NR	DLRoB	NR	DHRoB	DLRoB	PLRoB	DLRoB	3
			RIZ1 methyltransferase expression	PLRoB	NR	DLRoB	PLRoB	DLRoB	DLRoB	PLRoB	DLRoB	1
			RIZ1 silencing, proliferation	PLRoB	NR	PHRoB	PHRoB	DLRoB	NR	PLRoB	PLRoB	3
			RIZ1 overexpression, proliferation	PLRoB	NR	PHRoB	PHRoB	DLRoB	NR	PLRoB	PLRoB	3
	1066		Hypomethylation,PAX2 promoter, human normal endometrial cells	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	1
			Hypomethylation,PAX2 promoter, human Type I EC cells	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	1
	326	miRNA	miR203 knock out	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	DLRoB	PLRoB	1
	405		miR181a & miR98	DLRoB	PLRoB	PLRoB	NR	PHRoB	PLRoB	DLRoB	DLRoB	3
	667		miR145, mouse non cancer cells	DLRoB	DLRoB	PLRoB	NR	DLRoB	DLRoB	DLRoB	DLRoB	3
			miR143, miR145, 2 human cell lines	DLRoB	DLRoB	PLRoB	NR	DLRoB	DLRoB	DLRoB	DLRoB	3
	669		miR646, 2 human cell lines	DLRoB	DLRoB	DLRoB	PLRoB	DLRoB	DLRoB	DLRoB	PLRoB	3
	1418		miR200c	DLRoB	PLRoB	PLRoB	PLRoB	DLRoB	PLRoB	DLRoB	PLRoB	1
	669	Lnc RNA	Lnc_RNA, HOTAIR, 2 human cell lines	DLRoB	DLRoB	DLRoB	PLRoB	DLRoB	DLRoB	DLRoB	PHRoB	1

In light green are evidenced the endpoints in Tier 1. Appraisal for the same endpoint in different cell lines resulting in identical result have been merged, number of cell lines and origin has been indicated. DLRoB: Definitely Low RoB, PLRoB: Probably Low RoB, PHRoB: Probably High RoB, DHRoB: Definitely High RoB, NR: not reported. Apparisal questions are reported in Annex A.

3.3.2.2. Critical appraisal results - per prioritised KEs

See also tables 10, 11, 12 for details on the different lines of evidence (i.e., human, in vitro and in vivo) and box 1 for an overview of the evidence retrieved for each cluster (i.e., prioritised KEs).

"EPIGENETIC MODULATION"

ENDPOINT ACETYLATION

Histone acetylation is usually associated to an increased transcription, due the removal of positive charges and therefore the relaxion of the chromatin (euchromatin), whereas histone deacetylation is related to transcription repression, due to the formation of heterochromatin (Watson et al., 2014). In this cluster, only 1 record was identified for the *in vitro* line of evidence (Ref. ID# 296), 2 for the human line of evidence (Ref. ID# 296 and 426) and 2 for the *in vivo* line of evidence (Ref. ID# 296 and 494). Ref. ID# 296 is common to the three lines of evidence and was judged Tier 3 for human evidence and in tier 1 for both *in vivo* and *in vitro* evidence.

In study **Ref. ID#296** Human: (Tier 3) the gene expression of ATP-citrate lyase (ACLY) was investigated in the Cancer Genome Atlas (TGCA) and Sixth People Hospital recruited population. ACLY is an enzyme that catalyzes ATP-dependent citrate cleavage to produce acetyl-CoA. An increase in ACLY expression was observed in tissues from UA patients vs tissue from healthy population. The type of uterine adenocarcinoma was not specified but a significant correlation (r=0.484) between ACLY expression and BMI (Body Mass Index) was observed in the UA cohort. mRNA expression is paired to increased number of ACLY protein positive cells in UA and correlate to tumour grade. Nuclear localized of ACLY increased histone acetylation levels. The presence of p-ACLY in the nucleus directly correlate (r=0.503; n=156) with the level of histone H3 acetylation in fixed tumour specimens. In TGCA population ACLY resulted positively correlated with the expression of dihydroorotate dehydrogenase (DOHOD), an enzyme involved in the synthesis of pyrimidine and promoting proliferation.

In the same study, *In vitro* (Tier 1) the authors demonstrated that the silencing of ACLY in HEC-1A and ECC-1 cells decreases the DOHOD mRNA expression. Authors demonstrated that the effect is reversed by treatment with TSA, an inhibitor of histone deacetylases. Accordingly, the selective inhibition of H3K27 and H3K9

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acetylation by C646 reduced DOHOD mRNA expression and ChIP experiments showed a decreased interaction of H3K27ac antibodies with DOHOD promoter in HEC-1A and ECC-1 cells silenced for ACLY. Furthermore, silencing of ACLY in HEC-1A and ECC-1 cells decreases proliferation. Silencing of DOHOD decreased cell proliferation while its overexpression restored proliferation in ACLY silenced cells. Moreover, *In vivo* (Tier 1) the authors demonstrated that ACLY knockdown inhibits cell proliferation in ECC-1 cells *in vitro* and attenuates tumour growth (tumour volume and growth) in nude mice. ACLY regulated the downstream gene *dhod* to promote UA proliferation via histone acetylation.

In conclusion, DHODH is involved in the synthesis of pyrimidine and in tumour progression. In cancer cells, it is postulated that the phosphorylation and activation of ACLY are regulated by Akt phosphorylation on Ser 455 residues. Elevated phosphorylation of ACLY promotes cancer cell survival and development by increasing lactate secretion and glucose utilization. Sequela of events: Obesity-related factors, like estradiol, insulin and leptin, increase the expression of ACLY by STAT3 phosphorylation in UA. In addition, obesity-related factors also promoted nuclear translocation of ACLY by Akt phosphorylation, and nuclear-localized ACLY enhanced histone H3 acetylation levels and increased the expression of DHODH to promote UA progression.

Study **Ref. ID#426** (Human: Tier 1) investigated histone deacethylase 3 (HDAC3) in ER-α positive .A An increased mRNA expression of histone deacethylase 3 (HDAC3) and no effect on HDAC6 and HDAC9 mRNA was reported.

In Study **Ref. ID#494** (*in vivo*: Tier 3), the effects of histone deacetylase blockers, trichostatin A and sodium butyrate, on proliferative and morphogenetic reactions in the uterus under long-term estrogen treatment were examined. The effects of histone deacetylase inhibitors were found only in estrogen-treated mice and were not documented in control animals receiving olive oil instead of estradiol. In the case of low beta-catenin concentration, cell–cell interactions are less solid, which provides a foundation for cancer development. Histone deacetylase inhibitors exert an increased proliferative and morphogenetic effects of estradiol (34% increase with trichostatin A and 17% with sodium butyrate).

Conclusion for endpoint acetylation:

This endpoint was judged **LIKELY,** as two studies out of three, one of which across three lines of evidence (Human: tier3, *in vivo*: tier 1, *in vitro*: tier1), support a role of histone acetylation in the control of estrogen-dependent processes, thus, favoring proliferation, morphogenetic alterations, and tumour progression.

ENDPOINT METHYLATION

Hypomethylation of DNA repeats has been shown to promote tumour formation or progression by fostering DNA rearrangements. Beside DNA methylation, also methylation and demethylation of histones have been shown to turn the genes in DNA "off" (restricting access to the DNA) and "on,” (allowing transcription factors and other proteins to access the DNA), respectively.

- 5 studies were identified in human line of evidence reporting data on expression of methyltransferase, methylation status of specific gene promoters or global methylation (Ref. ID# 33, 426, 1066, 918, and 453), 2 of which (Ref. ID# 1066 and 426) in tier 1. Ref. ID# 47 reports only on gene expression of TFF3 whose promoter is demonstrated to be hypomethylated *in vitro*. For this reason, it will be discussed in this section.

- 5 studies were identified for the *in vitro* line of evidence (Ref. ID# 47, 337, 453, 918, and 1066). Among these papers, Ref. ID# 1066, 453, 918 (for RIZ1 expression) were judged tier 1.
- 4 studies were identified for the in vivo line of evidence (Ref. ID# 47, 317, 1042, 1066). Among these papers, Ref. ID# 1042 was judged tier 1.

Ref. ID# 47, 918 and 1066 are common to the three lines of evidence and ID# 453 is common to human and *in vitro* lined of evidence.

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Study **Ref. ID# 47**

In vitro: (Tier 3) TAM dose-dependently increases the expression of Trefoil factor 3 (TFF3) promoter activity in ER-α + (Ishikawa and ECC-1) but not ER-α - (RL95/AN3). In addition, increasing concentrations of TAM increases luminescence in an estrogen response element reporter assay. *TFF3* is an estrogen responsive gene, is one of three members grouped in the trefoil factor family that normally functions to protect the intestinal mucosal surface and promote epithelial healing in times of injury. TFF3 promotes cell survival and cell cycle progression, angiogenesis and metastatic dissemination in various cancers, acting through signaling mediators such as epidermal growth factor receptor (EGFR), AKT, mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 3 (STAT3) and the E-CADHERIN CATENIN complex. TAM exposure was able to increase mRNA expression in a dose dependent manner coupled with an increase protein expression of TFF3 in Ishikawa cells. TAM-stimulated upregulation of *TFF3* through hypomethylation of its proximal promoter region corresponding to binding sites for the transcription factors c-JUN and SP1. TAM treatment resulted in increased expression of the ER-responsive *TFF3* gene, which in turn promoted UA oncogenicity. Forced expression of *TFF3* gene in estrogen receptor positive (ER+) UA cells significantly increased cell cycle progression, cell survival, anchorage-independent growth, invasiveness and tumour growth in xenograft models.

Human: (Tier 3) immunohistochemical evaluation in endometrial carcinoma specimens, as by IHC, showed a significantly high % of cells expressing TFF3 protein. TFF3 expression in specimens (both endometrioid and non-endometrioid) did not correlate with ER expression. % of cells expressing high TFF3 protein was similar to the % expressing low TFF3 in simple endometrial hyperplasia and complex endometrial hyperplasia.

*In vivo (*Tier 3) forced expression of *TFF3* gene in Ishikawa cell enhances tumour formation in immunodeficient mice as assessed by tumour volume, cell proliferation (ki67 positive cells) and reduced apoptosis (TUNEL labeling).

Study Ref. ID# 1066

Human: (Tier 1) showed, by two different techniques (methylation specific PCR and bisulphite DNA), hypomethylation of PAX2-promoter in Type I uterine adenocarcinoma specimens at stage I and II compared to normal tissue where the promoter is hypermethylated. PAX2 is a factor that accompanies high rate in cell division and is expressed in different tumours. Co-localization of PAX2 and ER-α is evident in sections of human endometrial cancer samples as well as level of ER-α expression correlates to levels of PAX expression, suggesting that PAX is a downstream target of ER-α in endometrial cells.

In vitro (Tier 1) PAX2 reactivation in normal endometrial cells is triggered by the methyltransferase inhibitor 5-aza-deoxycytidine, confirming the need to reduce the methylation marks of PAX promoter to favor its expression. No direct evidence of the involvement of ER-α in hypomethylation of PAX2 promoter was reported but hypomethylation was necessary to allow the interaction of ER-α with the promoter. The role of PAX2 in Ishikawa and ECC-1 cells proliferation has been demonstrated with gain and loss of function experiments. Overexpression of PAX2 increased the percentage of Ishikawa and ECC-1 cells in S/G2/M phases and potentiated TAM and E2 induced proliferation. Silencing of PAX counteracted all these effects. B.

In vivo studies (Tier 3) investigated the effect of PAX2 on the growth of transplanted ECC-1 tumours in nude mice. Hypomethylation-linked activation of PAX2 mediates tamoxifen-stimulated endometrial carcinogenesis. In athymic mice that received a transplant of ECC-1 tumours with unchanged PAX2 expression (neither overexpression nor knockdown), both E2 and tamoxifen stimulated tumour growth. In athymic mice that received a transplant of ECC-1 tumours that had PAX2 overexpression, tumour growth was observed even with no treatment, and treatment with estradiol or tamoxifen further enhanced the ECC-1 tumour growth. In athymic mice that received tumour transplants with PAX2 knockdown, tumour growth stimulation by estradiol and tamoxifen was greatly attenuated.

Study Ref. ID# 918

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Human: (Tier 3) The role of RIZ1 in endometrial cancer was investigated. RIZ1 was frequently downregulated in endometrial cancer. RIZ1, retinoblastoma protein-interacting zinc finger protein 1, is a nuclear methyltransferase protein. It methylates histone H3 and is important in transcriptional repression. This study indicated, by immunohistochemistry, a decrease in the expression of methyltransferase retinoblastoma protein-interacting zinc finger protein 1 (RIZ1) in endometrial carcinoma. A progressive decrease is observed with the increase of tumour grade. Although both endometrioid and non- endometrioid specimens are analyzed most of the negative cells for RIZ1 are positive for ER-α.

In vitro (Tier 3) investigation reports that the overexpression of ER-ɑ significantly decreased RIZ-1 expression (mRNA and protein) and ER-α silencing increased it (mRNA and protein) in both in RL95-2 or Ishikawa cells. Accordingly, RIZ-1 increased expression (mRNA and protein) is observed in RL95-2 or Ishikawa cells exposed to ICI 182780, an ER-α selective antagonist. Overexpression of RIZ-1 decrease while its silencing increases HEC-1B and RL95-2 cells proliferation respectively. Inconsistency: RIZ-1 decreased expression is also evident in SPEC2 and KLE cells considered representative of type II (ER insensitive) endometrial adenocarcinoma.

In vivo (Tier 1): RIZ1 remarkably suppressed tumour proliferation, metastasis and invasion, supporting that RIZ1 as tumour suppressor. Moreover, ER-〈 signaling had a role in regulating RIZ1 expression.

In conclusion, the following scenario could be hypothesized: E2 binds to ER-α, downregulated RIZ1, which will lead to hypomethylation and increased transcription, which correlated with tumour progression.

Study Ref. ID# 453

Human: (Tier 3) The role of HOPX and the status of promoter methylation was investigated. HOPX is a homeobox gene whose loss of expression has been reported for several types of cancers. This study reported an increase in the methylation of CpG islands of HOPX and decreased mRNA and protein expression specifically in uterine endometrial epithelium (not in stroma) of endometrioid endometrial cancer (Type I). *HOPX* gene silencing was also observed in normal endometrium adjacent to endometrioid adenocarcinoma regions, suggesting that *HOPX* methylation may represent early changes leading to initiation.

In vitro (Tier 1): Ishikawa, HEC-1A, and HHUUA methylation of HOPX in the promoter region was analyzed. The hypermethylation of HOPX CpG region in the promoter of the gene correlate with the decrease of mRNA expression as well as protein of HOPX, compared to primary endometrial cells used as control. Reactivation of HOPX expression in tumour cell lines was induced by both trichostatin A (an HDAC inhibitor) and the methyltransferase inhibitor 5-aza-deoxycytidine, suggesting a role of both methylation and acetylation in the regulation of HOPX promoter. Forced expression of HOPX in Ishikawa cells resulted in a partial block of cell proliferation in response to estradiol while knockdown in immortalized human endometrial cells resulted in an accelerated proliferation. Forced expression of HOPX in Ishikawa and HHUA cells resulted in c-fos expression in HEC in response to E2 stimulation.

Only Human studies

Study Ref. ID# 33 (Tier 3) addresses the transcriptional regulation by tamoxifen (TAM) in human endometrial cells comparing normal and malignant tissues of women under treatment or not with TAM. The study reported that a higher global methylation on 5-methyl-cytosine occurred in endometrial vs control tissue independently on TAM treatment.

Study Ref. ID# 426 (Tier 1) reported, by qRT-PCR, an increase in mRNA expression of DNA methyltransferase-1 in UA tissues from stage I to III versus surrounding non tumour tissues.

Only In vivo studies

Study Ref. ID# 317 (Tier 3) did not focus on endometrial cancer, but investigated EZH2 expression, regulation, and its role in uterine development/function. Enhancer of zeste homolog 2 (EZH2) is a rate-limiting catalytic subunit of a histone methyltransferase, polycomb repressive complex, which silences gene activity through the repressive histone mark H3K27me3. Uterine EZH2 expression is developmentally and hormonally regulated, and its loss causes aberrant uterine epithelial proliferation, uterine hypertrophy, and cystic endometrial hyperplasia. Overexpression of EZH2 or EZH2 gain-of-function mutations occur frequently in

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endometrial cancer. Loss of-function mutations in EZH2 are also associated with abnormal growth/cell proliferation. EZH2 might be involved in the etiology of human uterine leiomyomas. Exposure of neonatal mice to DES altered uterine levels of the phosphorylated (inactive) form of EZH2, histone marks, and expression of key uterine proteins. Estrogen effects may be mediated through membrane ESR1, which activates the phosphotidylinositol-3-kinase (PI3K)/AKT pathway and phosphorylates and represses EZH2. Furthermore, EZH2 expression is induced by estrogens (e.g. DES, BPA) in mammary glands, possibly through estrogen response elements (ERE) in the EZH2 gene promoter.

Study Ref. ID# 1042 (Tier 1) a variety of *in vitro* and *in vivo* studies to identify the effects of KDM4 enzyme activity on AR signaling and UA progression were performed. KDM4 enzymes are important regulators of histone methylation. In clinical specimens, both KDM4B and KDM4A expression are significantly higher in EC tissues than that in normal endometrium. Histone demethylase KDM4 family KDM4B and KDM4A promote EC progression by regulating AR activity. KDM4 enzymes bind to AR to co-regulate transcription; depletion of any of the KDM4 proteins suppresses AR-mediated transcription in vitro and in vivo.

Only In vitro studies

Ref. ID# 337 (Tier 1) investigated the role of histone lysine specific demethylase 1 (LSD1) in UA. LSD1 is overexpressed in endometroid endometrial carcinoma and associated with tumour progression as well as poor prognosis. This protein expression is high in endometrial cancer cell lines and the treatment with E2 increases the level of LSD1 in dose- and time- dependent manner. E2 enhances LSD1 expression via GPR30/PI3K/AKT signaling pathway. Interestingly, no upregulation of PTEN was shown. LSD1-KD induces the deregulation of cyclin D1, and upregulation of P21 and cleaved caspase-3. In presence of E2 (when LSD1 is not silenced) there is an increase in level of pAKT, pERK, cyclin D1, Bcl-2, and weakened expression of P21 and cleaved caspase-3. Overexpression of cyclin D1 induced increase of proliferation. LSD1 act on the promoter of cyclin D1 demethylating it on H3K9me2 (no changes in H3K4me2). When LSD1 is silenced the total amount of H3K9me2 is increased on the promoter region of cyclin D1.

Conclusion for endpoint methylation:

Histone hypomethylation endpoint was judged LIKELY, as different studies (Ref. ID# 47, 371, 918, 1042, 1066), three of which with endpoints in Tier 1, support a role of hypomethylation favoring expression of genes critical in the development of endometrioid endometrial cancer, e.g. TFF3, PAX2, Cyclin D1. Contribution of estradiol in hypomethylation induction through ER-α is also LIKELY, as suggested by Ref. ID# 918, 371, 47 and 1066. Nevertheless, the contribution of receptors other than ER-α, e.g. GPR30 (Ref. ID# 337) or androgen receptors (Ref. ID# 1402), has to be considered. Regarding DNA methylation the involvement of ER-α is LIKELY as NOT. Although Ref. ID#453 (Tier 1 for *in vitro* line of evidence), suggest the involvement of hypermethylation in silencing oncogenes in endometrial adenocarcinoma and increase in DNMT1 in human UA specimen has been reported (Ref. ID#426), there is no evidence suggesting a direct involvement of estradiol and ER-α receptor.

ENDPOINT miRNA and LncRNA

MicroRNA (miRNA) is a class of posttranscriptional gene regulators acting through transcript degradation and/or translation suppression in the case of mRNAs (Hutzinger et al. 2011). miRNA has been shown to be a good biomarker for many diseases and some evidence showed that estrogen-induced response was partially mediated by miRNAs.

Long noncoding RNAs (lncRNAs) are a class of long non-protein-coding RNAs exceeding 200 nucleotides in length able to regulate numerous cellular functions in normal physiology and disease states. LncRNAs are known to have functions in cis by affecting the expression of neighboring genes and in trans by affecting genes located on different chromosomes. It is suggested that they coud play a decoy role competing on miRNA binding, thus reducing the regulatory effect of miRNAs (Paraskevopoulou et.al. 2016).

3 studies were identified for human evidence (Ref. ID# 405, 669 and 1418) of which Ref. ID# 669 in tier 1.

5 studies for in vitro evidence (Ref. ID# 326, 405, 667, 669 and 1418)

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4 studies for in vivo evidence (Ref. ID# 307, 326, 1062, 1600). Only reference ID# 326 was judged in Tier 1. Ref. ID# 307 investigated lncRNAs.

Ref. ID# 405, 669 and 1418 are common to human and *in vitro* lines of evidence and ID# 326 is common to *in vivo* and *in vitro* lines of evidence.

Study Ref. ID# 326

In vivo: (Tier 1) investigated miRNA expression in uterine samples from a standard 3-day uterotrophic assay using young female adult Lewis rats to identify E2-regulated miRNAs. Microarray analysis identified 47 E2 down-regulated miRNAs including miR-30a, and 25 E2-up-regulated miRNAs including miR-672, miR-203, and miR-146b. E2-upregulated miR-203 was selected for further analysis. Proliferation was reduced and G2-arrest was observed in all miR-203 deficient RUCA-I clones (Rat Uterus Carcinoma-I). Acer2, Zbtb20, Ptn, Rcbtb2, Mum1l1, Hmgn3, and Nfat5 possess one or more seed sequence matches in their 3-UTR that are predicted to be targets of miR-203. These data demonstrate the importance of E2 regulated miRNAs in general, and miR-203 in particular, for E2 regulated gene expression in the etiology of endometrial carcinomas.

In vitro studies (Tier 1) Rat endometrial adenocarcinoma cell line RUCA-I were genetically manipulated to silence or overexpress miR-203. Deletion of miR-203 reduced proliferation and cells arrest their cycle in G2, this effect was partially rescued by transfection with miR-203 mimic. In particular it has been observed that the same genes downregulated in rat uterus by estradiol treatment in vivo were up-regulated following KO of miR-203 in RUCA-I cells. The expression of these genes affects fundamental aspects cell physiology which are likely to impact on the cell cycle and proliferation rate of these cells.

Study Ref. ID# 405

Human:(Tier 3) analyzed two different miRNA, miR-181a and miR-98, in UC tissues compared with peri-postmenopausal endometrial specimens. Higher levels of miRNA-181 in endometrial cancer grade I and II vs normal endometrial tissue in peri-and postmenopausal period were reported. miR-98 has a tendency to increase (no significance) with the grade of the tumour.

In vitro studies (Tier 3) In ishikawa cells E2 reduces miR-181a and increased miR-98 after 48 and 24 h treatment respectively. Gain of function of miR-181a resulted in a decreased expression of both mRNA and protein of DDX3X (a factor ruling proliferation), and decreased mRNA expression of cyclin E1 but with no effect on Ishikawa rate of division. miR-98 gain of function reduced the expression of CYP19A1 and TIMP3 and decreased proliferation in Ishikawa cells.

Study Ref. ID# 669

Human: (Tier 1) investigated the molecular mechanism of the LncRNA HOTAIR in the tumourigenic progression of endometrial carcinoma. Expression of HOTAIR together with miR-646 have been measured in human UA tissues, resulting in a significant increase of HOTAIR and decrease of miR-646 and a significant negative correlation in UA tissues compared to normal ones.

In vitro (tier 1). miR-646 overexpression attenuated E2-promoted cell proliferation, migration an invasion in two different cancer cell lines (Ishikawa and HEC1-A). This effect is possibly promoted by negatively regulating NPM1, a multifunctional protein with a role in tumourigenesis, as luciferase activity of reporter plasmids containing NPM1 was negatively regulated by miR-646. On the contrary, HOTAIR promoted NMP1 expression and cell proliferation. These effects are linked to a negative regulation of miR-646 by HOTAIR as supported by RNA-binding protein immunoprecipitation and RNA-pull down assays and by HOTAIR/miR-646 gain and loss of function experiments.

Study Ref. ID# 1418

Human: (Tier 3) addressed expression and functional aspects of miR-200c in normal endometrial tissues and grade I-III endometrial cancer reported higher expression of miR-200c in UA grade I and II vs normal endometrial tissue in peri-and postmenopausal period. The trend of expression decreases with the increase of the UA grade.

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In vitro (Tier 1). Exposure of Ishikawa cells to E2 or P4 did not modify the expression of miR-200c. Gain of function of miR-200c repressed the expression of factors involved in cell transformation (ZEBs), of factors involved in angiogenesis, decreased both the activity of KLF9 promoter and its expression (mRNA and protein) and that miR-200c increased Ishikawa cells proliferation.

Only in vivo studies

Study Ref. ID# 307 (Tier 3) aimed to identify the transcriptional responses of the uterus from ovariectomized mice to E2, by RNA-seq to obtain global expression profiles of protein-coding transcripts (mRNAs) and long noncoding RNAs (lncRNAs) following 0.5, 1, 2, and 6 hours of treatment. Subcutaneous injection of 100ng/animal E2 regulated the expression of some conserved lncRNAs that are predictive of low overall survival in endometrial carcinoma patients (e.g., H19, KCNQ1OT1, MIR17HG, and FTX). The E2-regulated mRNA and lncRNA expression profiles indicate an association between lncRNAs and mRNAs that regulate E2-driven pathways and reproductive phenotypes in the mouse, including possible role in the etiology of endometrial carcinomas.

Study Ref. ID# 1062 (Tier 3) aimed to investigate the molecular mechanism underlying the role of metformin (Met) in reducing the risk of endometrial hyperplasia (EH) in a mouse model induced by tamoxifen. Expression levels of lncRNA urothelial cancer associated 1 (UcA1), miRNA-144 and other factors along the transforming growth factor-β1 (TGF-β1)/protein kinase B (AKT) signaling pathway. Met reduces the risk of EH by reducing the expression levels of UcA1, TGF-β and p-AKT, while increasing the levels of miR-144 and active caspase-3 in a dose-dependent manner.

Study Ref. ID# 1600 (Tier 3) investigated E2-regulated miRNAs in the mouse uterus (E2; 50μg/kg bw). miR-451, miR-155, miR-335-5p, and miR-365, were identified as E2-regulated miRNAs in the uterus. miRNA-451 was upregulated already after 1.5 h, and all peaked at 9 h. Predicted targets of these miRNAs are genes involved in cell grow control, consistently with the main E2 function in the uterus. MiR-451 had similar strong responses to E2 in the uterus of both immature and ovariectomized mice, and, thus, it could be a potential biomarker for estrogenicity in the uterus.

Only in vitro studies

Study Ref. ID# 667 (Tier 3) aims to evaluate the effect of progesterone on miR-145/miR-143 expression in murine endometrial epithelial cells and consequent effects cell proliferation. The miR-145/miR-143 encoding genes are in close proximity to each other on mouse chromosome 18 (on human chromosome 5) and are believed to be co-transcribed in the same bicistronic transcript. Selective mimics for miR-145 and miR-143 reduced the proliferation activity and inhibit the expression of cyclin D2 protein in the mouse EECs when the cells were pre-treated with E2. The same behavior has been reported for Ishikawa and Hec-1b cells. Due to the aim of the study, E2 was used as control for P4 effect, and no controls for E2 effect on miRNAs and cyclin D2 expression were shown. This hampers the possibility to fully evaluate E2 action on the endpoints presented.

Conclusion on endpoints miRNA and LncRNAs

As all studies used a different design, data (expression of different miRNAs or lncRNAs) were not confirmed, therefore, this endpoint was judged so far INADEQUATE EVIDENCE. Nevertheless, among the different miRNA, miR-451 is of interest as it is involved in the regulation of various human physiological and pathological processes, including progression of tumourigenesis and drug resistance. miR-451 targets *Cdkn2d* (cyclin dependent kinase inhibitor 2D), which inhibit the kinase activity of CDK4/6. The upregulation of miR-451 should result in a decrease in Cdkn2d expression with subsequent increased CDK4/6 kinase activity and cells grow. It has been shown that miR-451 not only directly affects the biological functions of tumour cells but also indirectly affects tumour cell invasion and metastasis upon secretion into the tumour microenvironment via exosomes (Bai et al. 2019). Also, miR-203 is of interest as proliferation was reduced and G2-arrest was observed in all miR-203 deficient RUCA-I clones. Among the different LncRNA, HOTAIR/miR-646 interaction (tier 1) is of interest since the molecular mechanisms dissected in human cancer cell lines are consistent with the results observed in human specimens of endometrial carcinoma.

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ENDPOINT GENE EXPRESSION

Gene expression, a general endpoint functionally connected to "Epigenetic modulation", collects 5 records on factors involved in tumourigenesis (Ref. ID# 1066, 47, 499, 596, 1418) altered in UA specimen and 9 records on factors involved in the control of cell proliferation (Ref. ID# 47, 326, 426, 453, 499, 596, 667, 1066, 1418). For Ref. ID# 47 and 1066 there is evidence supported by *in vitro* studies that increased expression of the factors PAX2 and TFF3 is due to hypomethylation of the promoter, while HOPX hypermethylation reduces HOPX expression (for records Ref. ID# 453). These results match with what observed in human specimens obtained from EC. Other factors result under the control of different miRNAs (Ref. ID#326, 667, 1418), while for records Ref. ID# 499, 596, 1418 no specific marker of epigenetic modulation has been retrieved in our literature research for any line of evidence.

Part of the records clustered in this section also address "Epigenetic modulation" (I.e. Ref. ID#47, 326, 453, 667, 1066, 1418) and have been already described. Below the details of the records not yet described are reported.

Study **Ref. ID# 426** Human: (Tier 1) focuses on the expression and role of Kruppel factors in endometrial cancer development and/or progression, based on the observation that dysregulated expression of these proteins is associated with multiple types of human tumours. In particular KLF9 and KLF4 expression was lower in UA tissues compared to surrounding non tumour tissues.

In vitro (tier 1) silencing of KLF9 in Ishikawa cells promoted their proliferation.

Study **Ref. ID#499** Human: (Tier 3) reported HOXB13 mRNA expression increased in specimen obtained from endometrial cancer patients. HOXB13 belongs to the family of Homeobox genes that have been previously reported to be suppressed in endometrioid adenocarcinoma. The different results here reported might be due to the presence in the specimen obtained of stromal tissue and blood.

In vitro study (Tier 3) demonstrated that E2 induced a dose dependent increase in HOXB13 mRNA expression in ANCA3 cells. Anyway, not concordant results on E2 and HOXB13 mRNA expression were noted.

Study Ref. **ID#596**

Human: (tier 3) addresses the expression of different *Wnt* genes, hormonally regulated genes with a transforming role in breast epithelium, in normal human endometrium and carcinoma tissues. Results showed that 6 out of 7 *Wnt* genes were expressed in human endometrium and that downregulation of Wnt 4, 2, 3 and 5a may be associated with endometrial carcinoma.

In vitro (Tier 3) mRNA expression of different Wnt genes subtypes has been measured in three different endometrial carcinoma cell line although it is difficult to evaluate the relevance of the data because mRNA values have been provided as single measurement.

Only in vivo

In study **Ref. ID#572** (Tier 3) the expression of *Sox4* expression in the reproductive tissues of mice was investigated by Northern blot analysis and ribonuclease protection assays. Results presented here suggest that expression of the *SOX4* gene is under ovarian hormone control in the uterus and implicate Sox4 in the complex effects controlled by ovarian hormones in the female reproductive system. Its relevance in uterine adenocarcinoma is not discussed. These results suggest a role for E2 in down-regulation of Sox4 mRNA expression and a role for P4 in its induction at estrus. *SOX4* is a member of the SRY-related HMG-box (SOX) gene family. Other studies indicate aberrant expression of SOX4 in endometrial cancer and partially was related to hypermethylation of miR-129-2. Sox4 could enhance β-catenin/TCF4 transcription, through upregulation of TCF4 at the transcription level, without any direct β-catenin association. Increased SOX4 leads to inhibition of cell proliferation.

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Study **Ref. ID#1068** (Tier 3) investigated by in situ hybridization the expression of c-fos and the jun proto-oncogenes in several uterine tissues (myometrium, stroma, and luminal and glandular epithelium) in response to tamoxifen in rats. Persistent overexpression of c-fos and jun-B in the uterine endometrial epithelium was observed, which may contribute to the molecular mechanism underlying the uterine toxicity associated with chronic tamoxifen treatment. Regulation of fos and jun genes in the uterus by estradiol appears to be through the interaction of functional estrogen response elements with ER. Persistent expression of proto-oncogenes, which are typically expressed during the G,-G, transition, may suggest that tamoxifen causes uterine endometrial epithelial cells to increase the duration of the Gi phase of the cell cycle, or perhaps undergo a temporary G1 block, resulting in massive hypertrophy (during this phase, the cell grows in preparation for DNA replication).

Conclusion on endpoint gene expression

The research strategy applied in this project aimed at identifying records addressing "estrogenic activity", "Epigenetic modulation" "DNA-repair" and "Accumulation of mutation" in the framework of uterine adenocarcinoma. In this context, papers addressed different aspects associated with gene expression, which can contribute to our understanding of estrogen-induced uterine endometrial epithelium proliferation. However, since different aspects (genes) were considered in the different studies, with no confirmation, this cluster was judged **INADEQUATE EVIDENCE** for the role of the selected stressors in modulating each single identified gene. It is important to note that this judgment is strongly influenced by the strings used for the present research and might change developing an *ad hoc* search focused on each single identified gene.

MUTATED GENES

Only in vivo

ENDPOINTS: HYPERPLASIA (SIMPLE), HYPERPLASIA (ATYPICAL), HYPERPLASIA (GLANDULAR/STROMA RATIO), UTERINE WEIGHT, UTERINE ADENOCARCINOMA

In this cluster, 6 Tier 3 papers only in the *in vivo* line of evidence were identified (Ref. ID# 239, 954, 3792, 4611, 4234, 4260).

Study **Ref. ID#239** (Tier 3) investigated in BALB/c nude mice the role of different ERα splicing (ERα mutation) in endometrial cancer, which are matter of debate. While estradiol, tamoxifen, and progesterone (only at mRNA) regulate wild type and variant ERα mRNA and protein expression separately and differently and that this hormonal regulation probably occurs, via different mechanisms, at the transcriptional or posttranscriptional level, the paper failed to demonstrate that changed expression in ERα variants induced by estradiol or tamoxifen are associated with increased tumour grafts of a moderately differentiated human endometrial adenocarcinoma grown in nude mice.

Study **Ref. ID# 954** (Tier 3) aimed to identify components of the cell cycle in the uterus that are regulated by the E2-induced IGF1 signaling pathway IRS-1 WT and IRS-1 null mutant mice. The IGF1/IRS-1 pathway contributes to the mitogenic action of E2 by stimulation of cyclin B-associated. The activation of the IGF1/IRS-1 pathway by E2 can result in phosphorylation of endogenous histone H1 isoform at 24 h. IGF1R/IRS-1/PI3K pathway stimulates the rate of mitosis in the uterine epithelium in response to estrogens. PI3K/AKT functions in the G2/M transition of the cell cycle. The phosphorylation of histone H1 in vivo by a cyclin dependent kinase (cdk) is considered to destabilize H1–chromatin interactions and, thereby, facilitate a more open chromatin structure, that can be accessed by DNA regulatory elements possibly changing the transcriptomic status in a cancerous state. Thus, by regulating cyclin B-associated kinase activity an IGF1R/IRS-1/PI3K stimulated signaling cascade mediates hormonal effects on the state of chromatin condensation.

Study **Ref. ID# 4611** (Tier 3) reported that stromal deletion of the APC tumour suppressor in mice triggers the development of endometrial cancer. The Authors further investigated a possible modulatory role of estradiol on this mouse model. They observed that APC KO mice showed minimal response to E2 treatment and gained significantly less uterine weight compared with controls. The results indicate that de novo mutation or loss of heterozygosity in stromal APC is sufficient to induce endometrial hyperplasia and endometrial

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carcinogenesis by mechanisms that are consistent with unopposed estrogen signaling in the endometrial epithelium.

In a Tier 3 study (**Ref. ID# 4234**), Shing et al. investigated if the genetic loss of Wdr13 in mice led to mimicking of the human endometrial hyperplasia associated metabolic disorders making Wdr13 knockout female mice a potential animal model to study human endometrial hyperplasia. Wdr13 KO mice had an increased gland/stroma ratio in the respect of WT animals. KO mice treated with DES showed a decrease (normal) gland/stroma ratio. The same treatment in WT mice showed no effect.

In study **Ref. ID# 3792** (Tier 3), the relation between PTEN signaling and E2/estrogen receptor in endometrial tumourigenesis was investigated. The Authors used genetically engineered mice as a model to address this relationship. Three of five *Pten*$^{+/-}$ mice (60 %) treated with estradiol developed myoinvasive carcinomas, whereas the controls for this group developed only complex atypical hyperplasia.

In study **Ref. ID# 4260** (Tier 3), very few numerical (quantitative) data are reported. Most of the data are narratively reported. The Cre-loxP system was used to achieve PTEN inactivation within mouse endometrial epithelium. Mice that did not receive E2 injection notably developed endometrial neoplasia, complex atypical hyperplasia, or carcinoma (7/8, 87.5%). In contrast, hyperplastic but nonneoplastic endometrium was observed in E2-treated mice. Moreover, hyperplastic but nonneoplastic endometrium permitting clonal expansion of Pten-null cells was observed in E_2-treated mice. Results suggest that E2 clonally proliferates PTEN-null epithelial cells. Furthermore, it demonstrated that a decrease in estrogen levels may contribute to endometrial carcinogenesis after PTEN deletion. This phenomenon might explain why the incidence of human endometrial carcinoma increases with perimenopausal or post- menopausal status.

Conclusion on these endpoints:

The effect for hyperplasia (simple, atypical, glandular/stroma ratio) and uterine weight was judged **INADEQUATE EVIDENCE** since inconsistent and limited data were available. Some studies were carried out in different genetically engineered mouse models specifically investigating PTEN, APC and Wdr13.

Considering uterine adenocarcinoma, the effect for this endpoint was **judged INADEQUATE EVIDENCE**, since two tier 3 study (Ref. ID# 3792, 4260) only with paucity of data were available.

ENDPOINTS: lacI mutation, DNA adduct formation, P53 mutation, ras mutation, uterine weight

In this cluster, 4 Tier 3 paper were identified (Ref. ID# 2172, 2175, 2743, 3515).

In **Ref. ID# 2172** (Tier 3), Big Blue rats were treated with TAM or its proximate carcinogenic metabolite a-hydroxytamoxifen and the mutant frequency in the lacI transgene was determined in the uterus. Statistically significant decrease of mutant frequency was seen in TAM treated animals. These results suggest that induction of endometrial tumours in rats is not due to TAM genotoxicity.

Since the contribution of α-hydroxy metabolites of tamoxifen to the induction of endometrial tumours is presently unknown, Gamboa da Costa *et al*. (**Ref. ID# 2175**) in a Tier 3 study compared the extent of DNA adduct formation in liver and selected non-hepatic tissues (including uterus) of female Sprague–Dawley rats treated by gavage with tamoxifen, α-hydroxytamoxifen, *N*-desmethyltamoxifen, α-hydroxy-*N*-desmethyltamoxifen and *N,N*-didesmethyltamoxifen, or intraperitoneal injection with tamoxifen, α-hydroxytamoxifen, 3-hydroxytamoxifen and 4-hydroxytamoxifen. None of the compounds resulted in an increase in DNA adducts in uterus (data not shown). These results suggest that endometrial tumours in rats do not arise from the formation of tamoxifen– DNA adducts.

In a Tier 3 study (**Ref. ID# 2743**), Murase *et al*. investigated the occurrence of p53 and ras gene mutations in preneoplastic and neoplastic mouse endometrial lesions induced by N-methyl-N-nitrosourea and 17 beta-

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estradiol. The results suggest that ras gene mutations were not related to carcinogenesis and inactivation of p53 may occur with low frequency during the mouse endometrial carcinogenesis in this model.

As androgen actions mediated via the androgen receptor (AR) supports uterine growth and may modify uterine cancer risk, Choi *et al.* (**Ref. ID# 3515)** in a Tier 3 study hypothesized that a functional AR may increase PTEN inactivation induced uterine cancer. They used genetically engineered mice. Uterine weight was increased in PTEN KO mice upon ovariectomy and testosterone treatment. The study demonstrates that the global AR inactivation, reduces PTEN inactivation induced uterine carcinogenesis by decreasing stroma ERα expression, raising the hypothesis that antiandrogen therapy may have a role in the prevention or treatment of uterine carcinogenesis.

Conclusion on these endpoints:

The effect for these endpoints was judged **NOT LIKELY** since most of the studies show no effect or inadequate evidence. Three studies show an effect but in one (Ref. ID# 2172) the effect was decreasing, in another (RefID #2743) the effect was of rare occurrence and in the third (Ref. ID# 3515) testosterone was used as stressor. All studies considered in this cluster are Tier 3.

3.3.3. Characterisation of the MIE and KEs: uncertainty identification

Epigenetic modulation and uterine adenocarcinoma - The term "Epigenetic modulation" defines all the heritable modification of DNA or chromatin that does not alter DNA sequence itself. Examples of these modifications include methylation of the DNA and methylation and acetylation of histones. Other mechanisms with an impact on gene regulation depend on non-coding RNAs, such as micro-RNA (miRNA) and long-noncoding RNA. Cancer is a disease initiated and driven by genetic anomalies, but it is increasingly clear that epigenetic pathways also contribute to the malignant properties of cancer cells (Dawson et al. 2012). The characterization of the epigenome reveals widespread epigenetic alterations in cancers e.g. silencing of tumour suppressor genes by local hypermethylation or, in some tumours, DNA methylation changes (hyper or hypomethylation) throughout the genomes. Epigenetic modulation in cancer may also impact histones, through their methylation and acetylation near genes that influence cellular behavior. Altered miR and LncRNA expression have been also reported. All these changes in the epigenome influence many of the hallmarks of cancer, such as malignant self-renewal, differentiation blockade, evasion of cell death, and tissue invasiveness.

Methylation- (both hyper and hypomethylation), acetylation as well as dysregulation of miRNAs and LncRNAs have been all revealed in human tissues of endometrial carcinoma, different human cancer cell lines sensible to estrogens and in few animal studies. Endpoints that indicate a dysregulation of epigenetic modulation in this type of tumours are represented by altered expression of enzymes involved in the control of histone acetylation, methylation through the alteration of specific methyltransferases and demethylases as well as the occurrence of global histone acetylation or changes in both the acetylation and methylation status of specific genes promoters.

Appraisal of the records retrieved indicate as **LIKELY the occurrence of acetylation and hypomethylation** among the epigenetic mechanisms identified, possibly under the control of estradiol (see paragraph below Estradiol-ERα-epigenetic modulation). This observation is in accordance with the promotion of gene expression. Indeed, histone acetylation is generally associated to an increased transcription, due to the removal of positive charges and therefore the relaxion of the chromatin (euchromatin) (Watson et al., 2014) and a similar effect is reached by a reduction of the methylated status usually of genes promoter region. Furthermore, a crosstalk between methylated DNA regions and histone deacetylation exists (Lee et al., 2020). Indeed, histone deacetylases are part of a multiprotein transcription repression complex and they are associated to Sin3A and methyl-CpG binding proteins (MeCPs). MeCPs can bind to CpG islands which are methylated, leading to the repression of the transcription of that gene (Cho et al., 2004; Song et al., 2013). Loss of methylation mark is usually linked to loss of the association complex containing MeCP2, mSin3A and HDAC1. This mechanism is recruited in human endometrial primary cancer cells to promote the expression of

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PAX2, a factor involved in the control of proliferation (Wu et al, 2005 RefID 1066, Tier 1 for human and *in vitro* and tier3 for *in vivo*).

Estradiol-ERα-epigenetic modulation – Estradiol may acts recruiting different receptors. Two main classes are represented by ERα and ERβ, which are member of the nuclear receptor family of intracellular receptors. In addition, there is a group of membrane receptors, among which the most studied is GPR30, which are G protein-coupled receptors. In the uterus, estradiol acts primarily through ER-α (Walker et al. 2004). Several records provide direct evidence of estradiol triggered epigenetic modulation. Less clear is the direct involvement of ERα.

Evidence that supports E2-induced epigenetic modulation are reported in:

RefID 47 – TAM induced hypomethylation of TFF3 promoter in ER-α + cells and increased TFF3promoter activity is observed in two different endometrial cancer human cell lines positive to ER-α but not in two different endometrial cancer human cell lines negative to ER-α

RefID 918 – decrease expression of RIZ1 is under the control of ER-α. As such overexpression of ER-α decreases RIZ1 expression (as observed in tumour specimens from humans) while ER-α a selective inhibitor ICI 182780 increases it.

RefID 326 – E2 induced miR-203 in vivo in a 3day uterotrophic assay in rats

RefID 307 and 1600 – E2 induced different miRNAs in mouse uterus

RefID 337 – E2 increases in Ishikawa cells LSD1, a histone lysine specific demethylase recruiting GP30 receptor. This observation introduces an uncertainty on the recruitment of ER-α since not directly addressed with the use of specific inhibitors or by silencing the receptor.

RefID 317 – the existence of estrogen response element on gene promoter of specific histone methyltransferase

Estradiol – epigenetic modulation – endometrial cells/endometrium – The prevalent activity, functionally connected to epigenetic modulation in the records retrieved and analyzed, is cell proliferation and tumour growth, through the dysregulation of several different factors. For some of these factors evidence of altered expression is reported both in human specimens and human cancer cell lines (Tab. 1). Involvement of tumour growth have been supported in animal models using xenografts.

Table 13 – Growth and transcription factors linked to deregulated proliferation in human UA specimen and endometrial cancer tumour cells: link to epigenetic modulation

Growth factor	Specimen type	ER-α	Cell type	ER-α	Epigenetic modulation	Essentiality of epigenetic modulation	RefID
Increased expression (mRNA and protein)							
TFF3 Increased expression	UA	Positive and negative	Ishikawa and ECC-1 Higher protein expression	Positive	TAM induced TFF3 promoter hypomethylation only in ERa positive cells	no	47
PAX2 increased expression	Type I	positive	Ishikawa,ECC-1, primary human endometrial type I cancer cells, primary human normal endometrial cells treated with methylase inhibitor	Positive	Hypomethytion of the promoter and reduction of the binding of a repressor factor with deacetylase activity	Yes on the role of hypomethylation in PAX2 expression	1066

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NMP1 Increased expression	UA	na	Ishikawa, HEC-1A,	na	HOTAIR (lncRNA)	Yes on the role of HOTAIR in NMP1 expression	669
DOHOD increased (mRNA)	UA	na	ECC-1, HEC-1A	na	Decreased acetylation at histone H3K27 in DOHOD promoter	Yes on the role of acetylation in DOHOD expression	296
Decreased expression (mRNA and protein)							
KLF9	UA	Positive	na	na	DNMT1 & HDAC3 (mRNA) overexpressed	no	426
RIZ-1 Decreased expression	UA	Positive	Ishikawa, RL95-2	Positive	Decreased levels of a methyltransferase (RIZ-1)	Yes on the role of RIZ-1 in proliferation	918
HOPX Decreased expression	Endometrioid adenocarcinoma	na	Ishikawa, HEC-1A, HHUA	na	Increased methylation and decreased acetylation (the latter only in Ishikawa)	Yes on the role of methylation in HOPX expression	453

na: not assessed

The search strategy adopted in the present tender was intended to provide a broad overview of factors and KEs involved in estrogen-dependent uterine adenocarcinoma. As a result, the identified growth factors may represent potential biomarkers plausibly linked to uterine adenocarcinoma under both estrogenic and epigenetic control. A search tailored on each of these factors would be instrumental to improve the confidence on this data.

Uterotrophic bioassay: definition and limits - A well-known screening *in vivo* method used to detect an estrogenic activity directed to the uterus and linked to (non-atypical) hyperplasia is represented by the uterotrophic bioassay. This is a short-term *in vivo* screening assay in female rodents proposed by the OECD for chemicals that interact with the estrogen receptor (ER). It is based on the increase in uterine weight (or uterotrophic response) and evaluates the ability of a chemical to elicit biological activities consistent with agonists or antagonists of natural estrogens. The assay is intended to be included in a battery of *in vitro* and *in vivo* tests to identify substances with potential to interact with the endocrine system, ultimately leading to risk assessments for human health or the environment. Graduated test substance doses are administered for a minimum of three consecutive days. The animals are necropsied approximately 24 hours after the last dose. For estrogen agonists, the mean uterine weight of the treated animal groups relative to the vehicle group is assessed for a statistically significant increase. Histological findings reported due to an estrogenic action in the uterus are stromal edema and hyperplasia with eosinophil infiltration, along with luminal epithelial cells hypertrophy and hyperplasia. Being a short term assay the uterotrophic bioassay is not able to elicit long term effects, namely pre-neoplasic (such as atypical hyperplasia) or neoplastic lesions.

Human studies - All appraised records in human line of evidence are Diagnostic Research Studies (DRS). These studies are designed to look for better ways to identify a particular disorder or condition and are based on analysis of human tissues obtained from groups characterized by a specific disease. The comparison group is usually represented by tissues derived from people without the disease. In the specific case of uterine adenocarcinoma, clinicopathologic studies and molecular analysis support the classification of endometrial carcinoma in two wide categories identified as Type I and Type II, which are characterized by a different pathogenesis. Type I arise in the setting of endometrial hyperplasia and is considered estrogen dependent due to expression of ER and because unopposed estrogen stimulation has been identified as a risk factor. Tumour type represents an uncertainty in our research. Indeed, authors refer to endometrial carcinoma without specifying the type (e.g. type II, serous, clear cells) in most of the studies. This uncertainty is considered to have a medium impact because Type I endometrial carcinoma accounts for approximately 60-70% of all cases and thus results prevalent (Murali et al. 2014).

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There are three histologic grades of endometrioid carcinoma, grade 1: well differentiated, grade 2: moderately differentiated and grade 3: poorly differentiated. Moreover, uterine adenocarcinoma can be classified in IV stages that define the extension of the developing tumour inside and outside the uterus. When reported, analysis on human tissues is performed in specimen from cases of different grade and/or stages. It is not excluded that some of the endpoint considered may be differently regulated in the different tumour grades/stages.

In the records retrieved evaluation if hyperplastic tissue was almost absent. Thus, our evaluation is to be considered as representative of an already transformed (tumoural) tissue. The same consideration applies to data obtained from *in vitro* studies essentially performed in human endometrial cancer cell lines. This has to be considered as an uncertainty to the possibility to extend the identified pathways to a healthy organism/normal cell. To this regard is interesting the observation reported in RefID 1066, where treatment of primary normal human endometrial cells with TAM 5μM or E2 100nM for 3h does not trigger PAX2 promoter activation, contrary to what observed in primary Type I human endometrial cells (Wu et al 2005, RefID 1066). TAM and E2 reactivation of PAX promoter in normal cells is possible in the presence of the methyltransferase inhibitor 5-aza-deoxycytidine. Nevertheless, two limits hamper the conclusion that demethylation pathways are not activated by E2 in normal cells, the choice of an experimental paradigm (TAM and E2 dose and 3h exposure) tested on cancer cells and the lack of a dose and time response in normal cells.

An advantage of diagnostic research studies is that the endpoints observed in human specimens have also been studied in cancer cell lines for their mechanisms, providing evidence of essentiality for some of the KERs developed in the presented AOP. For some of these records, essentiality of KERs is also supported in *in vivo* studies. DRS have thus the advantage to be concerned with endpoints (KEs) and adverse outcome relevant in a population of interest and to include heterogenous individuals. In addition, the association to mechanistic *in vitro* and *in vivo* studies overcome the limit of observational studies that are usually not designed to provide direct evidence of causality. The functional analysis provided with *in vitro* studies allowed to develop KERs in the AOP anchored to endpoints (KEs) considered relevant for humans.

Stroma and stromal cells. Prolonged estrogenic stimulation forces simple columnar epithelium (glandular cells) to proliferate resulting in endometrial hyperplasia, defined as an increased proliferation of the endometrial glands resulting in an increased gland-to-stroma ratio, that may then develop to uterine adenocarcinoma. Although stromal cells/tissue have not been considered in our analysis, it is important to consider that they may represent an important modulator of uterine tissue microenvironment. Tanwar et al. (2021, RefID 4611, tier 3) reported that genetic manipulation of stromal tissue in a mouse model is sufficient to induce endometrial hyperplasia and endometrial carcinogenesis by mechanisms that are consistent with unopposed estrogen signalling in the endometrial epithelium. Thus, the activation of certain pathways might be different in the presence or the absence of stromal cells. In addition, different components of uterine endometrium can express different factors, e.g. HOPX is expressed in the epithelium but not in stroma (Yamaguchi et al. 2009, RefID 453, tier 3). This may impact on the evaluation of specific endpoints in human specimens or animal tissues if a distinction between epithelium and stroma is not performed.

AO, Uterine adenocarcinoma - Incidence of spontaneous uterine adenocarcinomas in rodents is low in most of the strains (for details see EFSA 2022 in preparation, Annex A) including Fisher 344 (F344) and Sprague-Dawley rats (5.4 and 0.9% respectively, Harleman et al 2012) that represent the most common rat strains used in toxicology studies and especially in chronic toxicity and carcinogenicity studies. This may be reflected in a low sensitivity of these animal models in developing uterine adenocarcinoma following treatments with the substance under test. Some strains of rats (BDII/Han, DA/Han, Han:Wistar, Donryu) have a high incidence of spontaneous uterine adenocarcinoma but are seldom used in *in vivo* studies. Moreover, several genetically engineered mice (GEM) with a high incidence of uterine adenocarcinoma have been recently developed (e.g. PTEN knock-out mouse models).

A study analyzing different pesticides in combined chronic toxicity and carcinogenicity studies in rats (Wistar or Fischer) and in mice, identified seven pesticides increasing the incidence of endometrial adenocarcinoma in rats but not in mice (Yoshida et al. 2015).

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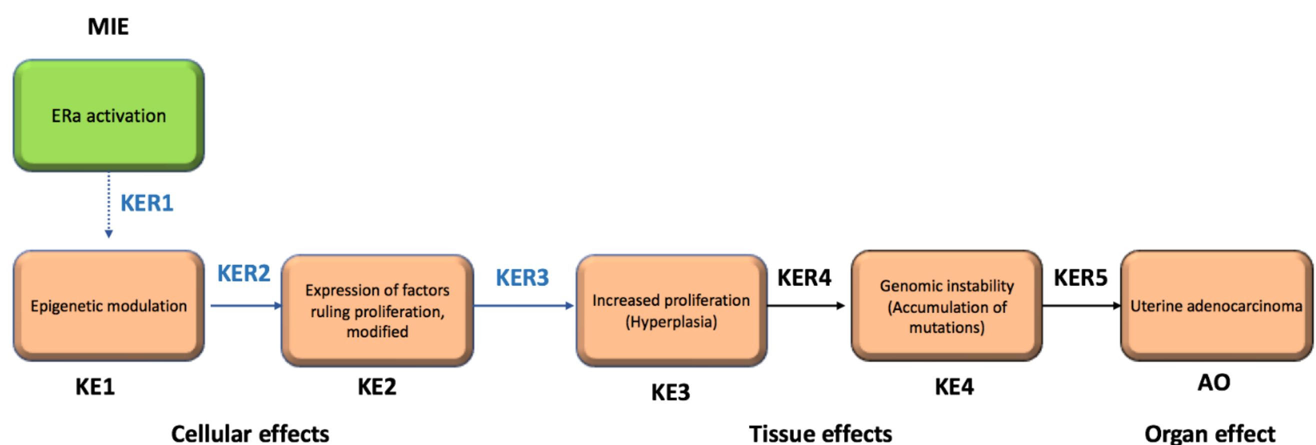
All the studies retrieved in the present research addressing uterine adenocarcinoma after E2 or TAM exposure were performed in mice or in Sprague Dawley and Wistar rats. The species used in these studies may give reasons for the paucity of data obtained and their inconsistency. This is reflected in the last relationship of the developed AOP (KER 5, Genomic instability-accumulation of mutation leading to uterine adenocarcinoma), populated with human data for which there is sufficient evidence for the carcinogenicity of estradiol and TAM.

3.4. Adverse Outcome Pathway development

Starting from the postulated AOP and following the methodology described in the previous sections, the following AOP has been designed and developed:

Activation of uterine estrogen receptor-alfa leading to endometrial adenocarcinoma, via epigenetic modulation



"Epigenetic modulation" was included in the AOP based on:

the information and data retrieved from the systematic approach addressing "Epigenetic modulation", "Accumulation of mutation" and "DNA repair"

the results from RoB in accordance with the protocol

Information and data extracted from the studies included in this group also populated KE2, part of KE3 and the connecting KERs (blue lines) as described in 2.2.2.3.

Due to the paucity of the studies retrieved and eligible for appraisal (n=1, Fig. 7) "DNA repair" was not further considered.

"Accumulation of mutation" and "Uterine adenocarcinoma" were developed based on human studies since considered well established (black lines) and thus based on seminal studies (Annex A 4.4).

For prioritized KEs/KERs, empirical evidence, essentiality and concordance table have been derived from records/endpoints with a Tier 1 and Tier 2 score in RoB analysis for each line of evidence or Tier 3 score when presenting concordant results in three (human, *in vivo* and *in vitro*) or at least in two lines of evidence (human and *in vitro*, human and *in vivo* or *in vivo* and *in vitro*), unless specified.

A list of the appraised records included to the developed AOP and excluded is reported in Annex H.

The detailed AOP is described in Annex I. KERs quantification in the framework of the AOP network on uterine adenocarcinoma and regulatory implications of the network are addressed in EFSA PPR Panel et al. 2023.

4. Conclusions

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Endocrine disruptors (EDs) may act as exogenous substances promoting target tissue proliferation leading to cancer development. Uterine (endometrial) adenocarcinoma is a common human malignancy, of which Type I endometrioid adenocarcinoma represents an estrogen dependent form. Uterine adenocarcinoma can also develop in laboratory animals as a consequence of an elevated estrogen:progesterone ratio (strains Donryu, DA/Han, BDII/Han). Aim of this work was to contribute with EFSA in developing an AOP network for uterine adenocarcinoma in mammals with the task to support regulatory applications to identify substances with and endocrine disruptor activity. To accomplish this task a transparent and trackable evidence-based approach was used. An evidence based AOP starting from **activation of uterine estrogen receptor-alfa leading to endometrial adenocarcinoma, via epigenetic modulation** was postulated, based on human, *in vivo* and *in vitro* lines of evidence. Epigenetic modulation and the related KERs were prioritized for appraisal and weight of evidence analysis. Among the epigenetic markers identified as possibly relevant in uterine adenocarcinoma and appraised, i.e. acetylation, methylation, miRNA and LncRNA, only acetylation and hypomethylation of the promoter of specific factors promoting cell proliferation were judged likely in term of involvement in uterine adenocarcinoma under the control of estrogens. From the analysis of diagnostic research studies, where human specimens are analyzed in parallel to mechanistic *in vitro* and *in vivo* studies, it has been possible to identify a list of factors involved in the promotion of endometrial cell proliferation and tumour growth, relevant for human uterine adenocarcinoma. Some uncertainties have been identified, the most important is the lack of data addressing the identified endpoints in non-cancer cells. An additional uncertainty is due to the lack of empirical data that provide correlative and/or causal relationships between the i**ncreased proliferation (KE4) and Genomic instability (accumulation of mutations) (KE5) to the AO.** The inclusion of additional stressors and the refinement of the search with *ad hoc* strings to address specific KEs will decrease the uncertainty. The AOP included in this report focus on in uterus pathways and was integrated in a complete AOP network considering extra-uterine pathways converging on ER-α activation and published in EFSA PPR Panel et al. 2023. As such, in the same EFSA scientific opinion is reported the quantification of the KERs certainty (Scientific Opinion and Annex C in EFSA PPR Panel et al. 2023). Based on the analysis of the uncertainty few recommendations have been postulated. According to EFSA requirements, the present AOP was submitted to OECD and will be uploaded in AOP-Wiki.

5. Recommendations

For the optimization of an evidence-based approach to develop AOP:

1- Use of machine learning techniques to identify unknown MIE and KEs: it is important to set a group of experts in the biological domain of interest to evaluate the results obtained

2- It is recommended the use of an evidence-based approach

3- It is recommended to adopt a simplified model of the NTP appraisal tool to develop AOPs, focusing on a restricted number of key questions to improve the efficiency of the process

For the optimization of an AOP network addressing uterine adenocarcinoma in mammals:

4- It is recommended to develop and *ad hoc* search strategy to address pre-neoplastic lesions (such as atypical and complex hyperplasia) to identify KEs relevant in a pre-tumoural status. This would further support the involvement of the analysed pathway in tumour progression

5- ER signalling is strongly tissue dependent. It is thus recommended to use cells representative of the uterine endometrium and an adequate comparator (same tissue and cell type) to develop an *in vitro* battery addressing MIEs/KEs related to uterine adenocarcinoma. Mammary epithelial cells or endometrial stromal cells are not suitable comparators. This approach will facilitate the recognition of selective estrogen receptor modulators (SERMs), a category of compounds that function as agonists or antagonists for ERs in a target gene-specific and tissue-specific fashion.

6- Stromal cells exert important modulator action on endometrium epithelial cells functions. It is recommended to enrich the AOP network considering the possible interaction between these two different cell types

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7- It is recommended to expand the stressor-based approach to furtherly test the presented AOP (e.g. diethylstilbestrol; ethynilestradiol) in *in vivo* and *in vitro* studies reducing the level of uncertainty

8- The uncertainty identified in the postulated AOP should be further explored with an ad hoc search strategy and addressed by including additional empirical data

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Abbreviations

AO	Adverse Outcome
AOPs	Adverse Outcome Pathways
AR	Androgen Receptor
ALAN	As Likely As Not
ACLY	ATP-Citrate Lyase
BMI	BMI
TGCA	The Cancer Genome Atlas
Cdkn2d	Cyclin Dependent Kinase Inhibitor 2D
Cdk	Cyclin Dependent Kinase
DRS	Diagnostic Research Studies
DES	Diethyilstilbestrol
DOHOD	Dihydroorotate Dehydrogenase
E2	17β–estradiol
ED	Endocrine Disruptors
EH	Endometrial Hyperplasia
EZH2	Enhancer of Zeste Homolog 2
EGFR	Epidermal Growth Factor Receptor
Epi	Epigenetics
ER	Estrogen Receptor
ER+	Estrogen Receptor Positive
ERE	Estrogen Response Elements
GEM	Genetically Engineered Mice
HDAC3	Histone Deacethylase 3
KERs	Key Event Relationships
KEs	Key Events

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LNcRNA	Long Noncoding RNAs
sLSD1	Lysine Specific Demethylase 1
Qs	Main Assessment Questions
Mesh	Medical Subject Headings
Met	Metformin
MeCps	Methyl-Cpg Binding Proteins
RIZ1	Retinoblastoma Protein-Interacting Zinc Finger Protein 1 (RIZ1)
miRNA	MicroRNA
MAPK	Mitogen-Activated Protein Kinase
MoA	Mode of Action
MIEs	Molecular Initiating Events
PI3K	Phosphotidylinositol-3-Kinase
RoB	Risk of Bias
RUCA-I	RUCA-I
SERMs	Selective Estrogen Receptor Modulators
STAT3	Signal Transducer and Activator of Transcription 3
SOX	SRY-Box transcription factors
SQs	Sub-Questions
TAM	TAM
TGF-B1	Transforming Growth Factor-B1
TFF3	Trefoil Factor 3
UA	Uterine adenocarcinoma
UCA1	Urothelial Cancer Associated 1
WHO	World Health Organisation

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Appendix A – List of Annexes

Annex A - Protocol

Annex B - Documenting the search process for topic modelling

Annex C - Topics

Annex D - Tabular presentation of Topics

Annex E - Strings main search

Annex F1 - Human data extraction

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Annex F3 - In vitro data extraction

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Annex G1.2 - In vivo appraisal

Annex G1.3 - In vitro appraisal

Annex H- Appraised records IN_OUT

Annex I - AOP

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