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## Review

# Epigenetic transgenerational actions of endocrine disruptors

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### ABSTRACT

Environmental factors have a significant impact on biology. Therefore, environmental toxicants through similar mechanisms can modulate biological systems to influence physiology and promote disease states. The majority of environmental toxicants do not have the capacity to modulate DNA sequence, but can alter the epigenome. In the event an environmental toxicant such as an endocrine disruptor modifies the epigenome of a somatic cell, this may promote disease in the individual exposed, but not be transmitted to the next generation. In the event a toxicant modifies the epigenome of the germ line permanently, then the disease promoted can become transgenerationally transmitted to subsequent progeny. The current review focuses on the ability of environmental factors such as endocrine disruptors to promote transgenerational phenotypes.

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## 1. Introduction

An integral part of biology is the ability of environmental factors to influence and regulate biological processes. No organism develops or functions without environmental impacts on basic biological systems. The ability of environmental factors to influence biology is represented from broad processes such as evolutionary biology to specific processes such as the development of organ systems. Examples range from basic environmental factors such as light and temperature requirements for the survival of an organism, to more specific individual nutritional or environmental compound actions on specific cellular processes. The environment is a critical element that is integrated into the molecular and cellular biology of any organism. Although one of the critical building blocks of biology is DNA and the genome sequence, the ability of environmental fac-

tors to regulate genome activity is also a critical element of biology not completely appreciated in this era of molecular biology and genetics. Highly conserved and efficient molecular processes have evolved allowing the environment to directly regulate genome activity independent of alterations in the basic genome sequence and genetics of the organism. The current review will expand on the mechanisms of how environmental factors influence biological systems and can promote abnormal physiology associated with disease.

The majority of environmental factors and toxicants do not have the ability to alter DNA sequence or promote genetic mutations [1–3]. This is due in large part to the stability of the genome. The DNA sequence developed a general resistance to change to maintain genome stability during evolution. Therefore, many environmental factors promote abnormal phenotypes or disease, independent of any change in DNA sequence. Interestingly, often early life exposures lead to later life adult onset abnormal physiology and disease [4]. This toxicology is not mediated through basic genetic mechanisms, but alterations in molecular processes

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**Table 1**  
Environmental epigenetics terminology.

Term	Definition
Environmental actions on somatic cells	Allows tissue specific toxicology and critical for adult onset disease in the individual exposed, but not capable of transmitting a transgenerational phenotype
Environmental actions on germ cells	Allows transmission between generations and in the absence of direct exposure promotes a transgenerational phenotype
Multigenerational phenotypes	Coincident direct exposure of multiple generations to an environmental factor or toxicant promoting a toxicology in the multiple generations exposed
Transgenerational phenotypes	After the initial exposure, the transgenerational phenotype is transmitted through the germ line in the absence of direct exposure
Epigenetics	Molecular factors and processes around the DNA that regulate genome activity that are independent of DNA sequence and are mitotically stable

that influence genome activity, such as epigenetics [1,5,6]. The majority of environmental factors act on somatic tissues and influence the physiology of the individual exposed. However, in some cases these environmental factors promote a heritable or familial transmission of the disease phenotype in a non-Mendelian manner. The heritable transmission of toxicology phenotypes is referred to as transgenerational inheritance [1,3,7]. Although the vast majority of environmental exposures involve somatic cells and cannot promote a transgenerational phenotype, in the event a germ-line epimutation is involved then the exposure has the potential to promote a transgenerational phenotype (Table 1). Therefore, transgenerational phenomena will be a small subset of toxicology involving direct germ-line actions of environmental factors.

Environmental and toxicology studies often involve a correlation between exposure and the development of an abnormal phenotype or disease. The future of these fields lies in elucidation of the molecular and cellular mechanisms involved in the actions of the environmental factor or toxicant. A basic understanding in the molecular mechanisms involved will dramatically facilitate risk assessment, provide diagnostics for exposure and develop potential treatments for exposures and adult onset disease. Although exposure susceptibility and genetics will be important molecular factors, alternate mechanism such as the role of epigenetics will be critical to consider in future research.

**2. Environmental factors and toxicology**

Epidemiology studies for decades have suggested a major impact of environment on biology and disease. Examples include the regional differences in disease frequency [8] and the identification of a number of diseases related to environmental exposure to endocrine disrupting chemicals [9], that cannot be explained by Mendelian genetic processes, Table 2. A more direct group of studies involves the observation that identical twins with similar genetics have different disease frequencies [10], suggesting an

**Table 2**  
Environmental impact on disease etiology.

Regional disease frequencies	[8]
Low frequency of genetic component of disease	[11]
Increases in disease frequencies	[26]
Identical twins and variable disease frequency	[10]
Environmental exposures and disease	[9]

**Table 3**  
Environmental factors associated with disease states.

Nutrition	
Caloric restriction	[15]
Fat content	[16]
Plant compounds—phytoestrogens	[17]
Environmental compounds	
Pesticides	[19]
Fungicides	[20]
Plastics	[18]
Stress	
Anxiety induction	[54]

environmental factor and not genetic processes promoting disease. Another epidemiological observation is that the percentage of disease that is known to be due to genetic abnormalities is relatively small for nearly all diseases, Table 2. For example, for breast cancer approximately 5% of the disease is due to known genetic mutations, while the majority has no known genetic cause [11]. Almost half of the tumor suppressor genes that cause familial cancers via mutations can also be inactivated with promoter hypermethylation [12]. Although there are several disease states that do derive in part from specific genetic mutations, the majority of disease states have not been shown to have a complete genetic link. The assumption for the past several decades is that there exist genetic mutations or susceptibilities yet to be identified, but through relatively rigorous molecular approaches few have been identified. The recent genome wide association studies (GWAS) have shown that very low percentage, generally less than 1%, of any disease is due to a specific genetic polymorphism [13]. Observations suggest that genetics will not be the only causal factor in disease etiology and environment must be an important factor to consider in conjunction with epigenetic mechanisms.

The final epidemiology example discussed is the phenomena of non-Mendelian or familial disease states [14]. Often common disease states such as breast cancer, prostate cancer, diabetes and obesity do not involve classic genetic transmission or heritable characteristics. Instead they appear familial, but do not involve Mendelian genetic transmission. Examples include Autism and many neurological disorders, cancer and forms of metabolic disease. This familial association has been well known for several decades, but the mechanisms involved are unknown. Often environmental factors have been identified or suggested, but the mechanism appears not to involve classic genetics. The existence of such familial disease further supports the role of the environment in disease etiology, as well as suggests potential roles of non-genetic mechanisms.

The types of environmental factors that have been shown to promote or influence disease involve common items such as nutrition, as well as factors such as environmental compounds or toxicants, Table 3. Nutrition can be a factor both in the amount and in the type of nutrients. Caloric restriction or high fat diets can influence disease, as well as diets with high concentrations of phytoestrogens or plant compounds [15–17]. Environmental compounds such as plastics, pesticides and fungicides also have been shown to promote disease and act as environmental toxicants [18–20]. The current society is exposed to hundreds of different compounds on a daily basis, such that their potential impact on biology and disease needs to be considered. In addition to these specific compounds and nutrients, other factors such as stress and behavioral considerations influence disease as well [21,22], Table 3. Although numerous environmental factors are involved, the current review will focus on environmental compounds and toxicants (endocrine disruptors).

A critical element to consider in any environmental factor exposure and disease is the concept of the fetal basis of adult onset disease [23]. The most sensitive period to environmental factors

is during the active initial development of the organism and tissue. Most organ systems in mammals develop during the fetal period, such that exposures during this time often promote multiple disease phenotypes later in life. Exposures during pubertal development often promote disease in organs such as the mammary glands or prostate that develop during puberty [24]. Therefore, the sensitive development period to consider for an organ or associated disease is during active development. The adult period is generally resistant to environmental exposures due to most organ systems being developed and inability to modify cellular differentiation. In considering the effects of environmental factors or toxicants on disease etiology, the developmental aspects of the exposure need to be considered when studying the biology of the organ system influenced.

### 3. Endocrine disruptors

Endocrine disruptors can be classified according to the nature of its endocrine actions. For example, anti-androgenic, androgenic, estrogenic, arylhydrocarbon receptor agonists, inhibitors of steroid hormone synthesis, anti-thyroid substances, and retinoid agonists. Based on usage in agriculture and daily life, endocrine disruptors can be separated into classes of chemicals including pesticides (DDT and methoxychlor), fungicides (vinclozolin), herbicides (atrazine), industrial chemicals (PCBs, dioxins), plastics (phthalates, bisphenol A, alkylphenols) and plant hormones (phytoestrogens). Some pharmaceuticals, personal care products and nutraceuticals are also known endocrine disruptors [25].

One of the first studies describing endocrine disruptor actions showed that alligators exposed to an organochlorine pesticide, dieldrin, presented many reproductive and endocrine problems [26]. Another initial study demonstrated birds exposed to the organochlorine pesticide DDT experienced reproductive failure [27]. Phytoestrogens were discovered to be endocrine disruptors when consumption of clover impaired fertility in sheep [28]. In humans, prenatal exposure to the estrogenic diethylstilbestrol (DES) was linked with the development of a rare form of vaginal cancer in the adult [29–32] and this effect has been replicated in experimental animal models. For example, exposure of rodents to DES at the perinatal period produced developmental toxicity, neoplasia, and more subtle endpoints of reproductive dysfunction [30–32]. The plastic compounds bisphenol A (BPA) and phthalates are more recently studied endocrine disruptors. Although several environmental compounds or therapeutics can induce genetic mutations [33,34], the vast majority of endocrine disruptors do not alter DNA sequence. The major action of endocrine disruptors is on the endocrine system and in regards to long term developmental effects appears to involve alterations in the epigenome. A number of disease states are promoted by endocrine disruptors. The concept of the fetal basis of adult onset disease is a critical factor to consider regarding the effects of the endocrine disruptors. A number of endocrine disruptors have been shown to have a significant role in causing adult onset diseases in later life following perinatal exposure, confirming the Barker hypothesis that is the concept that adult diseases have a fetal (early developmental) origin [23,35]. Since the endocrine system is essential for the development of a large number of tissues and biological processes, abnormal actions of endocrine actions during early development can have dramatic effects later in life on disease etiology. For example, abnormal androgen exposure during early gestation perturbs multiple organ system programming and leads to disease such as polycystic ovaries in adult women [36]. Perinatal and pubertal exposure to estradiol and bisphenol A alters the prostate epigenome and increases susceptibility to carcinogenesis in adult males [37]. Susceptibility to cancer may be a result of developmen-

tal exposures rather than exposures existing at or near the time of tumor detection [38]. Therefore, endocrine disruptors can induce abnormal development during fetal or early life exposures that then leads to adult onset diseases. How an early life endocrine disruptor can promote an adult onset effect in an organ system, long after the compound is removed, is presumed to involve in part epigenetic mechanisms and will be discussed below.

### 4. Transgenerational phenomena

The actions of an environmental factor or toxicant to promote an altered phenotype or disease can affect the individual exposed through the somatic cells. If the germ cell is directly affected, then a transgenerational phenomena is possible. In many cases exposure of a gestating female allows multiple generations to be exposed [39], Table 1. This does not constitute a transgenerational phenotype, but a multigenerational exposure.

A classic example of a multigenerational phenotype involves the pharmaceutical agent with estrogen agonist activity diethylstilbestrol (DES) [40,41]. Exposure of a gestating female to DES was found to promote an abnormal reproductive tract and gonadal dysfunction in the F1 generation males and females, as well as abnormal female reproductive tract function in the F2 generation [42]. Interestingly, the phenotype of the F1 and F2 generations has differences. Recent studies have started to emphasize the transgenerational aspect after early environmental exposures [43]. F3 generation rodent models have not observed a major phenotype [40,42]. It is possible that DES promotes a transgenerational phenotype, but extended generations need to be investigated [42]. Another example of a multigenerational exposure is a study with flutamide [44]. This anti-androgenic endocrine disruptor after exposure of a gestating female promoted an F1 generation abnormality in the testis and F2 generation effects in skeletal development, but no F3 generation effects [44]. Again the F1 and F2 generation phenotypes were distinct. In contrast, another endocrine disruptor vinclozolin did promote a transgenerational phenotype in the F3 generation [44]. Environmental factors that promote a toxicology for multiple generations involving direct exposure of the individual, the fetus, or germline have been observed for numerous agents [1,4], Table 4. These multigenerational exposures and phenotypes are not transgenerational phenotypes, although critical to consider in assessing the toxicology of an environmental agent, Table 4.

Transgenerational phenotypes require transmission of germ line alterations between generations. These transgenerational phenotypes occur in the absence of direct exposure, Table 1. Somatic cell targets are critical and common in toxicology to promote adult onset disease and phenotypes, but are not able to transmit the phenotype transgenerationally without continued direct exposure

**Table 4**

Studies on multi-generational epigenetic actions of environmental signals.

Environmental signal	Reference
Bis-phenol A	[96,97]
Diethylstilbestrol	[41–43,98]
Flutamide	[44]
Maternal depression	[99]
Food restriction	[100]
Maternal care	[101]
Airborne polycyclic aromatic hydrocarbons	[102]
Maternal cocaine	[103]
Pesticides	[104]
Phytoestrogens	[96,105,106]

Studies included in this table have evaluated the effects of early exposure to environmental signals for the F1 and/or F2 generations. In such cases, the organisms somatic tissues (F1) or germ-line (F2) cells were directly exposed.

[45]. Therefore, the critical target cell for transgenerational phenotypes and toxicology is the germ-line. One of the initial studies to demonstrate epigenetic transgenerational effects of an endocrine disruptor involved the analysis of vinclozolin actions on the male germ line of rats. Vinclozolin is a fungicide commonly used in agriculture that is known for its anti-androgenic endocrine disrupting action [46]. Exposing a pregnant rat to either vinclozolin or methoxychlor during embryonic days 8–14, a critical period for gonadal sex differentiation and testis morphogenesis, produces transgenerational defects in spermatogenic capacity, which are transmitted through four generations (F1 to F4) [47]. The transgenerational phenotypes observed in these animals also include adult onset diseases such as male infertility [47,48], increased frequencies of tumors, prostate disease, kidney diseases and immune abnormalities that develop as males age [49]. Changes in behavior and learning capacity have also been observed following vinclozolin exposure [50–54], including transgenerational changes in mate preference [51] and anxiety behavior [54]. Transgenerational effects on tissue transcriptomes have also been observed. For example, in the embryonic testis transcriptome a subset of genes have their expression altered in a consistent manner in males from the F1 through the F3 generation [55]. The actions of vinclozolin to promote this transgenerational phenotype appears to be epigenetic through alterations in DNA methylation of the male germ line [1,47,56]. Since these initial observations with vinclozolin, other agents that promote transgenerational phenotypes include actions of BPA on testis function [57] and nutrition on obesity [58].

Crucial to obtain a transgenerational phenotype is the action of environmental factors on the germ line and gonadal development [3,56]. During mammalian development the primordial germ cells migrate down the genital ridge towards the newly formed gonad prior to sex determination [59–61]. The germ cells develop into a male or female germ cell lineage at the initial stages of gonadal sex determination. The female germ-line then enters meiosis in the developing embryonic ovary while male germ cells continue to proliferate until immediately prior to birth when they resume proliferation after birth until puberty [62]. The female germ-line forms from oogenesis during follicle development that generate oocytes. The male germ-line, in turn, develops from spermatogonial stem cells and undergoes spermatogenesis that originate spermatozoa in the testis. The critical period for epigenetic regulation of the germ line is during the period of primordial germ cell migration and gonadal sex determination. Permanent alteration in the epigenetic programming of the germ line appears to be the mechanism involved in the transgenerational phenotype [1,3,47,56].

In addition to the transgenerational phenotype that involves a single generation exposure and an epigenetic modification of the germ line for transmission to multiple generations, there are examples of transgenerational phenotypes that involve a programmed environmental factor at each generation to promote a transgenerational phenotype [63]. The best example of this is the impact of maternal behavior and early postnatal life exposures [64]. A mother rat that licks and has an increased maternal care for the pups appears to program an epigenetic event during brain development that promotes the same maternal behavior in that female, such that she promotes the same maternal behavior and propagates the behavior transgenerationally [64,65]. Therefore, the continued environmental event is required to transmit the transgenerational phenotype. In order to distinguish these transgenerational processes that require a persistent transgenerational environmental exposure from those requiring only a single generation exposure we have recently proposed a clarification of the term transgenerational epigenetics, separating them into intrinsic and extrinsic categories [45]. An intrinsic transgenerational process requires a germ-line involvement, permanent alteration in the germ cell epigenome, and only one exposure to the environmental factor.

**Table 5**

Examples of epigenetic processes.

DNA methylations	Methyl cytosine at CpG sites [68,69]
Histone modifications	Methylation and acetylation at lysine residues [70,107]
Chromatin structure	Loop and bend structures and nuclear matrix associations [108]
Non-coding RNA	Small RNA influencing RNA stability and gene expression [72–74]

An extrinsic epigenetic transgenerational process involves an epigenetic alteration in a somatic tissue and requires exposure at each generation in order to maintain the transgenerational phenotype [45]. Therefore, the intrinsic and extrinsic epigenetic transgenerational phenomena are distinguished by the involvement of the germ-line and an isolated exposure versus a somatic cell effect and continued generational exposures. The mechanisms behind these transgenerational processes would be epigenetic in nature.

## 5. Epigenetics and epigenetic technology

Conrad Waddington in the 1940s coined the term epigenetics [66,67] during his gene–environment interaction studies associated with phenotype change [66,67]. The definition of epigenetics has evolved over the past decades with more refined understanding of the molecular mechanisms involved [3]. We propose epigenetics is defined as “molecular factors and processes around DNA that regulate genome activity independent of DNA sequence and are mitotically stable”, Table 1. The first epigenetic molecular factor identified was DNA methylation in the 1970s [68], Table 5. In the late 1980s X-chromosome inactivation was shown to involve DNA methylation and in the early 1990s imprinted genes were shown to involve DNA methylation [69]. The next epigenetic factor identified was histone modifications in the mid 1990s [70]. In 2000 small RNAs were identified [71–74] and in 2005 one of the first whole epigenome analysis was performed [75], Table 5. Therefore, the majority of epigenetic marks have been identified recently [3], and likely to be expanded in the future. Alterations in these normal epigenetic marks, in particular DNA methylation, have been shown to be associated with several disease states, Table 6. These include Angelman, Prader–Willi, Beckwith–Weidemann, Silver–Russell and Fragile X Syndromes [76–79]. Therefore, the link between epigenetic alterations or abnormalities with disease has been established in previous studies. Improvements in the technology to investigate epigenetic marks is required to allow a significant advance in understanding the role of epigenetics in medicine and biology.

One of the initial methods developed to evaluate DNA methylation and epigenetic changes was bisulfite DNA sequencing [80]. Combinations of bisulfite sequencing with a variety of other methodologies such as methylation restriction enzyme analysis is commonly used [81]. Current epigenetic methods can be separated in three categories: global methylation, local methylation and genome-wide methylation (Table 7). Global methylation [82–84] together with restriction enzyme analyses [85] were the first methods developed. A limitation to global methylation is that only major changes can be detected and local changes in DNA methylation can-

**Table 6**

Epigenetic diseases.

Angelman syndrome
Prader–Willi syndrome
Beckwith–Weidemann syndrome
Fragile X syndrome
Brain disorders—Autism, schizophrenia, Rhetts syndrome
Cancer (chromosome stability)



**Table 7**  
Techniques for measuring DNA methylation and epigenome.

Category	Technical basis	References
Global methylation	HPLC	[83]
	Radioactive incorporation	[84]
	Antibody labeling and cytometry	[82]
Local methylation	Restriction enzyme digestion	[85]
	Combined restriction enzyme and bisulfite conversion	[81]
	Bisulfite conversion and sequencing	[86,87,90]
	Bisulfite conversion and pyrosequencing	[88]
	Bisulfite conversion and mass spectrometry	[89]
Genome-wide methylation	MeDIP–Chip	[56,91]
	ChIP–Seq	[94]

not be detected. The majority of regulatory epigenetic mechanisms involve small local changes in DNA methylation not reflected in global analysis procedures. The most common current local methylation analysis to detect changes in DNA methylation involves bisulfite conversion of cytosine to uracil (converted to thymidine after PCR) unless the cytosine is methylated. After bisulfite conversion the DNA [80] is either directly sequenced [86] or subcloned and individual clones sequenced [87]. More advanced procedures performed after bisulfite conversion are pyrosequencing [88] or mass spectrometry analysis [89]. Bisulfite conversion based methods have been previously considered the gold-standard in studies involving DNA methylation. These methods have the advantage of interrogating DNA methylation at a CpG base pair resolution. Bisulfite conversion followed by analysis of individual subclones allows one DNA molecule to be analyzed in each subclone sequence [80]. However, one disadvantage is that the number of subclones and molecules analyzed is generally small enough to not allow for good statistical analysis, thus clone bias can be produced in the selection of subclones and DNA sequence analyzed. Digital bisulfite sequencing simplifies this technique by substituting the process of cloning by random dilutions to allow for the amplification of single molecules [90]. Pyrosequencing [88] has the advantage of providing the mean DNA methylation percentage for a CpG site, but allows for only short regions of DNA to be evaluated per amplicon, in average 50–150 base pair in size. This procedure provides the average mean DNA methylation percent to be assessed, but is limited by the span of CpGs to be analyzed. Not all DNA sequences can be interrogated with pyrosequencing. A more recent procedure is after bisulfite conversion to perform mass spectrometry to assess DNA methylation [89]. Although one disadvantage is that some CpGs measured in one sample are not measured in others, limiting the comparison between treatments, generally longer stretches of DNA (500–600 bp) can be interrogated. The limitations to the classic bisulfite clonal analysis needs to be seriously considered, such that the more advanced pyrosequencing and mass spectrometry should be used more commonly in the future.

In regards to genome-wide methods, one of the most useful sample preparation procedures is chromatin immunoprecipitation with specific antibodies to epigenetic marks. One of the most commonly used is the methyl cytosine antibody to immunoprecipitate methylated DNA fragments (MeDIP) [91]. Other chromatin immunoprecipitations (ChIP) can be performed with specific histone modifications and DNA binding protein antibodies. Therefore, the MeDIP method, for example with methylated DNA, can enrich DNA in a sample through immunoprecipitation for genome wide analysis [56]. One of the first genome wide analyses developed used tiling arrays of the genome in microarray chip hybridizations [91]. This procedure is termed a MeDIP–Chip or ChIP–

Chip analysis [56]. This powerful tool has been used to map the methylome in *Arabidopsis thaliana* [92] and human breast cancer metastasis [93]. This method has the obvious advantage of being able to scan for epigenetic changes in the whole genome. False positives can arise in MeDIP–Chip analysis such that confirmation of differential methylation sites with the local methylation tools previously described is needed [56]. It is not possible to map with more than a few hundred base pair resolution using MeDIP–Chip analysis, so the base pair resolution requires follow up analysis [56]. A more recent and promising tool that is able to overcome this limitation and allows genome wide analysis at the base pair CpG resolution is high throughput sequencing in combination with chromatin immunoprecipitation termed ChIP–Seq [94]. Base pair resolution DNA methylation measured by bisulfite conversion followed by high throughput sequencing has been used in *Arabidopsis* [94]. ChIP–Seq has also been used to identify patterns of histone modification in human CD4+T cells [95]. In the event methylated DNA immunoprecipitation (MeDIP) is used the ChIP–Seq can be used to simplify the genome and sequence analysis for methylated DNA. Although, MeDIP–Chip, ChIP–Chip and ChIP–Seq procedures are not wide spread, they will be critical in the future to map genome wide changes in the epigenome. A list of these methods is shown in Table 7.

## 6. Summary

Epigenetics has a critical role in mediating the actions of environmental factors on biology and disease. Elucidation of the actions of environmental toxicants such as endocrine disruptors will involve the use of epigenetic mechanisms and marks. Early developmental stages are more sensitive to environmental factors and need to be considered when studying adult onset disease. Therefore, epigenetics will be a critical mechanism in understanding the fetal basis of adult onset disease and in disease etiology. When somatic cells are the target for an epigenetic mutation, these will be critical for the disease of the individual exposed, but not be transmitted to the next generation. However, in the event the germ line is permanently modified through an epimutation a transgenerational phenotype can develop. The specific mechanisms of how epigenetics can be modified in the germ line need to be clarified. The potential impact of such epigenetic transgenerational phenomena in environmental toxicology and disease etiology are anticipated to be critical to elucidate in the future.

## Conflict of interest

There are no conflict of interest.

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