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Review Article

Genetically modified mouse models addressing gonadotropin function

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ABSTRACT

The development of genetically modified animals has been useful to understand the mechanisms involved in the regulation of the gonadotropin function. It is well known that alterations in the secretion of a single hormone is capable of producing profound reproductive abnormalities. Human chorionic gonadotropin (hCG) is a glycoprotein hormone normally secreted by the human placenta, and structurally and functionally it is related to pituitary LH. LH and hCG bind to the same LH/hCG receptor, and hCG is often used as an analog of LH to boost gonadotropin action. There are many physiological and pathological conditions where LH/hCG levels and actions are elevated. In order to understand how elevated LH/hCG levels may impact on the hypothalamic–pituitary–gonadal axis we have developed a transgenic mouse model with chronic hCG hypersecretion. Female mice develop many gonadal and extragonadal phenotypes including obesity, infertility, hyperprolactinemia, and pituitary and mammary gland tumors. This article summarizes recent findings on the mechanisms involved in pituitary gland tumorigenesis and hyperprolactinemia in the female mice hypersecreting hCG, in particular the relationship of progesterone with the hyperprolactinemic condition of the model. In addition, we describe the role of hyperprolactinemia as the main cause of infertility and the phenotypic abnormalities in these mice, and the use of dopamine agonists bromocriptine and cabergoline to normalize these conditions.

1. Introduction

The gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are produced by the pituitary gland as heterodimers, composed of a common α-subunit and the hormone-specific β-subunit. These hormones have a key role in ovarian and testicular functions by acting through binding to their specific seven-transmembrane domain G-protein coupled receptors [1]. In the gonads, LH receptors are located

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in testicular Leydig cells and in ovarian theca, granulosa and luteal cells [2]. FSH receptors are expressed in testicular Sertoli cells and in granulosa cells of the ovary [3]. Although primarily expressed in gonads, LH receptors are also found in numerous extragonadal tissues [4,5]. However, their physiological significance is still unclear [6]. The placental analog of LH, human chorionic gonadotropin (hCG) interacts with the same LH/hCG receptor, and functions as an LH agonist with a longer half-life and higher biopotency than its pituitary counterpart [7].

There exist physiological and pathophysiological conditions where gonadotropin secretion and/or action are elevated. For instance, human pregnancy is characterized by a transient production of very high levels of hCG by the placenta during the first trimester, which is essential for the maintenance of progesterone production by the corpus luteum gravidarum, and to prepare the uterus for implantation and placental development [7]. hCG is also needed to stimulate fetal testicular testosterone production for masculinization of the male fetus [8]. There are pituitary gonadotrope adenomas, but they rarely produce high gonadotropin levels [9]. On the other hand, postmenopause is a physiological situation where women are exposed to chronically elevated levels of gonadotropins for decades, and this exposure is proposed to be a risk factor for developing ovarian [10–12] and adrenal tumor [13]. A number of articles reported the expression of hCG by a variety of cancers, such as common carcinomas, bladder, lung, pancreatic and colorectal tumors with poor prognosis [14–16]. Finally, activating mutations of gonadotropin receptors, such as those of LH, lead to chronic elevation of the gonadotropin action, with the most conspicuous phenotype of gonadotropin independent male-limited precocious puberty (testotoxicosis) [17].

Several lines of investigation in humans or different animal models have shown that changes in the action of one single hormone, either its secretion or its signaling, are able to affect the integrity of the hypothalamic–pituitary–gonadal (HPG) axis and ultimately cause infertility [17]. Increased LH/hCG action alters the endocrine balance and reproductive function in mice and humans of both sexes. Studies on genetically modified mice provide new insights into the pathophysiological role of LH/hCG in gonadal and extra-gonadal function. We have developed a transgenic mouse model overexpressing the hCGβ subunit that produces chronically elevated levels of bioactive hCG [18,19]. Multiple endocrine alterations were observed in these mice [18–25]. Profound alterations are found in function of the HPG axis, and particularly females present with increased levels of prolactin (PRL) along the lifetime and develop pituitary lactotrope adenomas in later adulthood [18,24,25]. Our recent investigations have focused on the molecular mechanisms by which elevated hCG levels affect PRL regulation in females, and the ways of reversing it by dopamine receptor agonists, such as bromocriptine and cabergoline. This review summarizes the main findings of these studies.

2. Generation of transgenic mice producing high levels of hCG

We generated a transgenic mouse model with hCGβ overexpression by using a conventional pronuclear microinjection technique [18,19]. The mice harboring the hCGβ subunit coding sequence under the human ubiquitin C promoter (hCGβ+ mice) display ubiquitous expression of the transgene both in fetal life and adulthood. hCGβ associates with the endogenously expressed glycoprotein hormone common α-subunit (*Cga*) in the pituitary gland, and produces moderate levels of the bioactive dimer. The dimerization process is expected to occur mainly in gonadotropes and thyrotropes but possibly also in some extra-pituitary tissues, such as the ovary, where *Cga* expression has been detected [26]. Circulating LH/hCG bioactivity is increased about 30-fold in females [18], but only 3- to 4-fold in males [19]. This difference is likely the result of sexually dimorphic regulation of the *Cga* gene in the pituitary gland [27]. The transgenic line was established, and transgenic mice can be produced by breeding the fertile males with wild-type (WT) FVB/N females, since transgenic females are infertile.

3. Hormone profile and reproductive aspects of female transgenic mice

As a consequence of hCG hypersecretion, hCGβ+ females show an altered serum hormone profile with increased levels of estradiol, progesterone and testosterone at peripuberty. From 2 months of age onwards, serum progesterone and testosterone levels continue increasing, but the peripubertally high estradiol rapidly reverts to normal throughout the rest of life [18]. Moreover, no significant differences were observed in FSH levels of hCGβ+ females as compared with WT females at any age studied [18,25]. These hCGβ+ females were also hyperprolactinemic, reaching up to 600-fold increases in serum PRL at the age of 10–12 months. With respect to reproduction, the hCGβ+ females exhibit precocious puberty, with vaginal opening at 21–22 days of age, which is 5–7 days earlier than in WT females. Transgenic female mice also demonstrated alterations in estrous cycles with constant diestrus-type pattern from 45 days onwards. A persistent diestrus has been observed in other animal models with hormonal alterations, such as mice with increased levels of PRL or progesterone, or pseudopregnancy [28,29]. Moreover, mice hypersecreting LH exhibit precocious puberty, and vaginal smears obtained from these females showed a persistent presence of leukocytes [27,30]. In addition, the ovarian morphology of hCGβ+ mice demonstrated the presence of occassional hemorrhagic cysts from the age of 2 months, and enlarged ovaries with massive luteinization resembling luteomas after 6 months of age [18,25]. Furthermore, luteinized unruptured follicles are a frequent histological finding on the hCGβ+ ovaries.

The luteotropic action of PRL in rodents is well known, since this hormone is responsible for progesterone production and corpus luteum maintenance in murine pregnancy [31]. The excessive luteinization observed in transgenic females may be due to the elevated levels of hCG and PRL, which caused a state of constant progesterone overproduction. Elevated LH levels are related with increased androgens concentration and this may be a cause for follicular cyst development [32,33].

4. Pituitary macroprolactinomas in hCGβ+ female mice

Prolactinomas are the most frequent pituitary tumors with high incidence in women of reproductive age. Like humans, mice and rats present with high incidence of prolactinoma formation, especially with aging. Additionally, pituitary tumors can be induced by estrogen administration in rats of both sexes [34].

The main regulatory pathway for PRL secretion is the inhibitory action of dopamine released from the hypothalamus [35,36]. In knockout mice for dopamine receptor (*Dr2* KO), the absence of negative regulation of dopamine induces hyperprolactinemia and lactotrope hyperplasia, and later in life these animals develop pituitary adenomas [37].

For patients with hyperprolactinemia, different treatment options are available, such as surgery, radiotherapy or dopamine agonist administration, depending on the clinical situation, the underlying cause of the elevated PRL level and the presence of hypogonadism and infertility. Within dopamine receptor agonists, bromocriptine, cabergoline, pergolide and quinagolide are the most commonly used [38]. Although bromocriptine has been used for the past 30 years, it does not have a long-lasting effect and it presents with common side effects such as nausea, vomiting, postural hypotension and headache [39]. For this reason the newer compound cabergoline has been used with better results. This compound has been demonstrated to be more effective in reducing PRL levels and pituitary weight [40,41]. Moreover, cabergoline administration restored fertility in hyperprolactinemic women and women resistant to bromocriptine [42].

Among the extragonadal phenotypes, hCGβ+ females show enlarged pituitary glands from 2 months of age, with persistent growth thereafter. The pituitary growth was clearly hyperplastic at the age of 6 months, and development of pituitary tumors was evident by 12 months of age, reaching up to 100 mg of weight. This uncontrolled pituitary growth was accompanied by exaggerated increase in PRL levels, as mentioned above. At the macroscopic level, the pituitary tumors were nodular and irregular in shape, presenting with suprasellar expansion and hemorrhage. All adenomas studied showed strong immunohistochemical reaction for PRL. Immunohistochemical reactions for GH, ACTH and TSHβ were only observed in cells scattered around the tumor nodules [18]. The increased levels of serum PRL and the immunopositive signal for PRL in the tumor thus confirmed the diagnosis of prolactinoma.

Increased estrogen production is a risk factor for prolactinoma development in humans, and there is evidence of progression of microprolactinomas to macroprolactinoma during estrogen therapy [43]. During pregnancy, prolactinomas show increased growth due to elevated levels of estradiol [44]. However, all women are not responsive to estrogen action in the same way, and like humans, not all animal models have the same sensitivity to estrogen-induced prolactinomas. Fischer-344 rats are especially sensitive to the tumor-promoting effects of estrogens. The mechanism by which estrogen induces prolactinomas involves the down-regulation of D2R and up-regulation of TGF-β isoform gene expression. This hormone also stimulates angiogenic factors and extends intracellular communication between lactotropes and folliculo-stellate cells [34]. In our model, estrogen levels are elevated only transiently during peripuberty, but androgens are persistently elevated, and this hormone may be a source for locally estrogen production [45], which may be involved in the induction of prolactinomas.

5. The role of high concentration of progesterone in the development of prolactinomas

In addition to the well-known role of estrogens in the development of prolactinomas in humans and some animal models, the participation of other ovarian hormones such as progesterone in promoting tumorigenesis is less defined. Previous reports demonstrated that progesterone receptor is expressed in mouse lactotrope cells [46] and human prolactinomas [47]. Since the pituitary adenomas develop in hCGβ+ females in the presence of normal estrogen but constantly elevated levels of progesterone throughout life, we studied the role of progesterone and its possible cooperative action with estrogens in promoting prolactinomas in these mice [24]. Thus, the first approach was to subject hCGβ+ females to a long-term treatment with tamoxifen and mifepristone, i.e. estrogen and progesterone antagonists respectively, as well as gonadectomy. The results showed that all the treatments were effective in reducing pituitary weight as well as PRL levels. On the other hand, hormone replacement after gonadectomy in hCGβ+ females was more effective in reverting pituitary weight when estrogen and progesterone were administered together than estrogen alone. However, combination of the two hormones was not able to completely reverse PRL levels in gonadectomized transgenic females [24]. The next step was to exclude the possibility of indirect effect of progesterone through its pituitary or extra-pituitary conversion to estrogen. To this end, somatomammotrope GH3 cells, which are not able to convert progesterone to estrogen, were stimulated with progesterone alone or with a combination of progesterone and estrogen in different concentrations. In agreement with the results obtained on transgenic females, a significant enhancement of cell proliferation was observed only when the cells were cultured in the presence of both hormones. In addition, the antiprogestin mifepristone significantly decreased cell proliferation in the presence of estrogen and progesterone. This finding is in agreement with the decrease in pituitary weight observed in hCGβ+ females treated with the same antagonist [24].

We then elucidated the molecular mechanism by which progesterone induces pituitary adenomas in the presence of physiological levels of estrogens. It has been demonstrated that the absence or overexpression of factors that control cell cycle are capable of inducing pituitary tumorigenesis [48]. One key pathway involved in the G1/S control consists of the CyclinD1 (Ccdn1)/Cyclin-dependent kinase 4 (CDK4)/retinoblastoma protein (pRB) cascade, where the CDK/cyclin complex mediates phosphorylation of RB protein, which evokes the activation of E2F1. This active form is able to induce the gene expression of factors necessary for S and M phases of the cell cycle [48]. It has been demonstrated that an increase in *Ccdn1* as well as a partial deletion of pRB leads to pituitary adenoma formation [49]. Additionally, mice deficient in both RB and E2F1 presented with decreased tumor

development [50]. Besides, the CDK inhibitors, such us $P27^{Kip1}$ and $P18^{INK4C}$, confirm the two separated branches that act to suppress pituitary adenoma formation [51]. Indeed, in our mouse model, elevated levels of progesterone in response to hCG hypersecretion induced the up-regulation of *Pcna*, *Ccnd1* and *E2f1* expression, leading to a loss of control of cell division and an increase in proliferation, which resulted in pituitary adenoma formation in the hCGβ+ female mice [24]. These results were further confirmed by using primary cell culture from pituitaries of hCGβ+ females stimulated with progesterone, estrogen and a combination of both hormones. In this experiment, only the stimulus with both hormones was able to up-regulate *Ccnd1* [24].

6. The role of hyperprolactinemia in the phenotype and fertility of hCGβ+ females

PRL is a multifaceted hormone that participates in many different processes. It has an important role in the regulation of reproductive function, but it is also involved in controlling metabolism, growth, immunoregulation and behavior [31,52]. Differently from humans, PRL exerts a luteotropic action on the rodent ovary and is essential for the corpus luteum function during pregnancy or pseudopregnancy [53]. Besides, in mice, development of the mammary gland and lactogenesis depend mostly on PRL during pregnancy [54]. On the other hand, in humans, elevated levels of PRL are associated with the presence of prolactinomas and cause fertility problems such us amenorrhea, anovulation, orgasmic dysfunctions and reduced libido [55].

In a recent study we found that hyperprolactinemia is essential for the phenotypic defects of the hCGβ+ females, since most of them are reversed by treatment with dopamine agonists with proven efficacy in hyperprolactinemia [25]. Therefore, hCGβ+ females were treated with the dopamine agonists cabergoline and bromocriptine. Long-term bromocriptine treatment of adult hCGβ+ females from 2 to 6 months of age was effective in the control of obesity, pituitary growth and disturbances of the hormone profile, demonstrating that

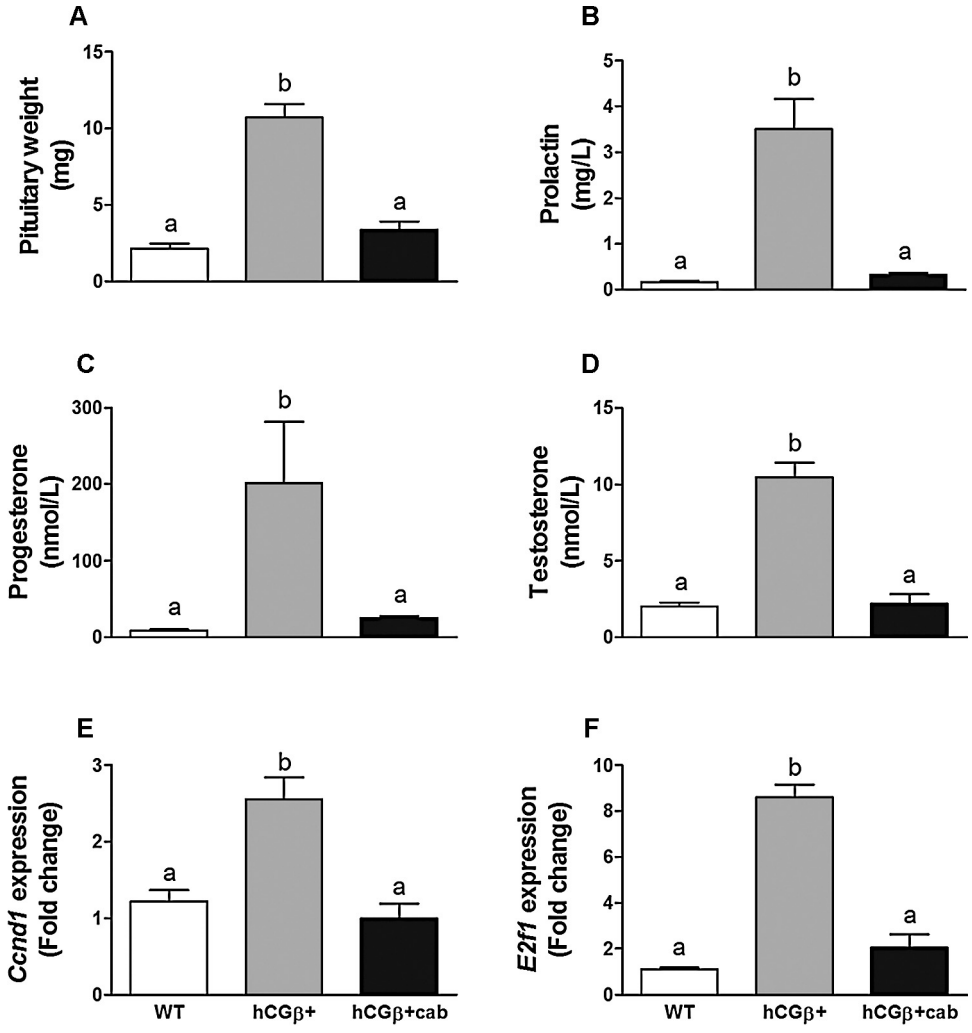

Fig. 1 – Effects of a 1-week treatment of cabergoline applied at 5 weeks of age on hCGβ+ females. Pituitary weights (A) and serum prolactin (B), progesterone (C) and testosterone (D) levels at the age of 6 months are shown (n = 4–8; mean ± SEM). The mRNA expression analysis of pituitary *Ccnd1* (E) and *E2f1* (F) was carried out by qRT-PCR (n = 4). One-way ANOVA, followed by Bonferroni's post hoc test was conducted. Different letters indicate significant differences of at least $p < 0.05$. Modified from Ratner et al. [25].

hyperprolactinemia is the main cause of the hCGβ+ female phenotype. Additionally, a 1-week administration of cabergoline to young hCGβ+ females (5-week-old) was also effective in the normalization of their phenotype, and in the recovery of fertility at adulthood [25]. A recent study, where the hCGβ+ mice were crossed with PRL receptor deficient mice (PRLR−/−), confirmed the importance of PRL in promoting the phenotypic alterations of hCGβ+ female mice [56]. In these double mutant females, the lack of PRLR in the presence of high bioactive hCG levels prevented the massive luteinization of the ovaries and the mammary tumor development in adulthood [56]. The importance of high levels of PRL in infertility was also demonstrated recently [57]. The administration of PRL to female mice led to anovulation by decreasing kisspeptin levels and consecuently GnRH secretion. In addition, the administration of kisspeptin concomitantly with PRL reverted the alterations, thus confirming the relationship between this neuropeptide and PRL. This finding opens up a new possible tool to treat infertility of those hyperprolactinemic women who do not tolerate classical dopamine agonist treatments [57].

The short-term treatment with cabergoline leads to a persistent elimination of hyperprolactinemia (even after 5 months), but only when administered to young females, since the same treatment administered at 3 months of age failed to rescue the hCGβ+ phenotype [25]. This shows that chronically elevated hCG levels from the early stages of sexual maturation induce persistent alterations of the pituitary–gonadal axis that are not reversed by a short treatment with cabergoline, once the dysfunctional phenotype is established in adulthood. Effectively, 1-week treatment of 5-week-old female mice with cabergoline restored the normal cyclicity and pregnancy success with normal timing of parturition.

The luteotropic action of PRL in WT mice stimulates progesterone production in response to pituitary LH by up-regulation of *Lhcgr* expression and inhibition of *Akr1c18*, the enzyme 20α-hydroxysteroid dehydrogenase, responsible for converting progesterone into biologically inactive 20α-dihydropregesterone [58]. The short-term treatment with cabergoline of the hCGβ+ mice prevented the development of ovarian cysts in early adulthood, and the massive luteinization in late adulthood. This was accompanied by normalization of *Lhcgr* and *Akr1c18* expression, as well as progesterone and testosterone synthesis [25].

It is interesting that a short-term cabergoline treatment is able to reverse the hyperprolactinemia of hCGβ+ females, even after many months, since the serum levels of PRL, progesterone and testosterone, and pituitary overgrowth, are normalized in adulthood (Fig. 1A–D) [25]. The expression of pituitary *Ccnd1* and *E2f1* (Fig. 1E and F) are suppressed in the cabergoline-treated hCGβ+ females, which correlates with the blockade of pituitary expansion. In this regard, the cabergoline treatment abolishes the main proliferative stimulus responsible for the pituitary growth and tumor development. Thus, once the PRL secretion is initially controlled by cabergoline, the massive luteinization of the ovary and the progesterone over-production can be prevented. In this regard, lactotrope proliferation may be suppressed both by a direct action on the pituitary by increasing the dopaminergic tone, and by an indirect effect by reducing the progesterone-induced tumorigenic signaling pathways [24].

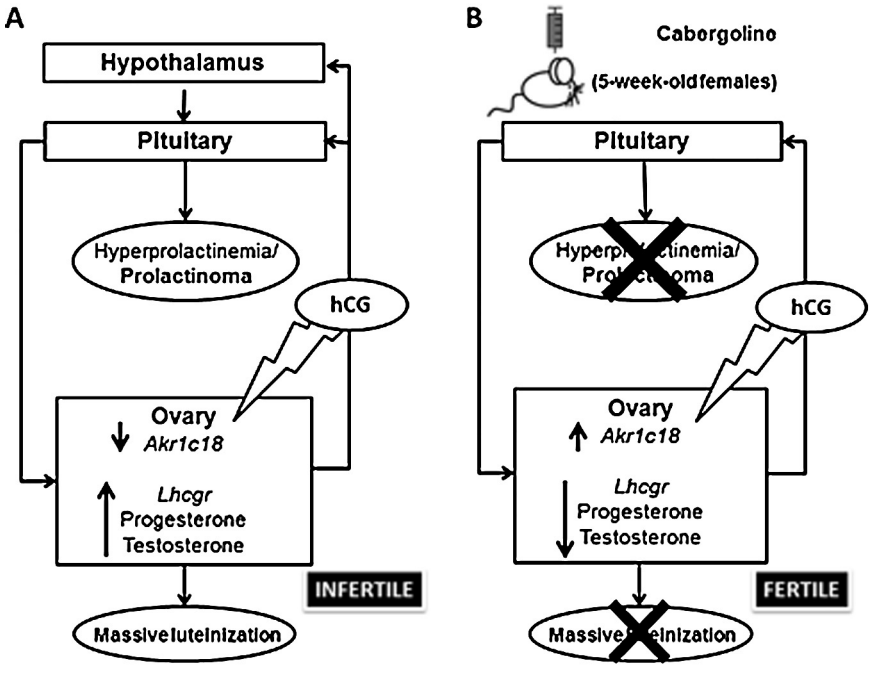

Fig. 2 – (A) Scheme showing the main phenotypic changes observed in hCGβ+ females. In response to hCG hypersecretion, the ovary produces elevated levels of steroid hormones. At the pituitary level, lactotrope adenomas and hyperprolactinemia are developed in adults. The luteotropic action of prolactin induces up-regulation of *Lhcgr* and inhibition of *20α-HSD* expression in the ovary, which leads to massive luteinization and elevated levels of progesterone. (B) A short-term cabergoline treatment applied to 5-week-old hCGβ+ females reverses infertility and normalizes the phenotype at adulthood.

7. Concluding remarks

Taken together, these studies indicate that chronically elevated hCG leads to multiple gonadal and extra-gonadal defects in females, including those of the ovary as the primary effect, whereas those found in the pituitary are due to secondary effects of the aberrant gonadal function (Fig. 2). The influence of increased PRL levels is crucial in the emergence of the phenotypic characteristics of this model.

The genetically modified mouse models have been useful to clarify the role of gonadotropins on the endocrine physiology and pathophysiology. They have also shed light on the involvement of gonadotropins in tumorigenic processes. Interestingly, the recovery of fertility of transgenic females with hCG hypersecretion are promising, since it will provide novel information about the potential functions of hCG in different organs during gestation. Lots of work is still needed to understand all gonadotropin actions and their role in various diseases and therapeutic potentials.

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Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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