# THE EFFECTS OF PUBERTAL EXPOSURE TO DIETARY SOY ISOFLAVONES ON THE BREAST AND REPRODUCTIVE TISSUES

BY

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| | |
|---|---|
| $16OHE_1$ | 16α-hydroxyestrone |
| 17βHSD | 17β-hydroxysteroidegenase |
| 2OHE | 2-hydroxyestrogens |
| AAALAC | Association for the assessment and accreditation of laboratory animal care |
| ABI | Applied biosystems |
| ACTB | Beta actin |
| ACUC | Animal care and use committee |
| ANOVA | Analysis of variance |
| ApoE | Apolipoprotein-E |
| ASF | Altered Schaedler flora |
| BMC | Bone mineral content |
| BMD | Bone mineral density |
| BPA | Bisphenol A |
| BRCA2 | Breast cancer 2, a tumor suppressor gene |
| CCND2 | Cyclin D2 |
| cDNA | Complementary deoxyribonucleic acid |
| CL | Casein and lactalbumin |
| CSN1S1 | Casein alpha s1 |
| CYP19 | Aromatase (Cytochrome P450 family 19) |
| CYP1A1 | Cytochrome P450 family 1, subfamily A, polypeptide 1 |
| CYP1B1 | Cytochrome P450 family 1, subfamily B, polypeptide 1 |
| CYP3A4 | Cytochrome P450 family 3, subfamily A, polypeptide 4 |
| DES | Diethylstilbestrol |
| DEXA | Dual energy X-ray Absorptiometry |
| DNMT | DNA methyltransferase |
| DMBA | 7, 12-dimethylbenz [a] anthracene |
| DOHAD | Developmental origins of health and disease |
| E1 | Estrone |

| | |
|---|---|
| E2 | 17-β Estradiol |
| EDC | Endocrine disrupting compound |
| EGFR | Epidermal growth factor receptor |
| ELF5 | E74-like factor 5 |
| ERα (ESR1) | Estrogen receptor alpha (gene) |
| ERβ (ESR2) | Estrogen receptor beta (gene) |
| ERE | Estrogen response element |
| EST | Estrogen sulfotransferase |
| FC | Fold-change |
| FOXA1 | Forkhead box A1 |
| FSH | Follicle stimulating hormone |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GSEA | Gene set enrichment analysis |
| GREB1 | Gene regulated by estrogen in breast cancer 1 |
| H&E | Hematoxylin and Eosin |
| HEC-1 | Human endometrial carcinoma cell line |
| HER2 | Human epidermal growth factor receptor 2 |
| HIF | High isoflavones |
| HPG | Hypothalamo-pituitary-gonadal |
| HSD17B1, HSD17B2 | Hydroxy steroid (17β) dehydrogenase 1 or 2, genes encoding 17βHSDs |
| HSD | (Tukey) honestly significant difference test |
| HT | Hormone (replacement) therapy |
| IF | Isoflavones |
| IGFBP-2 | Insulin-like growth factor binding protein 2 |
| IHC | Immunohistochemistry |
| KEGG | Kyoto encyclopedia of genes and genomes |
| LH | Luteinizing hormone |
| LIF | Low isoflavones |
| LSM | Least square means |
| MKI67 | Antigen identified by monoclonal antibody Ki-67, a proliferation marker |
| MNU | N-methyl-N-nitrosourea |
| mRNA | Messenger ribonucleic acid |

| | |
|---|---|
| MRPP | Multi response permutation procedures |
| MUC1 | Mucin-1 |
| O-DMA | O-desmethylangolensin |
| P4 | Progesterone |
| PGR | Progesterone receptor |
| PTEN | Phosphatase and tensin homolog |
| PUFA | Polyunsaturated fatty acid |
| RASSF1 | Ras association (RalGDS/AF-6) domain family member 1, a tumor suppressor gene |
| SHBG | Serum hormone binding globulin |
| STS | Steroid sulfatase |
| SULT1E1 | sulfotransferase family 1E, estrogen-preferring, member 1 (estrogen sulfotransferase-encoding gene) |
| TSS | Transcription start site |
| qRT-PCR | Quantitative realtime reverse transcription polymerase chain reaction |
| RIA | Radioimmunoassay |
| SEM | Standard error of the mean |
| SERM | Selective estrogen receptor modulator |
| STAT5A, STAT5B | Signal transducer and activator of transcription factor 5A or 5B |
| SPI | Soy protein isolate |
| TEB | Terminal end bud |
| TFF1 | Trefoil factor 1 |
| TR-FIA | Time-resolved fluorescence immunoassay |
| T-RFLP | Terminal restriction length polymorphism |

**ABSTRACT**

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THE EFFECTS OF PUBERTAL EXPOSURE TO DIETARY SOY ISOFLAVONES ON THE BREAST AND REPRODUCTIVE TISSUES

Dissertation under the direction of

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Diet has been thought to modify developmental regulation and subsequently influence later-life susceptibility to various diseases including cancer. Meta-analyses showed that the intake of soy, which contains the phytoestrogenic compounds isoflavones (IFs), is associated with reduced breast cancer risk. A similar relationship has been reported for soy and endometrial cancer, although the evidence is more limited. Interestingly, the inverse association between soy and breast cancer is more consistent when soy exposure occurs preceding puberty. Our primary aim was to investigate the effect of soy consumption beginning pre-adulthood on the breast and uterine tissues in relation to estrogen action. We utilized two animal models: cynomolgus macaques and apolipoprotein E-null mice. The macaque study longitudinally assessed tissue changes across pubertal development to model young girls consuming a North American diet with/without soy IF exposure. We found that soy intake did not alter pubertal growth, menarche, or uterine development. Soy exposure initiated at puberty promoted mammary gland differentiation which resulted in a breast composed of abundant mature lobular structures in adulthood, a phenotype consistent with low cancer risk. We observed a modestly lower expression of ERs and ER activity markers, suggesting that early soy exposure could potentially result in lower estrogen-responsiveness in the adult breast. These changes were not associated with differential circulating estradiol and progesterone, CpG methylation within the specific promoter regions examined, or expression of intramammary estrogen-metabolizing enzymes. To explain whether the soy effect is dependent on the primary IF metabolite equol, we conducted a

study whereby mice were modified to have equol-producing vs. non-equol-producing capability to model the low frequency of equol production observed in human populations. Mice were fed dietary soy with/without exogenous equol starting at peripubertal age. Equol, but not a soy diet *per se,* modulated estrogen-dependent uterine responses; the effect was less clear in the breast. Female equol producers had lower estrogenic reproductive tissue phenotypes compared to nonproducers, regardless of IF dose. Collectively, our findings indicate that pubertal exposure to soy may have a subtle effect in promoting breast differentiation and downregulating estrogen-responsiveness. Equol and/or equol-producing microbiota may influence estrogen-associated tissue phenotype and response to soy diet.

# INTRODUCTION

Over the past two decades, there has been a shift in the perception of how chronic diseases such as cardiovascular disease, type 2 diabetes, and cancer develop. It is more widely accepted that developmental periods where cells are differentiating and forming specific tissues (i.e. pregnancy, early childhood, puberty) serve as a critical window for setting trajectories for later life health and disease risk (1). This paradigm, known as the Developmental Origins of Health and Disease (DOHaD), was pioneered by David Barker and initially focused on epidemiological observations relating low birth weight with risk for cardiovascular disease during adulthood (2). The theory has since evolved; diet and nutrition is now considered to be one of the major factors that can potentially modify developmental regulation to subsequently affect later life susceptibility to various diseases. The mechanistic explanation, however, is not fully understood and requires investigation. The paradigm opens a new avenue of research for disease prevention by early life nutritional modulation.

The estimated incidence rate of cancer of the breast and corpus uteri in Asian countries is lower than in the Western countries (3). These cancers are hormone-modulated and the differences in lifestyle that affect the reproductive events are likely the major contributor to the inter-national variation. Interestingly, it is becoming more evident that dietary factors can also play a key role in modulating cancer risk (4, 5). One of the major components of diet consumed in Asian populations is soybean. Soybean is a member of the leguminosae family and a source for protein. Importantly, soybean contains numerous bioactive components such as phytic acid, phenolic acids, saponins, oligosaccharides, protease inhibitors, phytoestrols, $\alpha$-linoleic acid, vitamin E, soy peptides, and the most-widely studied soy component isoflavones (IFs) (6). IFs have drawn the most attention in regards to health because of their structural similarity to the hormone estrogen and their ability to bind to estrogen receptors (ERs) to elicit weak estrogenic properties, making them a phytoestrogen (7).

In this project, we sought to determine the effects of pre-adulthood exposure to dietary soy IF on breast and reproductive tract development in regards to generating low cancer-risk phenotypes. Our study mainly focused on the mechanisms associated with estrogen and ER-associated regulation.

**Estrogen Receptor-dependent transcription**

ER is a ligand-activated transcription factor in the nuclear receptor superfamily and consists of at least two subtypes alpha (α) and beta (β). Distribution and density of the ERs varies across tissues, and both subtypes are present in the mammary and reproductive tissues; these tissues are highly responsive to estrogen action. Classic ER signaling occurs through the binding of ligand to ER; the ligand-ER complex binds to an estrogen response elements (EREs) in the DNA, recruit coregulators and activate gene transcription (8, 9). In many tissues, ERβ has anti-proliferative effects in contrast to ERα that induces proliferation (10). Although transcriptional activity of ERα is relatively stronger, ERβ also plays a key role in regulating cell response to estrogens by modulating ERα transcriptional activity (11, 12). Therefore, the distinctive patterns of ERs can affect the magnitude of ligand effect, and ERα:ERβ ratio at a target site may be an important determinant for ligand action on the tissue. Other mechanisms of ER-mediated transcriptional signaling are ERE-independent genomic actions, ligand-independent genomic actions, and non-genomic actions (8). In this project, our assessment on ER pathway is limited to the classic ERE-dependent signaling pathway.

**Soy isoflavones: mechanisms of action**

The primary IFs in soybeans are genistein and daidzein; another IF called glycitein is also present in smaller amount. IFs are present as aglycones or glucoside conjugates (β-, acetyl- or malonyl-glucosides). The amount of IFs contained in soybean depends on various factors (e.g.

climate, soil condition, processing, storage conditions, etc.) and in general, average 1.5-2.0 mg/g with a wide range between 0.1-2.5 mg/g soybean (13, 14, 15). Notably, soy protein consumption in most Asian countries is approximately 35 g/day/person or equivalent to 25-50 mg IFs/day/person (as aglycones); in small proportion of the Asian population the intake can even be as high as 100 mg IF/day. In contrast, IF exposure in Western countries is less than 1 mg/day (in aglycone equivalents) (16, 17).

The chemical structure of IFs is similar to estradiol. Consequently, IFs can also bind to ER although with approximately 100 times weaker affinity than estradiol (7). Estradiol strongly binds to both ERs with ~2.5x higher affinity to ERα than that to ERβ, whereas IFs preferentially bind to ERβ than ERα. In the absence of estrogen, IFs are ER agonists that can elicit transcriptional efficacy comparable to that of estradiol. Genistein has been reported to be an ERα superagonist, which shows greater transcriptional efficacy than estradiol (18). IFs potency in inducing ER-mediated transcriptional activity, however, is less compared to estradiol (19). In the presence of estrogen, IFs have dual biologic effects; as estrogen agonist and antagonist depending on the amount of estrogen. With a lower dose of estrogen present, IF may show agonist effects. When estrogen is present in physiologic high dose (premenopausal-like), IF can inhibit estrogen action possibly by competitive binding to the receptor, more prominently with ERβ than with ERα (20).

Soy IF compounds work through various modes of action, although which are relevant to women's health remains controversial. While classified as a phytoestrogen, which by definition means a plant-derived estrogen, IF action more resembles a selective ER modulator (SERM) rather than a true estrogenic compound. IF can bind to ER and modulate ER-dependent pathways, eliciting estrogen agonist-antagonist effect depending on tissue location, the concentrations of circulating estrogens and ER (21, 22). Relevant to cancer protection, IFs have been shown to induce differentiation (23) and apoptosis (24), inhibit cell proliferation (25), protein tyrosine kinase (26) and angiogenesis (27), act as antioxidants (28), alter activity of enzymes for estrogen

synthesis (29) and metabolism (30), and have been associated with lower circulating and intra-tissue estrogen concentrations (31, 32, 33).

**Dietary soy and breast cancer risk**

Breast cancer is the most common cancer diagnosed and the leading cause of cancer death in women worldwide (4). The typical morphological progression of breast cancer starts from benign proliferative lesions such as atypical hyperplasia to pre-cancerous carcinoma *in situ*, microinvasive carcinoma, invasive carcinoma (mainly ductal carcinoma), and metastasized carcinoma; some steps may be omitted depending on the case (34). The mammary gland is responsive to reproductive hormones and growth factors. Consequently, hormone receptors such as ER and progesterone receptor (PGR), and human epidermal growth factor receptor-2 (HER2) have been used as breast cancer biomarkers to predict therapy responsiveness. Diagnosis of breast cancer also relies heavily on the tumor histological grade assessed by methods such as the Nottingham grading system, which is based on tubule formation, nuclear feature, and mitotic rate (35). With the vast development of high-throughput molecular approaches, studies have been conducted to further classify breast cancer subtypes and predict clinical outcomes based on transcriptomic and genetic signatures (36, 37). Overall, breast cancer prognosis is determined based on cancer stage (i.e. size and metastasis), histological grade and hormone receptor status.

Among well-established risk factors are those related to reproductive events such as age of menarche and menopause, nulliparity, lactation, and age of first pregnancy (38). These events are key stages of normal mammary gland development, which is a process highly influenced by endocrine factors including estrogen. In the breast, estrogen regulates normal cell growth, differentiation, and tissue-specific gene transcription. At the same time, this hormone and its metabolites are also categorized as carcinogens due to their ability to increase cell proliferation, decrease apoptosis, and react with the DNA to cause damage leading to the initiation and progression of breast cancer (39). Furthermore, mammary gland susceptibility to carcinogens is

also influenced by the gland's differentiation state and proliferative activity (40), highlighting the fundamental relationship between mammary gland development and breast cancer risk.

The lower incidence of breast cancer in Asian population has been associated with dietary habits such as the amount of soy consumption. Over the past two decades, numerous case-control and cohort studies have been conducted to assess the association between soy intake and breast cancer risk among Asian and Western populations. Independently, the studies showed mixed results whereby some showed lower breast cancer incidence with high intake of soy while others failed to show a significant association. Using a meta-analysis approach, there appeared to be a consistent association between soy consumption and risk reduction in breast cancer incidence. Trock et al. (41) analyzed 18 epidemiologic studies and found a small but significant reduction in breast cancer risk with soy intake. A similar result was found in the most recent meta-analysis of 18 prospective studies of breast cancer incidence and recurrence (42). When stratified by populations, however, the reduced risk of breast cancer incidence was only observed in the Asian populations. This observation is also consistent with another study (43) which reported an inverse trend of association between soy intake and breast cancer risk only among Asian populations (including Asian-Americans) with a dose-dependent association. The lack of association shown by the Western population data is thought to be related to the lower soy intake among the Western cohorts (an average of 0.8 mg IF/day) compared to the 20 mg IF/day in Asians. Interestingly, in Asians, adolescent intake showed a stronger effect on risk reduction than intake during adulthood. These results point out to the importance of IF dose and the timing of exposure for dietary soy to take effect. While the study suggested that soy intake is beneficial for both pre and postmenopausal breast cancer risk, other studies reported a significant reduced risk only in premenopausal (41) or postmenopausal (42, 44) breast cancer. A few other recent studies showed a significant inverse association between high soy intake and reduced risk of breast cancer incidence (45) and recurrence (46) in the Western populations. Interestingly, Anderson et al. (45) specifically showed a reduced risk of postmenopausal breast cancer only with adolescent soy

intake, further highlighting the importance of early exposure. Together with previous studies (47, 48, 49, 50, 51), there appear to be a consistent evidence indicating the protective effect of early life exposure rather than adulthood. Although the epidemiological findings are intriguing, the nature of human studies makes it difficult to eliminate potential confounders such as culture/lifestyle, ethnicity and genetic factors.

There have been numerous studies done in rodent models to assess the effect of eary life exposure to soy and/or IF (mainly genistein) on chemically-induced carcinogenesis amid mixed results. Lamartiniere et al. were the first to show a protective effect of pre-pubertal exposure to purified genistein against DMBA-induced mammary tumorigenesis in rats (52), while Constantinou et al. reported a similar finding with genistein but not daidzein in rats induced with MNU (53). Since then, more studies have been conducted using injection of genistein as well as by the dietary approach with soy protein isolate or IF-containing diet. Although there were few studies that showed the increase in carcinogen-induced tumors or pre-neoplastic lesions, the majority of them reported a reduction in mammary carcinogenesis with soy exposure suggesting early life exposure is required for genistein to reduce breast carcinogenesis (54, 55). A recent study conducted by National Toxicology Program assessed long term exposure of a very high dose of dietary genistein on Sprague-Dawley rats. They reported that genistein decreased benign fibro-adenomatous nodules but increased mammary adenomas/adenocarcinomas (56). It is also important to note that in contrast to the protective effect of early exposure to soy, genistein could promote the growth of existing tumors. Dietary genistein has been shown to stimulate the growth of MNU-induced estrogen-dependent mammary tumors in ovariectomized rats (57) and MCF-7 human breast cancer implanted in ovariectomized athymic nude mice (58). These findings further highlight the importance of exposure timing to determine the health effect of soy.

**Dietary soy and endometrial cancer risk**

In the current project, our focus on the reproductive tissue is limited to the uterus, mainly the uterine body which consists of endometrium, myometrium and perimetrium. Uterus is highly responsive to reproductive hormones; the balance between estrogen and progesterone is one of the keys in maintaining the healthy state of this tissue. Endometrial cancer is the most common cancer of the female reproductive organs found in the US (59), more often in post-menopausal women. In general, endometrial cancer is divided into two categories: types 1 and 2. Type 1 represents the majority of cases, low-grade histology, ER-positive, and primarily associated with exposure to unopposed estrogen. Type 2 cases are less common, non-endometrioid, high-grade histology, usually serous or clear cell, unrelated to estrogen exposure and have poor prognosis (60). The definite etiology of endometrial cancer is unknown; this cancer shares a lot of the same risk factors with breast cancer such as age of menarche and menopause, nulliparity, and the use of hormone therapy.

The attention on soy and endometrial cancer relationship has been greatly skewed towards the post-menopausal period due to the increasing use of soy as a natural alternative to synthetic hormone replacement therapy (HT) in post-menopausal women. The primary purpose of a HT is to relieve menopausal symptoms but importantly, there is an ongoing controversy on HT benefit for disease prevention and potential adverse risk depending on the formulation, route of administration and the timing of HT initiation (61). With the reluctancy of post-menopausal women to use synthetic HT, soy is often advertised as a natural alternative although the evidence for efficacy is not always consistent. At the same time, the SERM-like potential of IF also causes controversy regarding endometrial health and safety, which in part is due to how a SERM, like tamoxifen, can increase uterine hyperplasia and cancer risk. Furthermore, although the estrogenic activity of IF is much weaker than estradiol, there is a concern that IF can affect the uterus in a similar manner to unopposed estrogen (62).

The estimated incidence rate of endometrial cancer is lower in Asian countries than in the Western populations (3). Similar to breast cancer, phytoestrogens have also been postulated as an important diet factor associated with this finding. Epidemiological evidence for the association, however, is somewhat limited and less consistent. The first observational study on soy and endometrial cancer was conducted by Goodman et al., which reported a reduced risk with soy-rich food independent of the ethnicity of the studied population (63). Since then, two other case-control studies have reported inverse associations of IF (64) or soy food (65) intake and endometrial cancer risk. One study (64) suggested a stronger effect in postmenopausal women, and both studies indicate a more pronounced effect in obese women. In contrary, a more recent case-control study only found a significant inverse association between endometrial cancer and total IF consumption in lean women (66). More recently, a prospective study of multiethnic post-menopausal women showed a reduced risk of endometrial cancer with greater consumption of IF-containing foods (67).

In rodent models, the effect of soy IF on endometrial cancer has been mixed. There have been reports on protective effect of genistein and daidzein against estradiol-induced endometrial hyperplasia and adenocarcinoma with post-ovariectomized exposure (68, 69). In a recombinant mouse model of endometrial carcinoma (i.e. *PTEN* heterozygous mutant), neonatal exposure to genistein supressed carcinogenesis (70). On the other hand, genistein has been associated with increased uterine pre-cancerous lesions when mice were exposed post-ovariectomy (71), and adenocarcinoma incidence in mice exposed as neonates (72). Various studies have shown a dose-dependent uterotropic effect of genistein, although it requires 1000 times greater concentration to increase uterine weight similar to those seen by estradiol in mice (72) or rats (73).

**Soy interventional studies in women**

While observational studies generally suggest an inverse association between soy and the risk of breast and endometrial cancer, interventional studies have been inconclusive. The number of

studies on soy treatment and mammary health is very limited, whereas for the soy effect on endometrial cancer risk, the data mainly comes from clinical trials of soy supplement tablets/capsules being developed as treatment for menopausal symptoms.

The majority of short-term soy interventional studies reported no change in the cancer risk biomarkers. These biomarkers include nipple aspirate fluid (74), circulating steroid hormones (75, 76), endometrial thickness (75, 77), and markers for breast and uterine proliferation and steroid receptors (75, 78). A longer treatment period showed different effects with different treatment regimens. A 2 year intake of IF-rich food or supplement did not change mammographic density or circulating sex hormones (79, 80, 81), and 3 year period of supplementation with soy protein isolate (containing 91 mg IF) showed no effect on endometrial thickness, hyperplasia or cancer (82). In contrary, 5 years of daily supplementation with a tablet containing 150 mg IF was associated with higher occurrence of endometrial hyperplasia (83).

**"Timing of exposure" hypothesis**

Studies on immigrants showed that Asian women that migrated to the US and adopted western diets have increased breast cancer risk with rates comparable to that of American women, which further supports that lifestyle, not genetics, could be the main risk factor (84, 85, 86). Interestingly, among those Asian immigrants, the highest incidence rate was found in US-born Asians and residents who immigrated early in life potentially suggesting that early-life may be a critical window for intervention. A similar phenomenon was observed with endometrial cancer; although the incidence rate for US-born Asian women is still lower compared to white-Americans, it was still markedly higher compared to their Asia-born counterparts (87). These data are in line with the stronger and more consistent inverse association found between breast cancer and soy intake when the exposure occurs during childhood/adolescence (47, 49, 50, 51). Taken together, there is a strong indication that early initiation of consumption is important to benefit from soy, particularly when soy is consumed in periods before/during extensive breast

development or continuous throughout life (88). These findings are supported by rodent studies that show early-life, but not adult exposure to genistein reduces mammary tumorigenesis (55).

Epigenetics has been thought to be one of the mechanisms behind the early life soy effect. Environmental factors including diet can modulate gene expression without changing the DNA sequence. DNA methylation is an epigenetic mechanism that regulates gene expression by affecting the binding of methylation-sensitive DNA-binding proteins and interacting with various modifications of the histone proteins that regulate DNA accessibility for transcription (89). Increased methylation is mainly associated with silencing of genes, and DNA methylation levels and profile are highly dynamic throughout life. The overall sensitivity of epigenetics to stochastic and environmentally-induced changes are the highest during embryogenesis, remains high post-natally through adolescence, and is lower in adulthood (90). More recently, it was found that the most critical window for epigenetic modulation may not be the same for each tissue. Tissues such as the reproductive system can have an epigenetically-sensitive developmental period that extends into childhood or puberty (91).

During pre-natal life, maternal environment is the highest influencing factor of methylation. Starting from childhood onwards, more factors such as diet, lifestyle, stress, infections, microbiome, and environmental chemical and toxin exposure become important modifiers of the epigenetic profile (90, 91). These exposures may have profound effects on patterns of DNA methylation and long-term gene expression, which lead to altered tissue development and physiological function to affect future susceptibility to chronic diseases, including cancer (89, 92). It has been shown that soy IF diet altered DNA methylation of specific CpG sites within *Acta1* promoter in the pancreas but not the liver of mice (93). Another study found that dietary genistein treatment resulted in a partial change in the DNA methylation pattern of CpG islands of specific mouse genes in the prostate but not the liver (94). These findings indicate that IFs may affect methylation status of certain genes in a tissue-specific manner. Short-term treatment with dietary soy can increase promoter methylation level of breast cancer-related genes in healthy

mammary tissues of pre-menopausal women (95). Currently there is no evidence that can clearly associate early life soy exposure, DNA methylation and breast cancer risk. Nevertheless, the speculation on epigenetic modulation as the mechanism of soy action continues (55, 96, 97).

**The controversy of early life soy exposure**

The hypothesis on early life as a potential window for gaining the protective effect of soy is accompanied by a rise in safety concerns due to the fact that soy IF and other phytoestrogens are categorized as endocrine disrupting compounds (EDC) . The definition of EDC according to the U.S. Environment Protection Agency is an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior (98). It includes natural products or synthetic chemicals that mimic, enhance, or inhibit the action of hormones. Although the term EDC does not necessarily implicate absolute adverse health risk, it is more often associated with a compound's potential to elicit negative effects on the endocrine systems (99). Despite the potential health benefit of soy and lower estrogenic potency of IF compared to natural estrogens and synthetic estrogens (100), IFs are often compared to deleterious chemicals such as diethylstilbestrol (DES) and bisphenol A (BPA) (101). Early life exposure to those chemicals is associated with various adverse reproductive health risks. DES exposure is associated with a high lifetime risk of infertility, spontaneous abortion, stillbirth, cervical intraepithelial neoplasia and breast cancer (102). BPA effect is more controversial; it is less clear in humans but animal studies reported that maternal BPA exposure increased breast cancer risk, and altered mammary gland and reproductive tract development in mice (103, 104, 105) and macaques (106). Consequently, there has been an on-going concern regarding the safety of early life consumption of soy products. Human studies showed inconclusive results; epidemiological studies have associated pre-pubertal soy exposure with both accelerated (107) and delayed (108, 109) breast development. To our knowledge, up to now there is only one study

that assessed the association between early life soy exposure and the uterus, which found a nonsignificant increased risk of uterine fibroids (110) in adulthood.

With respect to mammary gland development and breast cancer, several rodent studies have reported that maternal and/or neo-natal exposure to IF, mainly genistein, increased susceptibility to mammary tumors (111), induced ductal hyperplasia (112), and disrupted mammary gland growth (101, 113). Not much is known about the adverse effect of pubertal exposure to soy on the mammary gland. For uterine development, subcutaneous injection with 50 mg/kg/day genistein in neonatal CD-1 mice was reported to induce uterine hyperplasia and squamous metaplasia by post-puberty and adulthood, comparable to that induced by DES exposure (notably, genistein dose was 50,000 times greater than DES dose) (101). Using the same model, neonatal exposure to lower dose of daidzein and genistein (i.e. <10 mg/kg) was also reported to result in endometrial hyperplasia and edema in the stroma by young adulthood (114). Other adverse effects of early exposure to genistein reported in rodent studies include altered estrous cyclicity (found with dose ranging from 0.5 to 100 mg/kg), disrupted ovarian development ($\geq$10 mg/kg), advanced puberty onset (in one study using 10 mg/kg), and abrupted fertility (with >50 mg/kg) (115, 116, 117, 118). Collectively, the two opposite health claims cause confusion regarding whether early-life soy consumption should be encouraged or avoided.

**The story of equol**

When ingested, IFs undergo further metabolism in the intestinal tract. Gut microbiota metabolize daidzein into compounds called O-desmethylangolensin (O-DMA) and equol. Most animal species can produce equol. Interestingly in humans, only ~30% of non-Asian non-vegetarian population is capable of producing this metabolite, whereas up to 60-70% of vegetarians (119) and Asians (120, 121) are equol producers. Factors that determine the ability to produce equol are not clear. Although inconclusive, some of the factors thought to be associated

with this phenotype are intestinal physiology, host genetics, and dietary factors such as fat, carbohydrate, green tea, PUFA, lactose, and soy (122, 123).

Equol has different ER binding affinity and potency compared to genistein and daidzein (19), and consequently the biological activity is also different (124). Equol exists in two enantiomeric forms: the S-(-) and R-(+) equol (from hereon shortened as S-equol and R-equol, respectively). S-equol is the natural form that is produced by the intestinal microbiota while R-equol is chemically synthetized. Chemical synthesis of equol from daidzein used to only yield the racemic mixture of both enantiomers. Therefore most equol studies, like our present work, have used the commercially available racemic form. It was not until recently the method for selective synthesis of the individual enantiomers was described, and animal studies led by Setchell and colleagues started using them for dietary treatment which showed that the biological action differed between the two enantiomers (122). They have reported that only R-equol appeared to have potential chemoprotective effect against DMBA-induced mammary tumor. The effect was observed only when exposed at peripubertal age (125) whereas neonatal exposure to R-equol enhanced mammary differentiation but lacked the chemoprotective effect (126). In the reproductive tissue, however, perinatal exposure to either enantiomer triggered hyperplasia of the uterus (127).

Interestingly, there have been studies in humans that indicate the differential response to soy consumption between equol producers and nonproducers (123, 128). Nevertheless, most of the published soy epidemiologic studies did not take the equol-producing phenotype into account. This unique metabolism factor could potentially contribute to the mixed results and inconsistencies observed for the past two decades. Notably, the development of equol-based supplement has been rising in the recent years despite the uncertainty regarding equol and the on-going controversy on the effect soy exposure, which further highlights the significance for investigating the effect of equol on the breast and uterus.

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## SPECIFIC AIMS

The aims of this project were:

- To determine dietary soy isoflavones (IFs) estrogenic effects on tissue phenotype

  a: utilizing a prepubertal nonhuman primate model

  b: utilizing a subadult mouse model

- To determine the effects of soy IFs on Estrogen Receptor (ER)-dependent gene transcription in the breast

  a: by measuring mRNA expression of markers for ER pathway

  b: by measuring promoter methylation of ER and ER-activity markers

  c: to identify novel pathways affected

- To determine the dependence on equol to attain estrogen-modulatory effect

The research hypotheses were:

- Soy IFs will antagonize estrogen effects on the breast and reproductive tissue
- Soy IFs will dampen estrogen-regulated gene transcription
- Estrogen-antagonizing effects of soy IF will only be observed in equol producers

## STUDY DESIGN

### Nonhuman primate study

Thirty female cynomolgus macaques (*Macaca fascicularis*) were imported from Institut Pertanian Bogor (Bogor, Indonesia) at the peripubertal age of approximately 1.5 years, as confirmed by dentition. The study was a parallel-arm trial of dietary soy versus no soy; animals were randomized to social groups of 4-5 on the basis of body weight. They received either a diet with soy protein containing soy IF (SOY, n=18), or a control diet devoid of soy IF with casein and lactalbumin as the protein source (CL, n=12). Macronutrient composition of the diets was designed to approximate a typical North American diet with 35% calories from fat (Appendix 1). The SOY diet was given in a daily dose of 10 mg/kg body weight, which was designed to provide the human equivalent of 120 mg/day of IF (as aglycones) assuming an average daily energy intake of 1800 Cal. Dietary treatment lasted for approximately 4.5 years. As shown in Figure 1, during this period breast biopsies were taken every six months starting at one month into treatment, with a total of 9 biopsies per animal spanning the period of pubertal development. Other measures at the time of biopsy included serum estradiol and progesterone, uterine size by ultrasound, bone mineral measures by Dual Energy X-Ray Absorptiometry (DEXA) scans, nipple length, trunk length, and body weight. Animals were swabbed daily for vaginal bleeding throughout the study, and the onset of menarche was defined as the initiation of regular monthly vaginal bleeding. Two animals died during the study due to causes unrelated to the dietary treatment, one at the beginning of the study (SOY) and one approaching year 4 of the study (CL); this latter animal did not have data from the final biopsy.

All procedures involving the animals were performed at the Wake Forest School of Medicine, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Procedures were conducted in compliance with state and federal laws and standards of the US Department of Health and Human Services and approved by the Wake Forest University Animal Care and Use Committee (ACUC).

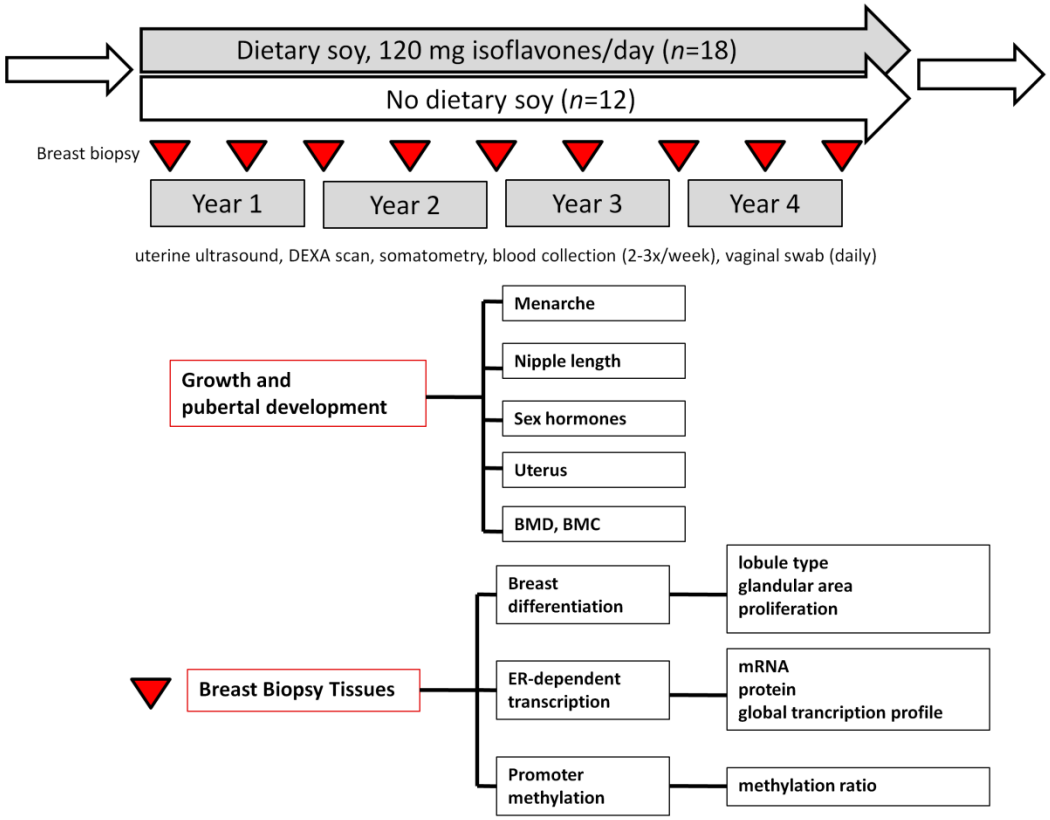


**Figure 1.** A parallel-arm study design for assessment of pubertal development in the cynomolgus macaque fed a high soy or control diet.

Main outcomes for this study were:

- Mammary ductal and lobular morphogenesis
- Lobular differentiation in the breast
- Mammary gland epithelial cell proliferation
- Expression and promoter methylation of ERs and ER-regulated markers
- Expression of estrogen-metabolizing enzymes
- Uterine development including total uterine area, endometrial area and thickness
- Puberty characteristics including timing of menarche, ovarian hormone levels, and nipple length progression

Specific methods are described in the respective chapters.

**Mouse study**

The study was part of an atherosclerosis-related project. The mice used in the study lacked the apolipoprotein E (apoE) gene and were susceptible to atherosclerosis; the parent study was aimed to investigate the role of equol in mediating atheroprotective effects of soy. All breeding and procedures involving animals were done at Taconic Laboratories in their AAALAC-accredited facilities in Germantown and Rensselaer, NY and were approved by the Taconic institutional ACUC. Tissue-related procedures were performed at Wake Forest School of Medicine. The gnotobiotic apoE-null mouse breeding colony was initially established by exposing 7-week-old germ-free mice to the Altered Schaedler Flora (ASF), which was a cocktail of eight, urease-negative, anaerobic bacteria, via aqueous fecal suspension from ASF-maintained donors. Preliminary assessment of serum equol concentrations indicated that mice harboring ASF were not equol producers.

Our study utilized the offspring ($n$=243) of apoE-null breeders harboring ASF. The animals were defined as equol nonproducers. Between 3-6 weeks of age, half of the colony ($n$=122) were exposed to intestinal microbiota from normal apoE-null mice via fecal material to generate an equol-producing phenotype (equol producer). Throughout the study, all animals were maintained under gnotobiotic conditions in plastic film isolators, and their microbiota status was monitored routinely by fecal culture. Microbial status of the nonproducers was verified under Taconic's Defined Flora microbiological health standard, whereas equol producers were maintained at the Murine Pathogen Free status.

Mice typically reach sexual maturation at around 5-8 weeks of age. Equol producers and nonproducers were randomly assigned to dietary treatment groups as described in Figure 2 starting the age of 6 weeks, with 9-11 animals in each group. The diets used one of three protein sources; 1) casein and lactalbumin (CL control), 2) alcohol-washed soy protein (low IF); or 3) intact soy protein (high IF). The total IF amounts in the diets were 0, 42, and 566 ppm, respectively. The ratio of daidzein:genistein:glycitein was 1:1.5:0.2. Additionally, the diets also

contained 291 ppm or no equol. This exogenous equol was given as a synthetic product chemically synthesized from daidzein and comprised of a 50/50 racemic R/S (±) mixture. A detailed description of the diet compositions can be found in Appendix 1. We used a dosing strategy for IF in mg/Cal to adjust for the metabolic rate difference between humans and mice, and to provide an IF dose relevant to human exposure. Human daily caloric consumption was considered to be 1800 Cal/day and our diet compositions were designed to create a dosing strategy of approximately 275 mg IF/1800 Cal for high IF, 20 mg IF/1800 Cal for low IF, and 140 mg equol/1800 Cal for exogenous equol.

Mice were humanely euthanized following 16 weeks of dietary treatment. Blood and tissue collection was performed. We identified the estrous cycle stage (proestrus, estrus, metestrus, and diestrus) of each animal based on histology of the vagina, uterus, and ovaries. Animals that did not meet all the criteria of a cycle stage were noted as "undetermined stage" and excluded from cycle-related analyses.

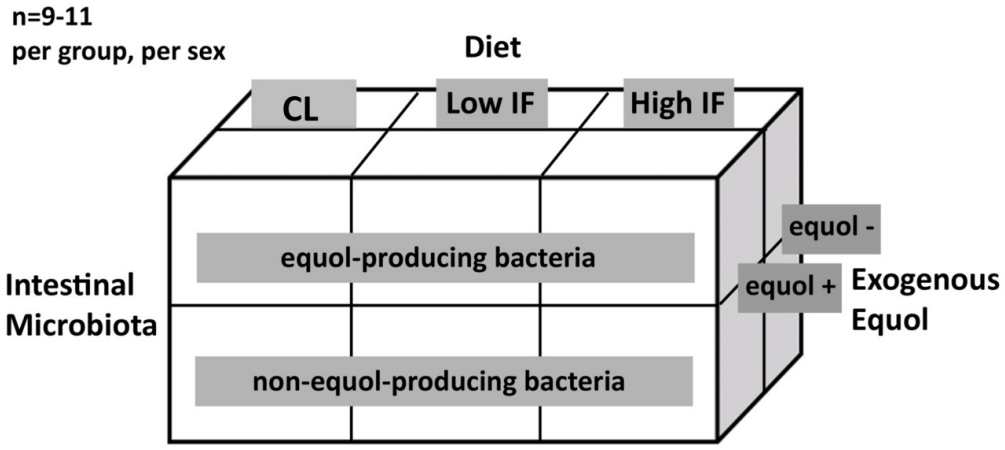


**Figure 2.** Three-way 3x2x2 factorial design to assess the effect of dietary soy isoflavones, intestinal microbiota status, and dietary racemic equol on apoE-null mice. CL, Casein and Lactalbumin; IF, Isoflavones

Main outcomes for this study were:

- Mammary gland total epithelial area
- Uterine tissue phenotype (wet weight, total uterine area, endometrial area, endometrial luminal epithelial height)
- Uterine epithelial cell proliferation
- Expression of sex steroid receptor (PGR) in the breast and uterine epithelial cells

Specific methods are described in the respective chapters.

# CHAPTER 1

## Dietary soy effects on mammary gland development during the pubertal transition in nonhuman primates

Fitriya N. Dewi, Charles E. Wood, Cynthia J. Lees, Cynthia J. Willson, Thomas C. Register, Janet A. Tooze, Adrian A. Franke, and J. Mark Cline

The following manuscript has been accepted for publication in Cancer Prevention Research. Stylistic variations result from the demands of the journal. Fitriya N. Dewi performed the experiments, completed the statistical analyses and data interpretation, and prepared the manuscript. Charles E. Wood provided guidance on the overall experiment and manuscript preparation. Cynthia J. Lees performed the breast biopsy surgery, DEXA and ultrasound assessments. Cynthia J. Willson performed histopathological review. Thomas C. Register provided guidance on the molecular endpoints. Janet A. Tooze provided guidance on the statistical approach and perfomed the mixed effects logistic regression with random effects. Adrian A. Franke performed measurements of serum isoflavonoid. J. Mark Cline was the principal investigator of the study, and provided signficant guidance throughout the project.

**Dietary soy effects on mammary gland development during the pubertal transition in nonhuman primates**

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**Running title:** Soy effects on pubertal breast development

**Keywords:** puberty, breast cancer, soy isoflavones, estrogen, macaque

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**Supplementary Materials:** Figures (2), Tables (2)

**Abstract**

While epidemiologic studies suggest that soy intake early in life may reduce breast cancer risk, there are also concerns that exposure to soy isoflavones during childhood may alter pubertal development and hormonal profiles. Here, we assessed the effect of a high-soy diet on pubertal breast development, sex hormones, and growth in a nonhuman primate model. Pubertal female cynomolgus monkeys were randomized to receive a diet modeled on a typical North American diet with one of two protein sources for ~4.5 years: i) casein/lactalbumin (CL, $n$=12, as control) or ii) soy protein isolate with a human equivalent dose of 120 mg/day isoflavones (SOY, $n$=17), which is comparable to approximately four servings of soy foods. Pubertal exposure to the SOY diet did not alter onset of menarche, indicators of growth and pubertal progression, or circulating estradiol and progesterone concentrations. Greater endometrial area was seen in the SOY group on the first of 4 postmenarchal ultrasound measurements ($P$<0.05). There was a subtle effect of diet on breast differentiation whereby the SOY group showed higher numbers of differentiated large-sized lobular units and a lower proportion with immature ducts following menarche ($P$<0.05). Numbers of small lobules and terminal end buds and mammary epithelial cell proliferation did not differ by diet. Expression of progesterone receptor was lower in immature lobules of soy-fed animals ($P$<0.05). Our findings suggest that consumption of soy starting before menarche may result in modest effects consistent with a more differentiated breast phenotype in adulthood.

## Introduction

Environmental exposures to hormonally active compounds during critical windows of development can influence breast cancer risk later in life (1). Soy protein contains a variety of bioactive compounds including isoflavones (IF), which are structurally similar to estrogen, bind to estrogen receptors (ERs), and elicit both estrogenic and anti-estrogenic responses depending on dose, tissue location, and estrogen context (2, 3). Soy intake has been widely studied as a potential dietary determinant of breast cancer risk, both as a natural chemopreventive approach as well as potential risk factor. Recent evidence suggests that timing of exposure may be critical to determine the magnitude and direction of soy effects. Several epidemiologic studies indicate that lower risk of breast cancer is observed when soy is consumed throughout life or before/during key periods of breast development (4, 5). These observations parallel those in rodents (6), supporting the idea that early soy exposure may alter breast phenotype later in life.

Puberty is a developmental process that involves a complex series of interactions between growth factors and sex hormones. Age at menarche, a key milestone of puberty, is a consistent predictor of later breast cancer risk in human observational studies, potentially due to hormonal and developmental factors (7, 8). In females, puberty is a key period for breast morphogenesis and differentiation. Ovarian hormones drive elongation and branching of rudimentary ducts and development of terminal end buds (TEBs) (9), which subsequently develop into lobuloalveolar structures. The lobules further undergo gradual maturation wherein the number of terminal ductular and alveolar units per lobule increases, resulting in a concomitantly larger size (10). The degree of lobular differentiation has been inversely related to cancer risk (11, 12), suggesting that modulation of breast developmental patterns may influence later life susceptibility to cancer. We hypothesized that pubertal exposure to soy would enhance mammary gland differentiation, potentially leading to a lower risk breast phenotype.

Previous studies have primarily used rodent models to assess environmental influences on breast development. While these models have contributed greatly to our understanding of

molecular signaling in the breast, there are important developmental and physiologic differences between rodents and primates. Most notably, pubertal breast development in rodents is mainly composed of ductal growth with scant lobular differentiation prior to pregnancy (13). Macaque monkeys serve as a valuable animal model for women's health due in part to their highly similar reproductive physiology, pubertal stages of development, and patterns of ductal and lobular morphogenesis in the breast (14, 15). Cynomolgus macaques also exhibit a nonseasonal 28-30 day menstrual cycle with ovarian hormone and tissue responses comparable to humans (16). The primary aim of the current study was to assess the effect of dietary soy exposure on breast development in cynomolgus macaques across the pubertal transition.

## Materials and Methods

### *Animals and Diet Treatment*

Thirty female cynomolgus macaques (*Macaca fascicularis*) were imported from the Institut Pertanian Bogor (Bogor, Indonesia) at approximately 1.5 years of age, as confirmed by dentition. Female macaques typically reach puberty at the age of 2-3 years. Animals were randomized to social groups of 4-5 on the basis of body weight to receive one of two diets for ~4.5 years: (i) a control diet with casein and lactalbumin as the protein source (CL, $n$=12) or (ii) a diet with isolated soy protein containing IF (provided by Solae, LLC) (SOY, $n$=18) with the human equivalent of 120 mg/day of IF (in aglycone equivalents), which is ~3-5 times higher than the typical consumption in Asian population (17) (Supplementary Table S1). Macronutrient composition of the diets approximated a typical North American diet with 35% calories from fat. Breast biopsies were taken every six months starting at one month into treatment, with a total of 9 biopsies per animal spanning the period of pubertal breast development. Other measures at the time of biopsy included serum sex hormones, uterine size by ultrasound, bone mineral measures by Dual Energy X-Ray Absorptiometry (DEXA) scans, nipple length, trunk length, and body weight. Animals were swabbed daily for vaginal bleeding throughout the study, and menarche

was defined as the initiation of regular monthly vaginal bleeding (16, 18). Following menarche, menstrual cycle stage of the animals (i.e., follicular or luteal) was determined retrospectively based on their menstrual bleeding calendar. Two animals died during the study due to causes unrelated to the dietary treatment, one at the beginning of the study (SOY) and one approaching year 4 of the study (CL); this latter animal did not have data from the final biopsy.

All procedures involving animals were performed at the Wake Forest School of Medicine, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Procedures were conducted in compliance with state and federal laws and standards of the US Department of Health and Human Services and approved by the Wake Forest University Animal Care and Use Committee.

*Serum Isoflavonoids*

Serum concentrations of the main isoflavones (genistein, daidzein) and isoflavone metabolite (equol) were measured using liquid chromatography electrospray ionization mass spectrometry at the laboratory of Dr. Adrian Franke (University of Hawaii Cancer Center), as described elsewhere (19). The measurements were performed on 18-hour fasted serum samples collected at the time of biopsy.

*Dual Energy X-Ray Absorptiometry Scans*

Whole body DEXA scanning was performed every six months using a Norland XR-46 Bone Densitometer (Norland Corp, Fort Atkinson, WI). Utilizing Norland Host Software, we generated measurements for whole body bone mineral content (BMC, in grams) and lumbar spine (lumbar vertebrae 2-4) bone mineral density (BMD, in grams per $cm^2$) (20). The coefficients of variation were 1.7% for BMC and 2.1% for BMD.

*Somatometry*

Trunk length, nipple length, endometrial thickness, and uterine and endometrial area were measured as indicators of growth and reproductive maturation. Trunk length was measured from the suprasternal notch to symphysis pubis using a Vernier Caliper (Fischer Scientific, Pittsburgh, PA). Nipple length was also measured by caliper. Ultrasound of the uterus was performed utilizing portable ultrasound devices equipped with 5.0-13 MHz linear transducers (SonoSite, Bothell, WA). Measurements of uterine area, endometrial area, and endometrial thickness were manually performed on the digitized images using NIH ImageJ (version 1.45q, available at *rsbweb.nih.gov/ij/*). Post-menarche analysis was adjusted for normal cyclical variation in endometrial thickness across the menstrual cycle, using menstrual cycle days 1-9 as the period of anticipated thinnest endometrium, with day 1 counted as the first day of menstrual bleeding (21).

*Hormone Assays*

Serum concentrations of estradiol (E2) and progesterone (P4) were measured by radioimmunoassay (RIA) using commercially available kits and protocols from Siemens Healthcare Diagnostics (Los Angeles, CA). Prior to menarche, serum hormone concentrations were measured in blood samples that were collected at the time of breast biopsy. One year after menarche, blood was collected at three consecutive menstrual cycles, twice during each menstrual cycle (at day 9-13 for E2 and day 18-25 for P4). The RIAs were performed at the Biomarkers Core Laboratory of the Yerkes National Primate Research Center of Emory University (Atlanta, GA) using standard procedures. The normal assay ranges were 2.85-546.00 pg/ml for E2 and 0.10-40.00 ng/ml for P4.

*Breast Biopsy Collection*

Serial breast biopsies were collected using methods described previously (22). The biopsy samples were wedge-shaped, ~200 mg in weight, 2-3 cm x 1 cm from nipple to the edge of the gland. Each sample was divided; one half was frozen for biomolecular work, and the other

half was placed on a fiberglass screen to prevent distortion, fixed at 4°C in 4% paraformaldehyde for 24 hours, and then transferred to 70% ethanol. Fixed tissues were then processed for whole mount staining, histology, and immunohistochemical staining. Hematoxylin and eosin (H&E)-stained slides were evaluated for developmental morphology and pathologic lesions blinded to experimental treatment by a pathologist (CJW) in consultation with a board-certified veterinary pathologist (JMC).

*Whole Mount Mammary Gland Staining*

Whole mounts were stained using 0.27% Toluidine Blue as previously described (15, 23). Whole mounts were photographed *in toto*, and the digital images were used for morphologic measures.

*Mammary Gland Morphometry*

. For measurement of mammary gland epithelial area, H&E-stained slides were digitized (Infinity 3 digital camera, Lumenera, Ottawa, Canada; Adobe Photoshop version 6.0, San Jose, CA) and measured with methods described previously (23) and modified as follows for developmental structures. Breast epithelium was subdivided into lobuloalveolar and ductal compartments. Lobules were categorized as immature (type 1) or mature (type 2 and 3) (10). Ducts were categorized as either immature (multiple luminal cell layers, columnar cell morphology, rounded myoepithelial cells, with/without a small lumen), transitional (multiple luminal cell layers with less stratification, more elongated morphology, increased lumen size, flattened myoepithelial cell borders), or mature (single luminal cell layer, cuboidal morphology, flattened myoepithelial cells). We did not histologically differentiate between TEBs and immature ducts on H&E images due to their overlapping features (15). Total epithelial area of the biopsy section and area of each lobule and ductal type were determined by manually tracing the structures using a computer-assisted technique with Image Pro-Plus Software (Media Cybernetics Inc., Bethesda, MD). Epithelial area was expressed as a percentage of the total area examined.

For whole mounts, mammary glandular structures were categorized as TEBs (tear drop-shaped, >100 μm in width), small-sized lobules (area ≤100,000 $\mu m^2$), or large-sized lobules (area >100,000 $\mu m^2$), and quantified across the entire tissue using NIH ImageJ. Structural counts were expressed as number of TEBs or lobules per $cm^2$.

*Quantitative Gene Expression*

Expression of mRNA for markers of mammary gland differentiation (casein alpha s1, *CSN1S1*; mucin-1, *MUC1*; E74-like factor 5, *ELF5*; signal transducer and activator of transcription factor 5A and 5B, *STAT5A* and *STAT5B*), proliferation (*MKI67),* and ER activity (progesterone receptor, *PGR*) was measured using quantitative real-time reverse-transcriptase PCR (qRT-PCR) based on standard methods described elsewhere (24). Amplification was performed using the ABI PRISM® 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA). Human or m*acaque-specific Taqman primer-probe assays were used to quantify target transcripts (Supplementary Table S2) with normalization to cynomolgus macaque-specific primer-probe sets of housekeeping genes GAPDH and ACTB. R*elative gene expression was determined using the ΔCt method calculated by ABI Relative Quantification 7500 Software v2.0.1 (Applied Biosystems, Foster City, CA). Stock mammary tissues and tumor samples were run in triplicate on each plate as external calibrators.

*Immunohistochemistry*

We used immunohistochemistry to assess protein expression and localization of markers for proliferation (Ki67) and estrogen activity (PGR) in mammary epithelial cells as previously described (22). Monoclonal antibodies used were anti-Ki67 (Ki67/MIB1; Dako, Carpinteria, CA) and anti-PGR (NCL-PGR; Novocastra Labs, Newcastle-upon-Tyne, UK), both with 1:100 dilution. Cell staining was quantified by a computer-assisted technique with grid filter where positively stained cells were scored based on staining intensity (+1, +2, or +3) to obtain a semi-quantitative measurement of staining intensity and distribution by H-score calculation (22).

*Data Analyses*

Data that were not normally distributed were transformed by logarithmic or square-root conversions to improve normality of the residuals. Data were back transformed to original scale for presentation of results; values are presented as least square means (LSM) $\pm$ standard error of the mean (SEM) or LSM (LSM-SEM, LSM+SEM) when standard errors were asymmetric. We used JMP (version 10.0.0, SAS Institute; Cary, NC) to fit a mixed model ANOVA with a random animal effect to model diet and time effects adjusted for body weight and menstrual cycle stage (post-menarche) to estimate and compare differences in serum isoflavonoid levels, hormone levels, somatometric measures, mammary gland differentiation, mammary epithelial cell proliferation, and ER activity markers in the breast, between SOY and CL groups over time. We also used SAS (version 9.2, SAS Institute; Cary, NC) to fit a mixed effects logistic regression with a random animal effect to model if a particular duct or lobule type was found at a given time point, and if there were differences by diet group, adjusted for body weight and menstrual cycle stage (post-menarche) (25). Menarche data were analyzed using a survival model and logistic regression to model whether onset occurred by 12 months of treatment adjusted for body weight. All outcomes were compared between monkeys of similar development stage across the pubertal transition, and the analyses were done separately for pre- and post-menarche. We also compared pre- vs. post-menarche. Multiple pairwise comparisons were done with Tukey Honestly Significant Difference (HSD) Test. Correlation between differentiation marker expression and large-sized lobule count was analyzed using a pairwise test for the significance of the Pearson Product-moment correlation coefficient.

## Results

### *Serum Isoflavonoid Concentrations*

Total serum isoflavonoid concentrations were significantly higher in SOY group compared to CL ($P<0.0001$). The mean concentration in SOY was 119 nmol/L (107.9, 131.2nmol/L) after overnight fasting, compared to 22 nmol/L (19.8, 25.2 nmol/L) in CL. Equol was the predominant circulating isoflavonoid, accounting for 70% of total isoflavonoids. This finding was similar to our previous work (22).

### *Pubertal Development*

Body weight, trunk length, BMC, and BMD increased across the treatment period (time effect $P<0.0001$) but did not differ between diet groups. Mean body weights were 1.76 $\pm$ 0.08 kg and 1.70 $\pm$ 0.07 kg prior to dietary treatment and 3.51 $\pm$ 0.17 kg and 3.79 $\pm$ 0.15 kg at the end of the study in the CL and SOY groups, respectively. Trunk length, BMC and BMD were associated with body weight ($P<0.0001$). While trunk length increased across both menarchal stages (data not shown), BMC and BMD only significantly increased after menarche (Supplementary Figure S1).

Timing of menarche varied widely across individuals with no effect of diet (Figure 1A). By month 12 of treatment, CL showed a nonsignificantly higher proportion of menstruating animals than SOY, and the onset was marginally associated with body weight ($P=0.07$). By month 18, ~70% of animals in each diet group had begun cycling.

Hormone status (Figure 1B,C) and markers for pubertal progression (Figure 2) were evaluated. Nipple length increased up to menarche ($P<0.0001$), which was partially driven by the change in body weight ($P<0.0001$). Uterine area also increased across pre-menarche ($P<0.01$) and was associated with body weight ($P<0.01$), whereas endometrial area ($P=0.07$) and thickness ($P=0.1$) did not significantly change. Importantly, there was no diet effect observed for any of these markers before menarche. Serum hormone concentrations were elevated after menarche

($P$<0.0001 in both diet groups) but remained unaffected by diet. Uterine area increased across post-menarche ($P$<0.01) with a significant diet x time interaction ($P$<0.05), and showed an association with body weight ($P$<0.05). Endometrial area and thickness were primarily determined by menstrual cycle day ($P$<0.01 and $P$=0.05, respectively). There was no diet effect observed on endometrial outcomes when covaried with menstrual cycle day. We found a significant diet x time interaction on endometrial area ($P$<0.05), with greater area in the SOY group during 1-6 months after menarche ($P$<0.05).

***Mammary Gland Differentiation***

Histological assessment revealed no significant abnormalities in mammary gland morphology throughout the study. Two animals (SOY) had a minimal lymphocytic perilobular infiltrate at one biopsy timepoint. A lobule in one animal (CL) contained abundant globular hypereosinophilic secretory material (eosinophilic secretory change), which was found only on the last biopsy.

Mammary epithelial features showed normal patterns of pubertal breast development (Figure 3A-C). Pre-menarchal breast contained predominantly ductal structures, with marginally greater areas of transitional and mature ducts in SOY relative to CL ($P$=0.05 and $P$=0.06, respectively) (Figure 3D). Following menarche, the relative area of immature and transitional ducts decreased ($P$<0.001), whereas that of lobules increased compared to pre-menarche ($P$<0.0001). After menarche (Figure 3F), there was a main effect of diet on immature ducts ($P$<0.05) with a significant diet x time interaction ($P$<0.05) whereby the area was greater in CL compared to SOY at 1-6 months post-menarche ($P$<0.01). In mixed model logistic regression, the proportion of post-menarchal monkeys that had immature ducts present in the breast was higher in CL relative to SOY (diet effect $P$<0.05). There was no diet effect on lobular area across the pubertal transition (Figure 3E, G). Histologically, total epithelial area reached 6% (4.8%, 8.0%) in CL and 10% (8.5%, 11.9%) in SOY by two years post-menarche. Similar to other pubertal

measures, body weight was associated with the areas of immature ducts, transitional ducts, immature lobules, and mature lobules ($P<0.05$ for all).

There was no diet effect on the numbers of TEBs and small-sized lobules at either pre- or post-menarche (Figure 4). Regardless of diet, the number of small-sized lobules after menarche was higher than that before menarche ($P<0.0001$), and there was an increasing pattern over time with a slight decrease by 1.5-2.0 years after menarche. The number of large-sized lobules significantly increased after menarche (time effect $P<0.01$), and there was an overall diet effect whereby the number was greater in SOY at this stage (diet effect $P<0.05$). This effect was mainly driven by whether or not animals had any large lobules present in the breast (diet effect $P<0.01$). Further, the difference was most evident at 1.5-2.0 years after menarche when the SOY group showed about 5-fold higher number of large-sized lobules compared to CL ($P<0.05$).

We did not find a diet effect on gene expression of differentiation markers before or after menarche (Table 1). There was a significant time effect on mRNA expression for *CSN1S1* ($P<0.0001$), *ELF5* ($P<0.0001$), *MUC1* ($P<0.01$), *STAT5A* ($P<0.0001$), and *STAT5B* ($P<0.001$). Post-menarchal increases in *CSN1S1* and *ELF5* were seen in both SOY ($P<0.001$) and CL ($P<0.01$), while *STAT5A* and *STAT5B* increased only in SOY ($P<0.01$). The mRNA expression of all markers was positively correlated with the number of large-sized lobules (r=0.35, $P<0.0001$ for *CSN1S1*; r=0.37, $P<0.0001$ for *ELF5*; r=0.25, $P<0.01$ for *MUC1*; r=0.34, $P<0.0001$ for *STAT5A*; r=0.27, $P<0.001$ for *STAT5B*). When stratified by dietary groups, correlations of *CSN1S1* and *ELF5* with large-sized lobule number were stronger in the SOY group, and correlations of *MUC1*, *STAT5A* and *STAT5B* with number of large-sized lobule were only significant in the SOY group. While body weight was significantly associated with lobule count ($P<0.0005$ for all lobule types), it was not associated with mRNA expression of differentiation markers.

***Epithelial cell proliferation was not affected by soy treatment***

Post-menarche *MKI67* mRNA expression was 1.7 fold higher than pre-menarche levels ($P<0.05$) but there was no effect of diet (Supplementary Figure S2). Ki67 protein expression in the ductal and lobular structures of the mammary gland also did not differ by diet, with or without menstrual cycle stage in the model. Before menarche, proliferation of the epithelial cells in the mature lobule compartment marginally increased over time ($P=0.06$). After menarche, the proliferation in the immature lobule compartment showed a non-significant decreasing pattern with time ($P=0.07$). We found a significant association of body weight and Ki67 expression in mature ducts ($P<0.05$), immature lobules ($P<0.005$), and mature lobules ($P<0.005$). This effect was interpreted to reflect the onset of ovarian activity at puberty.

***Estrogen activity in the breast decreased following menarche***

There was a significant time effect on *PGR* expression across the pubertal transition ($P<0.05$). Regardless of diet group, *PGR* mRNA expression decreased after menarche ($P<0.0001$) (Figure 5A). While there was no diet effect on *PGR* mRNA, PGR protein expression in immature lobules post-menarche was lower in the SOY group (diet effect $P<0.05$) (Figure 5B). The effect was mainly driven by a higher proportion of monkeys in CL (vs. SOY) that showed positively stained cells in the immature lobule (diet effect $P<0.05$). Localized PGR protein expression did not differ by menarche status (Figure 5C). Body weight was inversely associated with PGR expression in mature ducts ($P<0.05$), immature lobules ($P<0.01$), and mature lobules ($P<0.05$).

**Discussion**

In this study, we showed that pubertal soy intake did not alter onset of menarche, pubertal progression, circulating reproductive hormones, or mammary epithelial cell proliferation. Soy had a modest effect on post-menarchal breast differentiation as indicated by a higher number of large-sized lobules and fewer immature ducts. The numbers of TEB and small-sized lobules, and differentiation markers, however, did not differ significantly by diet. There was lower expression of PGR in immature lobules of the soy-fed animals after menarche, possibly indicating altered estrogen response.

Puberty is the major period for mammary gland morphogenesis in females. In humans, hormonal and growth factor signals induce the rudimentary ductal tree to elongate, branch, and form lobuloalveolar structures (9). In rodents, pubertal breast development is mainly a process of rapid ductal growth; and prominent lobuloalveolar growth only occurs during pregnancy (13, 26). Here we document for the first time the pattern of mammary gland development across the pubertal transition in the macaque model, showing a high degree of interindividual variation and morphologic similarities to humans. The human TEB is the leading edge of mammary gland growth that gives rise to new branches and alveolar buds that further cluster around a terminal duct, forming nascent lobules (10). Similarly, pre-menarchal macaque breast consists mainly of TEBs, ductal structures, and immature lobules. After menarche, fewer TEBs are present and the majority of the epithelial compartment is in the form of large ducts and lobuloalveolar structures. A large increase in macaque mammary lobular differentiation occurs around the time of menarche and more markedly around one year after menarche, similar to that reported in humans (10). Lobules type 1 and 2 become the predominating unit of the adolescent macaque breast, which is consistent with our prior findings in the adult nulliparous macaque (27). Large-sized lobules, composed of more mature type 2 and type 3 lobules, also become more abundant and cover the majority of the epithelial compartment. The adult macaque breast is comprised of 80% or more stroma, similar to that in nonlactating women (9).

Mammary differentiation is a determinant of breast cancer risk. Less differentiated progenitor-type cells in the terminal ducts are likely founder cells for the majority of ductal carcinomas, the most common type of human breast cancer (12, 26). Mammary stem and progenitor cells are present throughout life and they are targets for transformation. The fate of these cells is mainly regulated during key developmental events such as puberty and pregnancy (28). In human epidemiologic studies, nulliparity is associated with higher breast cancer risk, potentially due to less differentiation of susceptible progenitor cell populations (29, 30, 31). Experimentally*, in vitro* and rodent studies indicate that less differentiated mammary cells and lobules are more proliferative, susceptible targets of carcinogens, and prone to neoplastic transformation (12, 32). Recent evidence also points to epigenetic changes during development that may lead to carry-over effects on cancer risk (33, 34).

Several observational studies have reported that adolescent soy intake may reduce breast cancer risk (35, 36, 37). Mechanisms for this effect are unclear, and these studies are generally limited by recall and "healthy person" biases. A prominent hypothesis is that early soy exposure may enhance breast differentiation, potentially through ER agonist effects. Parenteral administration of purified genistein resulted in fewer terminal ducts and increased numbers of alveolar buds in a carcinogen-treated mouse model (38). In rats, early life exposure to dietary soy protein (39) or high dose genistein (40) reduced the number of TEBs. Our study extends these findings by evaluating developmental effects on the primate breast using soy/IF doses relevant to high dietary human exposures. Our findings suggest that soy intake before menarche may modestly enhance breast differentiation leading to a greater content of mature lobular structures following menarche. The markers *CSN1S1, ELF5, STAT5,* and *MUC1* have important roles in lobular epithelial differentiation (33, 41, 42), and we previously reported that these genes were highly expressed in the mammary gland of pregnant and lactating cynomolgus macaques (43). Here we confirmed their use as differentiation markers in the macaque breast. The positive correlations between the expression of these markers and lobular differentiation were generally

stronger in the soy-fed animals, further supporting the idea that soy may have a mild promotional effect on breast differentiation.

Soy IF share structural similarities to endogenous estrogens, bind to and transactivate ERs, and modulate proliferation of ER-responsive cells *in vitro*. This evidence has led to the idea that soy may alter ER-mediated activity in the developing breast through agonistic, antagonistic, or other hormone-disrupting effects. Here we found no evidence of strong ER agonist effects of a high-soy diet on systemic markers such as BMD or mammary gland markers such as epithelial cell proliferation. Interestingly, PGR immunolabeling in immature lobules was lower in soy-fed animals after menarche, suggesting that soy exposure may in some way diminish ER responsiveness in the type 1 lobule of adolescent breast. This was a modest effect specific to a single compartment, however, and it is unclear how such an alteration in ER activity may relate to differentiation patterns.

Although epidemiologic evidence generally suggests that soy intake may be beneficial for chemoprevention in some populations of women (4, 5), other rodent and cell culture studies have identified soy IF as potential endocrine-disrupting compounds due to ER or hormone modulating actions. Accordingly, there are concerns that exposure to soy during early life or critical stages of development may be potentially harmful for reproductive development (44). Our study showed that pubertal exposure to relatively high dietary levels of soy IF did not alter reproductive hormone concentrations or time to menarche in the macaque model. The IF dose used in the current study was ~60 times greater than those consumed by young girls in Germany (45) and the US (46); they found delayed breast development with higher daidzein intake (45) or urinary excretion (46). The total amount of circulating IF in our study doubled that in a study on Korean girls which reported an association of high soy IF with accelerated breast development (47). Equol was not assessed in those human studies. Here we also found no soy effect on nipple length or the prepubertal uterus. We did observe a transiently higher endometrial area in the SOY group at one time point, but no difference in uterine area or endometrial thickness; in the absence

of a histologic assessment this finding is difficult to interpret. However, we have in several studies shown a lack of uterotrophic effects of dietary IF at up to 500 mg/woman/day equivalent in adult female macaques (24).

Other than the species differences, several factors may explain the neutral or less-profound effect of IF in our study compared to the previous findings in rodents (44). One factor could be the difference in timing of exposure; in-utero/neo-natal may be a more sensitive period for modulation such as via epigenetic modification (48). Another is the delivery method, wherein prior rodent studies often used parenteral administration as opposed to dietary dosing strategy that mimics soy consumption in humans. Many studies also utilized genistein as purified aglycone which may elicit a different effect than the genistein, daidzein, and glycitein mixture of soy IF consumed within a protein matrix. In addition, equol was the predominating isoflavonoid found in our study. Equol is a natural product of daidzein metabolism by gut flora with distinct biological properties from genistein and daidzein (49). Notably, only ~30% of adult non-Asian and non-vegetarian populations have the ability to produce this metabolite (50), and human equol production, when present, is generally less robust than in rodents and macaques. This phenotype could potentially account for the differences between our findings and other studies.

Our results suggest that exposure to a soy diet beginning at puberty does not have overt effects on pubertal growth and development. If anything, pubertal soy exposure may have a subtle effect in enhancing mammary gland differentiation following menarche. Future studies are needed to determine whether such a phenotype may influence breast cancer risk later in life.

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**Authors' Contributions**

Conception and design: JMC, CEW, TCR

Development of methodology: JMC, CEW, CJL, TCR, FND

Data acquisition: FND, CJL, AAF

Histopathology: CJW, JMC

Analysis and interpretation of data: FND, JAT, JMC

**Table 1.** Gene expression of differentiation markers in the mammary gland of female cynomolgus macaques fed a high-soy (SOY) or casein/lactalbumin (CL)-based diet, as measured by qRT-PCR[a].

| Gene | Stage Effect | Diet Effect | Diet | Pubertal Stage | | Correlation with lobular maturation |
|---|---|---|---|---|---|---|
| | | | | Pre-menarche | Post-menarche | |
| *CSN1S1* | *P*<0.0001 | *P*=0.73 | CL | 1.00 (0.65, 1.53) | 5.23 (3.92, 6.99)[b] | r=0.36, *P*=0.004 |
| | | | SOY | 1.11 (0.77, 1.59) | 6.07 (4.75, 7.76)[c] | r=0.35, *P*=0.0003 |
| *ELF5* | *P*<0.0001 | *P*=0.22 | CL | 1.00 (0.74, 1.34) | 2.89 (2.39, 3.49)[b] | r=0.36, *P*=0.004 |
| | | | SOY | 1.16 (0.90, 1.49) | 4.51 (3.84, 5.31)[c] | r=0.37, *P*=0.0001 |
| *MUC1* | *P*=0.01 | *P*=0.63 | CL | 1.00 (0.60, 1.67) | 2.12 (1.40, 3.21) | r=0.23, *P*=0.07 |
| | | | SOY | 0.75 (0.49, 1.15) | 1.66 (1.17, 2.36) | r=0.29, *P*=0.004 |
| *STAT5A* | *P*=0.002 | *P*=0.61 | CL | 1.00 (0.80, 1.24) | 1.44 (1.26, 1.66) | r=0.19, *P*=0.13 |
| | | | SOY | 0.92 (0.77, 1.11) | 1.87 (1.66, 2.10)[b] | r=0.39, *P*=<0.0001 |
| *STAT5B* | *P*=0.0003 | *P*=0.48 | CL | 1.00 (0.84, 1.19) | 1.44 (1.29, 1.60) | r=0.14, *P*=0.27 |
| | | | SOY | 0.96 (0.83, 1.11) | 1.81 (1.65, 1.99)[b] | r=0.30, *P*=0.002 |

[a] Values are presented as fold-change of LSM (LSM-SEM, LSM+SEM) from pre-menarchal CL group;*n*=3-11 (CL) and 4-17 (SOY). Letter superscripts indicate difference from pre-menarchal relative expression with *P*<0.01 (b) or *P*<0.001 (c) by LSM Tukey's HSD test.

**Figure 1.**

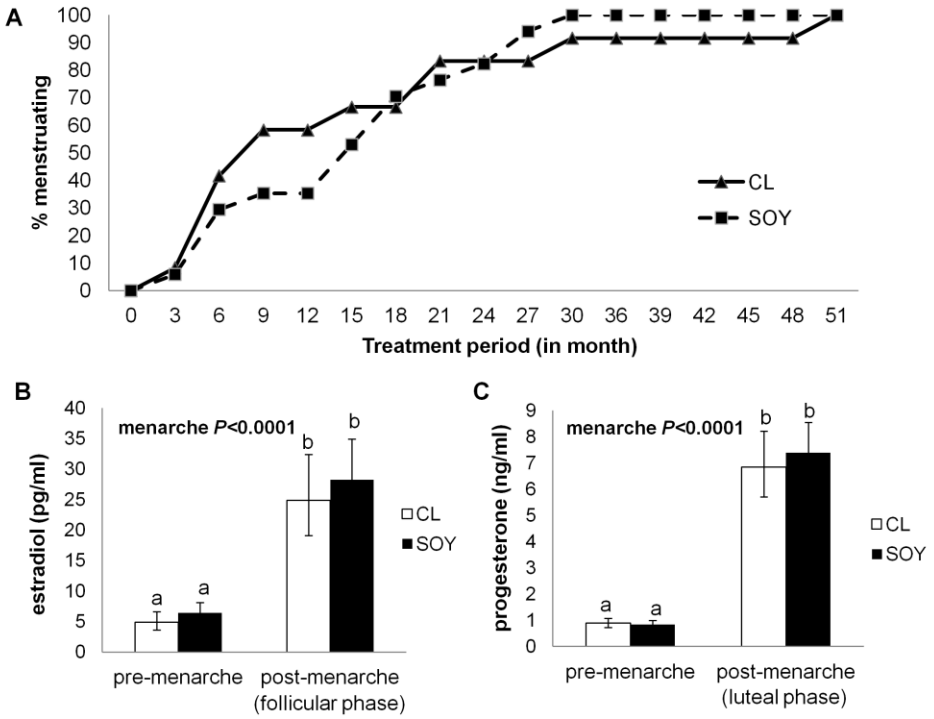


**Figure 1.** Onset of menarche and circulating reproductive hormones in cynomolgus macaques fed a high-soy (SOY) or casein-lactalbumin (CL)-based diet. A: The proportion of post-menarchal monkeys across the study period did not differ by diet. B,C: Serum concentrations of estradiol (B) and progesterone (C) were higher after menarche, with no dietary effect. Values are LSM for *n*=11-17 monkeys/group (error bars=SEM). The significant main effect is indicated in each panel. [a,b] Labeled means without a common letter differ ($P<0.0005$) by LSM Tukey's HSD test. Body weight was included in the model as a covariate.

Figure 2.

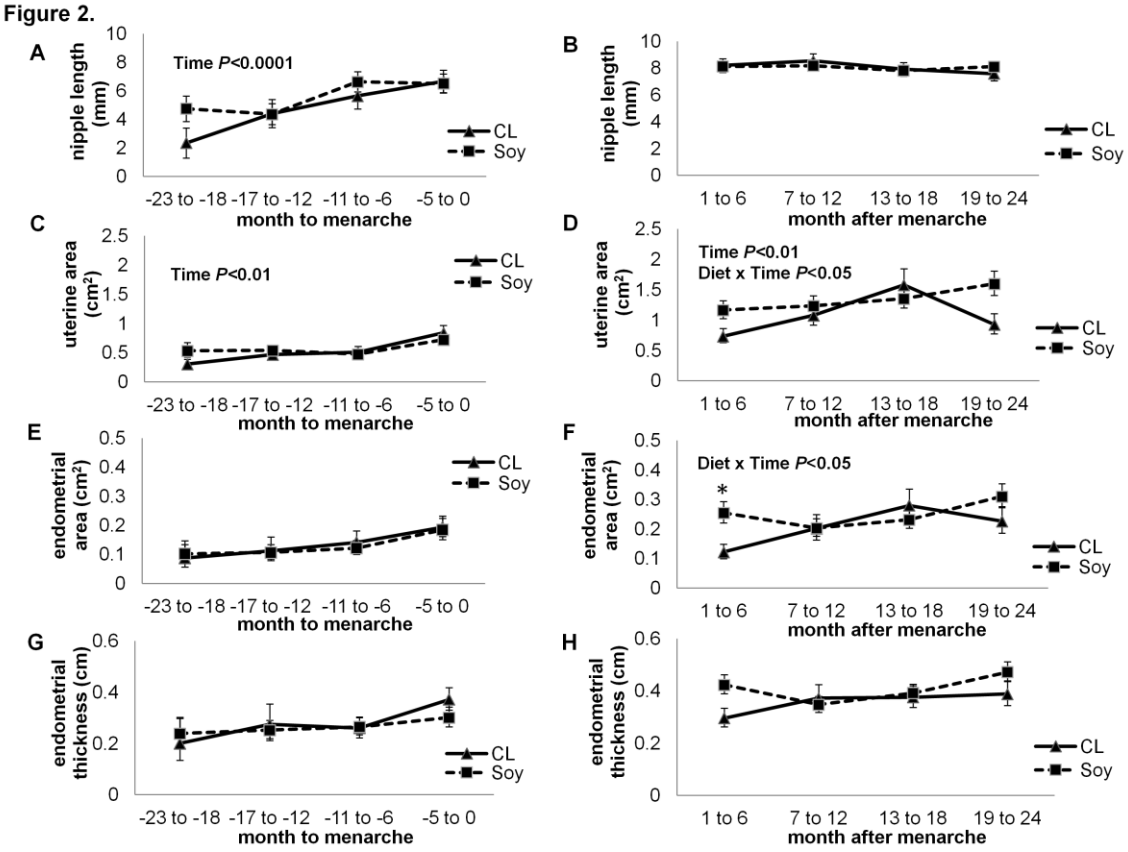


**Figure 2.** Measurement of puberty markers before (A,C,E,G) and after (B,D,F,H) menarche in cynomolgus macaques fed a soy (SOY) or casein-lactalbumin (CL) diet. A-B: Nipple length showed a significant increase prior to menarche independent of diet. C-D: Uterine area increased over time with no main effect of diet. E-H: Endometrial area (E,F) and thickness (G,H) did not significantly change over time albeit an increasing pattern. Values are LSM for *n*=4-12 (CL) or 5-17 (SOY), error bars=SEM. The significant main effect and interaction are indicated in each panel. Asterisk (*) indicates $P<0.05$ with LSM Tukey's HSD test for SOY compared to CL. Body weight was included in the model as a covariate except in nipple length analysis. Post-menarchal analysis also included menstrual cycle day (i.e. day 1-9 or after) in the model as a covariate.

Figure 3.

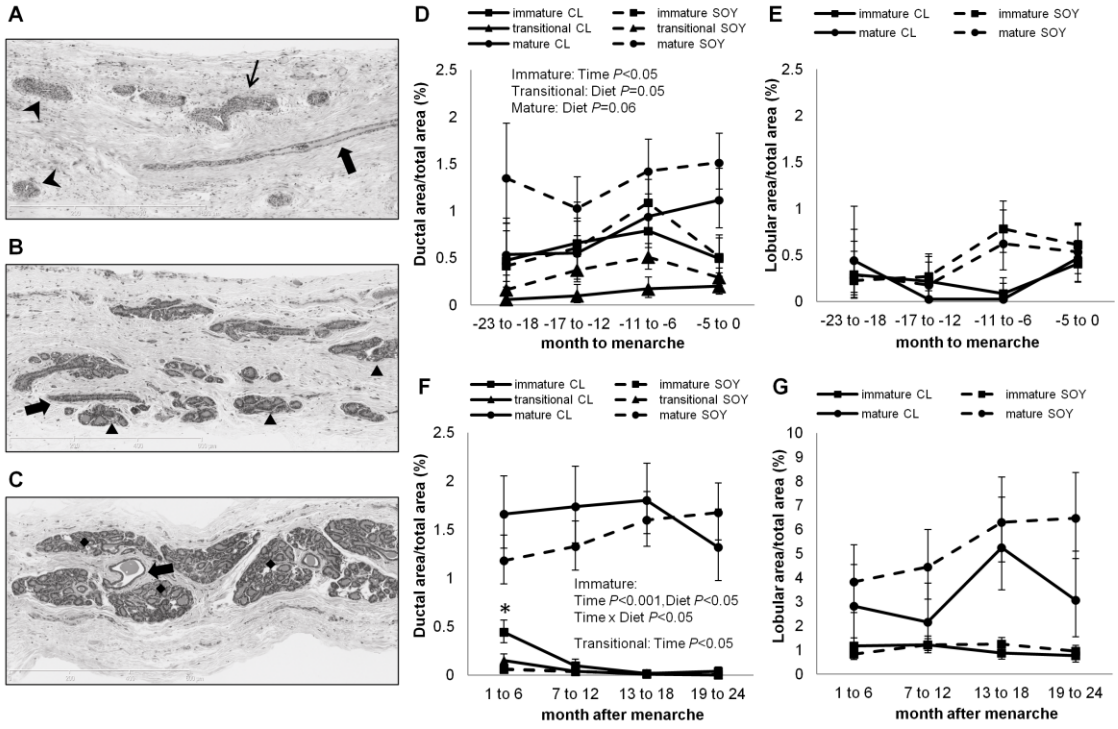


**Figure 3.** Pubertal mammary gland development of cynomolgus macaques fed a high-soy (SOY) or casein-lactalbumin (CL)-based diet. A-C: Pre-menarchal breast (A) composed of mostly ductal structures (immature, transitional, and mature types indicated as arrowheads, thin arrows, and thick arrows, respectively). Immature lobules (▲) were abundant approaching menarche (B) while mature lobules (◆) predominated post-menarche (C) (H&E staining, 10X magnification). D: Immature ducts were the only structures that showed a significant rise prior to menarche. E: There was no significant change in lobular area across pre-menarche. F: The proportion of immature and transitional ducts significantly decreased following menarche. G: The post-menarche breast contained primarily mature lobules. Values are LSM for $n$=4-12 (CL) or 5-17 (SOY), error bars=SEM. The significant main effects and interactions are indicated in each panel. Asterisk (*) indicates s difference between SOY and CL ($P$<0.01) by LSM Tukey's HSD test. Body weight and menstrual cycle stage (post-menarche) were included in the model as covariates.

Figure 4.

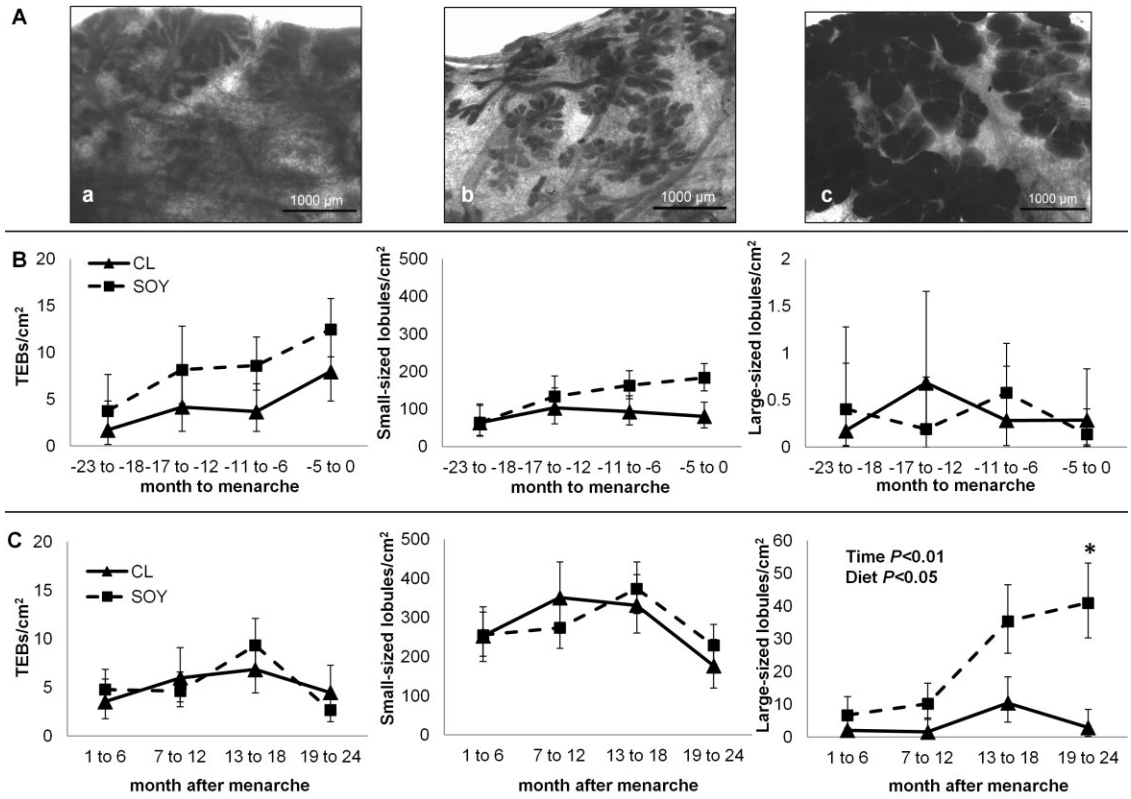


**Figure 4.** Quantification of TEBs and lobular structures in the breast of cynomolgus macaques fed a high-soy (SOY) or casein-lactalbumin (CL) diet across the pubertal transition. A: Whole mount images of the breast (toluidine blue staining) predominated by TEBs (a), small-sized lobule (b), and large-sized lobule (c). B: Before menarche, the lobular number did not differ between diet groups. C: After menarche, the amount of large-sized lobules significantly increased and was higher in the SOY group. Values are LSM for *n*=4-12 (CL) or 5-17 (SOY), error bars=SEM. The significant main effects are indicated in each panel. Asterisk (*) indicates a difference between SOY and CL ($P<0.05$) by LSM Tukey's HSD test. Body weight and menstrual cycle stage (post-menarche) were included in the model as covariates.

**Figure 5.**

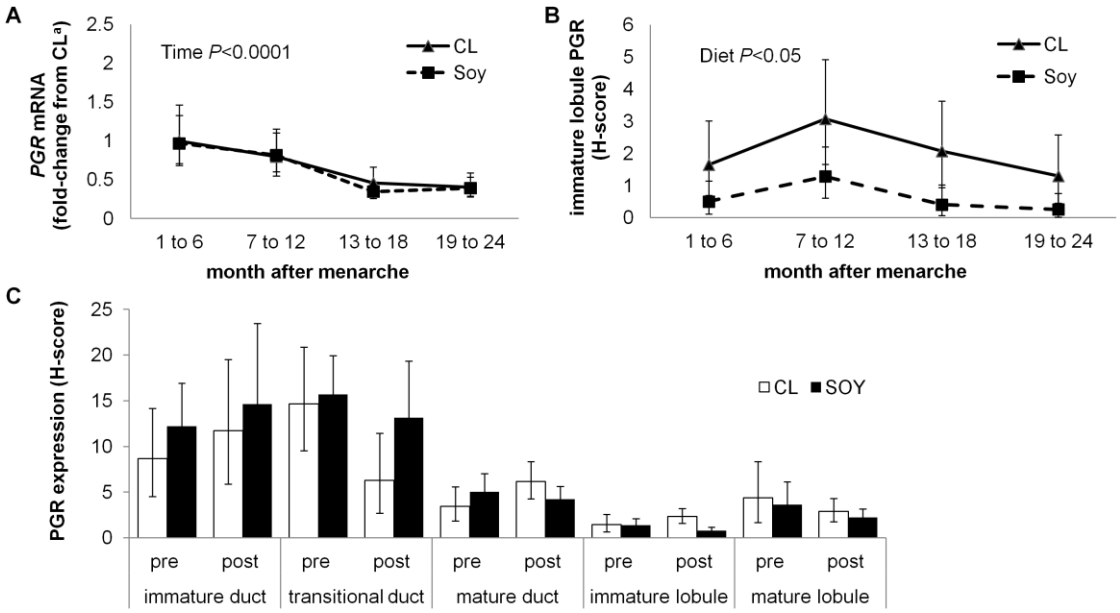


**Figure 5.** Progesterone receptor (PGR) expression as a marker for estrogen activity in the mammary tissue of cynomolgus macaques fed a high-soy (SOY) or casein-lactalbumin (CL) diet across the pubertal transition. A: mRNA expression of PGR decreased after menarche. B: Protein expression of PGR in the immature lobules of the breast was lower in the SOY group after menarche. C: Immunolocalization of PGR in the breast was similar before (pre) and after (post) menarche and not affected by diet. Values are presented as LSM and error bars=SEM. [a] Fold-change from CL at 1-6 months after menarche. The significant main effect is indicated in each panel. Body weight and cycle stage were included in the model as covariates.

## SUPPLEMENTARY MATERIAL

**Table S1.** Composition of diets used to assess the effect of high-soy diet on the breast of female cynomolgus macaques across the pubertal transition.

| INGREDIENT | Diet | |
|---|---|---|
| | CL[a] | SOY |
| | *g/100 g diet* | |
| Casein, USP | 8.50 | 1.30 |
| Lactalbumin | 8.50 | 1.30 |
| Soy Protein Isolate[b] | | 14.00 |
| DL-methionine | | 0.30 |
| Wheat Flour, self-rising | 35.76 | 35.76 |
| Dextrin | 9.00 | 8.90 |
| Sucrose | 7.00 | 7.00 |
| Alphacel | 8.03 | 8.72 |
| Lard | 5.00 | 5.00 |
| Beef Tallow | 4.00 | 4.00 |
| Butter, lightly salted | 3.10 | 3.10 |
| Safflower Oil (linoleic) | 3.00 | 2.56 |
| Crystalline Cholesterol | 0.061 | 0.061 |
| Vitamin Mixture[c] | 2.50 | 2.50 |
| Mineral mixture[d] | 5.00 | 5.00 |
| Calcium Carbonate | 0.400 | 0.357 |
| Calcium Phosphate, Monobasic | 0.150 | 0.145 |

[a] CL, Casein and lactalbumin-based diet (as control)

[b] Isolated soy protein with isoflavones (provided by Solae, LLC) containing 1.11 mg genistein, 0.48 mg daidzein, and 0.08 mg glycitein per gram of isolate (as aglycones). Soy protein isolate amount was determined to provide daily isoflavones (IF) dosing of 10 mg/kg body weight, which was the human equivalent of 120 mg/day of IF (in aglycone equivalents) assuming an average energy intake of 7530 kJ/d.

[c] Complete vitamin mixture (made by Harlan Laboratories) consisted of (g/kg diet): 0.045 retinyl palmitate; 0.0625 *dl*-α-tocopheryl acetate; 0.125 iso-inositol; 0.025 riboflavin; 0.05625 menadione sodium bisulfite complex; 0.125 *para*-aminobenzoic acid; 0.1125 niacin; 0.025 pyridoxine hydrochloride; 0.025 thiamine hydrochloride; 0.075 calcium pantothenate; 0.03375 vitamin B-12 in manitol; 0.0005 biotin; 0.00225 folic acid; 2.25 ascorbic acid; 1.875 choline chloride; 0.005 cholecalciferol; and 20.15725 dextrose monohydrate.

[d] Mineral mixture without Ca and P (made by Harlan Laboratories) provided (g/kg diet): 15.696 $K_2CO_3$; 8.119 NaCl; 7.195 $MgSO_4(H_2O)_7$; 0.3934 $FeSO_4$; 0.0676 $MnSO_4(H_2O)$; 0.0455 $ZnCl_2$; 0.0145 $CuSO_4$; 0.0039 KI; 0.0024 $(CH_3CO_2)_7Cr_3(OH)_2$; 0.0012 NaF; 0.0002 $Na_2SeO_3$; and 18.463 dextrin.

**SUPPLEMENTARY MATERIAL**

**Table S2.** Primer-probe sets for target genes evaluated by qRT-PCR.

| Gene ID | NCBI Refseq | Species | ABI Assay ID |
|---|---|---|---|
| *GAPDH* | DQ464111 | *Mf* | (custom)[a] |
| *ACTB* | DQ464112 | *Mf* | (custom)[b] |
| *MKI67* | NM_002417.3 | *Hs* | Hs00606991_m1 |
| *PGR* | NM_000926 | *Hs* | Hs00172183_m1 |
| *CSN1S1* | NM_001890 | *Hs* | Hs00157136_m1 |
| *MUC1* | NM_001018016 | *Hs* | Hs00159357_m1 |
| *ELF5* | NM_001194687 | *Mm* | Rh02838633_m1 |
| *STAT5A* | XM_001109557 | *Mm* | Rh02844604_m1 |
| *STAT5B* | NM_012448 | *Hs* | Hs00560035_m1 |

*Hs*, *Homo sapiens*; *Mf*, *Macaca fascicularis* (cynomolgus macaque); *Mm*, *Macaca mulatta* (rhesus macaque). [a]*GAPDH* (5'-3') TGCCCTCAATGACCACTTTGTC (forward), ACCCTGTTGCTGTAGCCAAAT (reverse), TCGTTGTCATACCAGGAAAT (probe). [b]ACTB (5'-3') ACCCCAAGGCCAACCG (forward), CCTGGATGGCCACGTACATG (reverse), AAGATGACCCAGATCATG (probe).

SUPPLEMENTARY MATERIAL

Figure S1.

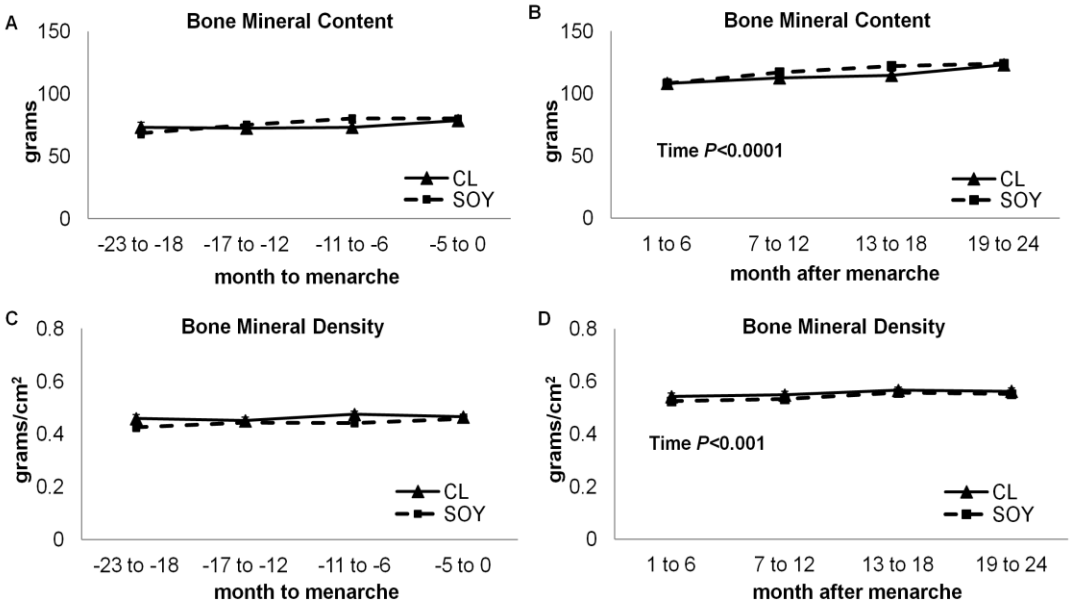


**Figure S1.** Bone mineral content (BMC) and bone mineral density (BMD) in the female cynomolgus macaque fed a soy or casein lactalbumin (CL) diet during pubertal development as measured by DEXA. A,C: During pre-menarche, BMC (A) and BMD (C) did not change significantly over time. B,D: After menarche, BMC (B) and BMD (D) increased over time regardless of dietary treatment. Values are LSM for *n*=4-12 (CL) or 5-17 (SOY), error bars=SEM. The significant main effects are indicated in each panel. Body weight was included in the model as a covariate.

**SUPPLEMENTARY MATERIAL**

**Figure S2.**

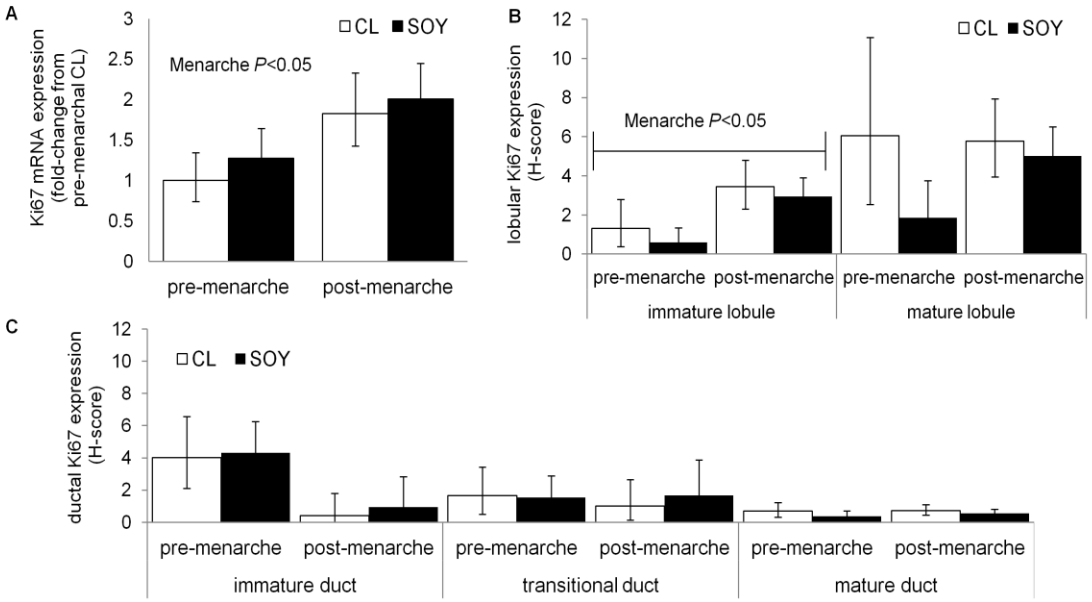


**Figure S2.** Epithelial cell proliferation in the mammary tissue of cynomolgus macaque was not affected by soy treatment across the pubertal transition. A: mRNA expression of proliferation marker Ki67 did not differ by diet, B,C: Protein expression (by immunohistochemistry) in the lobules (B) and (C) ductal structures. Immature lobule was the only structure that showed significantly higher localization of Ki67 at post-menarche relative to pre-menarche. Values are presented as least square means (LSM) and error bars are SEM. The significant main effect is indicated in each panel. Body weight and menstrual cycle stage (post-menarche) were included in the model as covariates.

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# CHAPTER 2

## Effect of pubertal exposure to dietary soy on estrogen receptor-responsiveness in the breast of cynomolgus macaques

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Fitriya N. Dewi performed the experiments, completed the statistical analyses and data interpretation, and prepared the manuscript. Charles E. Wood provided advice on the overall experiment and manuscript preparation. Cynthia J. Willson conducted the steroidogenic enzymes immunohistochemistry experiment. Cynthia J. Lees performed the breast biopsy. Thomas C. Register and Timothy D. Howard contributed in the development of assays. Zhiqing Huang and Susan K. Murphy generated pyrosequencing data. Janet A. Tooze provided guidance on the statistical approach. Lance D. Miller provided guidance on gene expression microarray experiment and analysis. Jeff W. Chou performed microarray data analysis. J. Mark Cline was the principal investigator of the study, and provided guidance throughout the project.

**Effects of pubertal exposure to dietary soy on estrogen receptor-responsiveness in the breast of cynomolgus macaques**

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**Abstract**

Endogenous estrogens are primary determinants of mammary gland development during puberty and breast cancer risk later in life. Soy foods contain the phytoestrogen isoflavones (IFs), which can elicit mixed estrogen agonist and antagonist effects, and timing of exposure may influence the health effects of soy. In this study, we evaluated mammary gland samples collected serially from pubertal female cynomolgus macaques fed either a control (casein/lactalbumin-based) diet ($n$=12) or a soy protein-based diet containing the human-equivalent dose of 120 mg IF/day ($n$=17) for ~4.5 years spanning menarche. We assessed estrogen receptor (ER) expression, activity, and promoter methylation, and markers of estrogen metabolism. Expression of ERα (*ESR1)* and classical ER response genes *(TFF1, PGR* and *GREB1*) decreased with maturity. Correlation between mRNA expression and promoter methylation was generally low for these genes, with an inverse relationship observed only for *TFF1*. ERβ expression was lower in the ductal structures of soy-fed animals ($P$<0.05). ERα mRNA was also lower with soy only after menarche ($P$<0.05), showing no diet effect on the protein expression or methylation of the specific promoter sites examined. Expression of GATA-3, an ERα-regulated luminal epithelial differentiation marker, correlated with ERα expression and appeared to be affected by soy. GREB1 was the only ER-activity marker that showed lower expression with soy after menarche. Expression of markers for estrogen-metabolizing enzymes did not differ by diet. Our results indicate that ER expression and activity decreases across puberty, independent of diet. Pubertal dietary soy exposure may exert transient effects on ER transcription after menarche, with subtle effects on differentiation and ER activity.

**Introduction**

Estrogen signaling plays a central role in regulation of normal cell growth and development of the mammary gland, and also influences progression of carcinogenesis (1). The biological effects of estrogen are primarily achieved through binding to Estrogen Receptors (ERs), which are ligand-regulated transcription factors consisting of subtypes $\alpha$ and $\beta$ (2). Isoflavones (IFs), an important bioactive component of soy foods, are also ligands to ERs. IFs exert mixed estrogen agonist-antagonist properties and tissue-specific physiologic effects, and thus are often categorized as a natural selective ER modulators (3). This characteristic along with others (4), have made soy the subject of interest in breast cancer research for the past two decades. Epidemiologic evidence suggests that soy intake is inversely associated with breast cancer risk, mortality, and recurrence (5, 6, 7) with unclear mechanism to explain the protective effect. Many in-vitro studies points to IF-estrogen interactions as the key path of soy action, mainly via ER-mediated estrogenic/anti-estrogenic effect, antiproliferation, and stimulation/inhibition of estrogen synthesis and metabolism (4). More recently, studies show that IF can also alter DNA methylation (8, 9), affecting transcription of various genes including those important to breast cancer (10, 11). Whether the epigenetic modulation by IF also involves genes associated with estrogen regulation is unclear.

Emerging evidence indicates that the early-life environment can establish trajectories of breast cancer risk extending into adulthood (12). Pre-puberty and adolescence are indicated as important windows for nutritional modulation to reduce later susceptibility to cancer (13, 14), possibly related to puberty being a significant event for mammary gland development. Prior studies using rodent models prepubertally exposed to genistein suggests that IFs may interact with estrogen to alter breast differentiation, proliferation, and epithelial cell fate (15, 16, 17). It is not understood, however, whether dietary soy intake at puberty can elicit similar effects in the human breast. The gaps in knowledge may be due to the methodological and ethical limitations for interrogating soy effects on the breast of healthy pubertal girls. Here we used a well-

characterized primate model with highly comparable genetic, endocrine and breast development profiles to humans to comprehensively assess dietary soy effects on ER activity and estrogen regulation in the breast across pubertal development.

## Materials and Methods

### *Diet and Animals*

All procedures involving the animals were performed at the Wake Forest School of Medicine, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Procedures were conducted in compliance with state and federal laws and standards of the US Department of Health and Human Services and approved by the Wake Forest University Animal Care and Use Committee.

This study utilized breast tissues collected from twenty-nine female cynomolgus macaques (*Macaca fascicularis*) during their pubertal development. The experimental design has been described previously (Study Design, Figure 1, page 30). Briefly, animals were obtained from the Institut Pertanian Bogor at the age of approximately 1.5 years and were randomized on the basis of body weight to receive one of two diets for ~4.5 years: (i) a control diet with casein and lactalbumin as the protein source (CL, $n$=12) or (ii) a diet with isolated soy protein containing IF (provided by Solae, LLC.) (SOY, $n$=17) with the human equivalent of 120 mg/day of IF. Throughout the study, all animals were swabbed daily for vaginal bleeding; the onset of menarche was defined as the initiation of regular monthly vaginal bleeding.

### *Breast Biopsy*

Serial breast biopsy samples were collected every six months spanning the period of pubertal development, using methods described previously (18). Briefly, animals were anesthetized with ketamine and xylazine, and a small sample of mammary gland was removed from a pre-selected breast quadrant by an experienced veterinarian. Biopsies were oriented

radially from the nipple to minimize effects on adjacent mammary gland structures. The site from which the tissue was removed was tattooed to prevent resampling at the same site. Each biopsy sample was divided; one half was frozen for biomolecular work, and the other half was fixed, trimmed, embedded in paraffin and sectioned for immunohistochemistry.

To control for the high inter-individual variation of puberty onset as in humans, all outcomes were compared between monkeys of similar developmental stage across the pubertal transition. Thus, after completion of the experiment we were able to categorize the biopsy samples into 8 timepoints based on their interval to the onset of menarche; from 18-23 months pre-menarche up to 19-24 months post-menarche. At each biopsy, a blood sample was also collected for assessment of circulating genistein, daidzein and the metabolite equol. Measurement was done using liquid chromatography electrospray ionization mass spectrometry at the laboratory of Dr. Adrian Franke (University of Hawaii Cancer Center), as described elsewhere (19). The serum IF levels at each timepoint are presented in Supplementary Figure 1.

***Quantitative Gene Expression***

Total RNA was extracted from frozen mammary tissues using Tri Reagent (Molecular Research Center, Cincinnati, OH) and purified using RNeasy Mini kit (Qiagen, Valencia, CA, USA). Quantitative real-time reverse transcriptase PCR (qRT-PCR) was used to measure mRNA expression of ERs (ERα, *ESR1*; ERβ, *ESR2*), classical estrogen-induced genes (trefoil factor 1, *TFF1*; growth regulation by estrogen in breast cancer 1, *GREB1*; progesterone receptor A & B; *PGR-A, PGR-B*; Insulin-like growth factor binding protein 2, *IGFBP-2*), epidermal growth factor receptor (*EGFR*), steroidogenic enzymes (steroid sulfatase, *STS;* aromatase, *CYP19*; estrogen sulfotransferase (EST) family 1E, *SULT1E1*; hydroxy steroid (17β) dehydrogenase 1 and 2, *HSD17B1* and *HSD17B2*) and enzymes for estrogen metabolism (*CYP1A1*, *CYP1B1*, and *CYP3A4*) using standard methods described previously (20). qRT-PCR reactions were performed on ABI PRISM® 7500 Fast Sequence Detection System (Applied Biosystems, Foster City,

CA), and relative expression was determined using the ΔCt method calculated by ABI Relative Quantification 7500 Software v2.0.1 (Applied Biosystems, Foster City, CA). Human or macaque-specific Taqman primer-probe assays were used as targets (Supplementary Table 1) and samples were normalized to mean values for housekeeping genes (GAPDH and ACTB) generated using cynomolgus macaque-specific primer-probe sets.

***Immunohistochemistry***

We assessed immunolocalization of ERs and GATA-3, a marker for luminal differentiation, in mammary gland epithelial cells on tissue sections from a subset of samples (timepoints 0-11 months before menarche, and 7-12 and 19-24 months after menarche) using a biotin-streptavidin staining method previously described (18). Monoclonal antibodies used were 1:15 anti-ERα (NCL-ER-LH1, Novocastra Labs, Newcastle-upon-Tyne, UK), 1:40 anti-ERβ (Clone 14C8, Thermo Scientific, Rockford, IL), and 1:50 anti-GATA-3 (Clone HG3-31: sc-268, Santa Cruz Biotechnology Inc., Santa Cruz, CA). Cell staining was quantified by a computer-assisted technique with grid filter where positively stained cells were scored based on staining intensity (+1, +2, or +3) to obtain a semi-quantitative measurement of staining intensity and distribution by H-score calculation (18). Based on the glandular features that changed across the menarchal transition, H-score data for immature/transitional ducts and mature lobules was limited to pre-menarchal or post-menarchal timepoints, respectively. Morphologic criteria for immature, transitional, and mature mammary gland structures are described elsewhere (21). Data for expression in mature ducts and immature lobules were obtained for both pre and post-menarche timepoints.

To evaluate changes in steroidogenic enzyme protein expression with respect to pubertal breast development, biopsies from two timepoints were used for immunohistochemistry: 12-17 months pre and post menarche. Antibodies and dilutions were used as follows: estrogen

sulfotransferase (EST) rabbit polyclonal (1:100, Biorbyt, Riverside, UK); HSD17B1 rabbit monoclonal (1:50, Epitomics, Burlingame, CA); HSD17B2 rabbit polyclonal (1:100, Proteintech, Chicago, IL); and STS rabbit polyclonal (1:100, Sigma-Aldrich, St. Louis, MO). Staining was scored qualitatively based on intensity (0, +1, +2, +3) for different tissue types (epithelial vs. stromal) by a veterinary pathologist (CJW) in consultation with a board-certified veterinary pathologist (JMC), and descriptive results are presented.

***Pyrosequencing***

We used breast biopsy samples from timepoints 0-5 months pre-menarche, and 7-12 and 19-24 months post-menarche for assessment of promoter methylation. Genomic DNA was extracted from the frozen biopsy specimens using DNeasy Kit (Qiagen, Valencia, CA). Extracted DNA was treated with sodium bisulfite using the Zymo EZ DNA Methylation Kit (Zymo Research, Irvine, CA) to convert unmethylated cytosines to uracils. Cynomolgus macaque-specific pyrosequencing assays (Supplementary Table 2) were designed for CpG sites around/near the Estrogen Responsive Element (ERE) of TFF1, GREB1 and PGR (half-site ERE), and CpG islands in the promoter regions of ESR1 (promoter B) and ESR2 (up to 300 bp upstream of transcriptional start site/TSS). The assays were designed using PyroMark Assay Design software (Qiagen, Valencia, CA), and the pyrosequencing was performed on Qiagen PyroMark Q96 MD Pyrosequencer with Pyro Q-CpG software (Qiagen, Valencia, CA) (22) at the Duke Epigenetics Research Laboratory, Duke University Medical Center.

***Gene microarray***

Breast biopsy samples from a subset of animals (n=4/group) from two timepoints (7-12 and 19-24 months after menarche) were used for microarray analysis utilizing the Affymetrix GeneAtlas System (Affymetrix, Santa Clara, CA). Extracted RNA was assessed for quality and integrity using a Nanodrop ND-2000 UV–VIS spectrophotometer (NanoDrop, Wilmington, DE)

and Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). The Ambion WT Expression kit (Life Technologies, Gaithersburg, MD) was used to generate sense-strand cDNA, and fragmentation and labelling of the cDNA was done using the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix, Santa Clara, CA). Samples were hybridized to Rhesus Gene 1.1 ST WT Array Strips. Microarray assays were done at the Microarray Shared Resource of the Comprehensive Cancer Center of Wake Forest University. Microarray data analysis was done using Partek Genomics Suite and the Limma package for R; RMA-normalized data were analyzed for difference in expression over time using paired t-test, and the expression was also compared by diet group using empirical Bayes at the two different timepoints.

Gene Set Enrichment Analysis (GSEA) was performed using GSEA-preranked method in the GSEA software version 2.0.13 with default parameters (23) on the gene lists generated from pair-wise comparisons by time (in each diet group). Using Batch SOURCE (http://puma.princeton.edu/cgi-bin/source/sourceResult), we aligned gene symbols from our dataset with human UniGene symbols; those that did not correspond with human genes were excluded. Each list consisted of 16,915 genes, and was ranked based on fold-change. The search was performed against curated KEGG gene sets available from molecular signature database (MsigDB) v4.0 (24). Enriched gene sets with false discovery rate (FDR) < 5% were considered significant.

***Statistical Analysis***

Logarithmic or square-root conversions were done where appropriate to improve normality of the residuals. Data were back transformed to original scale for presentation of results; values are presented as least square means (LSM) $\pm$ standard error of the mean (SEM) or LSM (LSM-SEM, LSM+SEM) when standard errors were asymmetric. All analyses were done across pubertal transition, and separately for pre- and post-menarche. For post-menarche, the menstrual cycle stage of the animals (i.e. follicular or luteal) during each biopsy was determined

retrospectively based on their menstrual bleeding calendar, and used as a covariate. We used JMP (version 10.0.0, SAS Institute; Cary, NC) to fit a mixed model ANOVA with a random animal effect to model main and interactive effects of diet and time adjusted for body weight and menstrual cycle stage (post-menarche) to estimate and compare differences in mRNA relative expression, protein expression, and methylation ratios in the breast, in the SOY and CL groups over time. Multiple pairwise comparisons were done with Tukey's Honestly Significant Difference (HSD) Test. Relationships between mRNA and protein or DNA methylation levels were examined by Spearman's rank correlation.

## Results

### *Estrogen receptors*

ESR1 mRNA (Figure 1A) decreased across the pubertal transition ($P<0.05$), and was inversely-associated with body weight ($P<0.001$). The expression was lower in the SOY group only after menarche ($P<0.01$ for diet effect), and there was an effect of cycle stage whereby the expression was higher in the follicular phase ($P<0.05$ vs. luteal). The protein expression of ERα in immature ducts showed a diet x time interaction ($P<0.05$) before menarche (Figure 1B). Following menarche, ERα expression in the mature duct ($P<0.05$) and immature lobules ($P=0.07$) decreased with time independent of diet, showing positive correlation with mRNA expression (Spearman's $\rho=0.25$, $P<0.05$ for immature lobules; Spearman's $\rho=0.50$, $P<0.0001$ for mature ducts). No diet or time effect was observed on ERα expression in transitional ducts or mature lobules. We also assessed methylation level of CpG sites around the *ESR1* promoter region B (Figure 1C) and found no effect of diet. Regardless of treatment, the methylation level was very low across the pubertal development; 2 of 4 CpG sites showed transient but significant increase over time.

For ERβ, we observed a main effect of diet on ESR2 mRNA expression across the pubertal transition ($P<0.05$) but the expression did not differ with time (Figure 2A). Before menarche, there was a marginal effect of diet ($P=0.08$) whereby the expression was lower in the SOY group. After menarche, however, diet and time effects were not observed. There was a main effect of diet on ERβ immunolocalization (Figure 2B) in the transitional duct before menarche ($P<0.05$), and on the mature duct after menarche ($P<0.05$) whereby SOY showed lower expression. Similar to *ESR1*, CpG methylation within 300 bp upstream of *ESR2* TSS was very low (<3%), and the means did not differ with diet or time (Figure 2C). Only one CpG site showed increasing methylation with time ($P<0.01$).

***Estrogen receptor activity markers***

Relative expression of ER-regulated markers *TFF1, PGR-A PGR-B,* and *GREB1* decreased across the pubertal transition ($P<0.001$ for time effect in all genes) (Figure 3A-D). For *TFF1* and *PGR*, however, the significant time effect disappeared when body weight was included in the model as a covariate. *TFF1*, *PGR-A* and *PGR-B* significantly decreased after menarche ($P<0.05$ for time effect) independent of diet, which was partially mediated by the change in body weight. *GREB1* expression marginally decreased after menarche ($P=0.08$); GREB1 was the only marker that showed a significant main effect of diet whereby the expression was lower in the SOY group post-menarche ($P<0.05$ vs. CL). Menstrual cycle had significant effect on *TFF1* ($P<0.01$), *GREB1* ($P<0.05$), and *PGR-B* ($P<0.0001$). The markers were higher in the follicular phase compared to the luteal phase. Body weight had an inverse association with post-menarchal *TFF1* ($P<0.05$), *PGR-A (*$P=0.05$), *PGR-B* ($P<0.01$) and *GREB1* ($P<0.05$). Additionally, we also assessed expression of the estrogen-induced gene *IGFBP2*, and marker for growth factor signaling *EGFR*. These markers did not show a significant change over time and with soy treatment (data not shown).

The pyrosequencing showed a significant time effect on *TFF1* promoter methylation ($P<0.0001$) (Figure 4A). Seven CpG sites flanking the ERE in the promoter showed increasing methylation level with time (by ~5-7%). There was an inverse correlation between *TFF1* methylation and mRNA expression (Spearman's $\rho= -0.31$, $P<0.01$). We also assessed CpG methylation around an ERE located at 1.6 kb upstream of the *GREB1* TSS and did not find a diet or time effect (Figure 4B). Consistently, there was no relationship between methylation of this region with *GREB1* expression. For *PGR* (Figure 4C), the CpG sites within the promoter regions for *PGR-A* and *PGR-B* showed a low level of methylation across pubertal transition (<10% and <5% for *PGR-A* and *PGR-B*, respectively), regardless of dietary treatment. *PGR-A* showed a very transient increase in methylation level with maturity ($P<0.05$ for time effect), and was associated with body weight ($P<0.05$). Among the 17 CpG sites that we assessed within the *PGR-A* promoter, only six sites showed a significant change with time, and one showed diet x time interaction ($P <0.05$). Methylation of *PGR-A* and *PGR-B* did not correlate with their respective mRNA expression.

***Luminal cell differentiation***

We assessed GATA-3 immunolocalization in the epithelial compartment of the mammary gland (Figure 5). GATA-3 was expressed in the luminal epithelium of the ductal and lobular structures throughout pubertal transition. GATA-3 expression in the immature duct did not differ by diet whereas for transitional duct, there was a diet x time interaction ($P<0.05$) reflected by a significant increase across pre-menarche only in the SOY group ($P<0.05$). Pre-menarchal GATA-3 expression in the mature duct and immature lobules showed a marginal increase with time ($P=0.06$ in both structures) independent of diet. After menarche, GATA-3 in the immature lobule was lower with soy relative to CL ($P<0.05$). This effect, however, disappeared when adjusted for menstrual cycle stage. For mature lobules, there was a time effect ($P<0.05$) with a significant diet x time interaction ($P<0.05$) wherein an increase of expression was only observed in the CL group

but not SOY. This effect also disappeared when cycle stage was included in the model. Except in the transitional duct, GATA-3 expression in each compartment was significantly correlated with ERα protein (Spearman's $\rho>0.3$, $P<0.05$), and mRNA expression (Spearman's $\rho>0.2$, $P\leq0.05$).

***Global gene expression***

We did not find a strong gene expression difference between diet groups and across 7-12 months and 19-24 months post-menarche (i.e. when the analysis was adjusted for multiple comparisons). For the purpose of screening for potential new markers, we used the raw *P*-values to identify genes that changed over time in each diet group, and genes that differed by diet at each timepoint. Results from this limited analysis suggested that mammary gland development in CL and SOY across the two timepoints may involve different genes, which were also associated with different KEGG pathways. The results are presented in Supplementary Figure 2.

***Enzymes for estrogen metabolism***

There was no diet effect on mRNA expression of genes involved in estrogen synthesis, bioactivation, and catabolism (Figure 6). A significant effect of time was observed for mRNA expression of several steroidogenic enzyme markers. For example, *SULT1E1* expression changed over time ($P<0.01$), which was driven by a decrease following menarche ($P<0.05$) with an inverse association with body weight ($P<0.01$). *STS* also had an inverse association with body weight ($P<0.01$) and was marginally decreased across the pubertal transition ($P=0.07$). Expression of aromatase (*CYP19)* was generally low with an increase after menarche ($P<0.01$) independent of diet. *HSD17B1* mRNA increased across the pubertal transition, particularly after menarche ($P<0.05$) and was positively associated with body weight ($P<0.05$). Despite an increasing pattern, we did not find a significant time effect on *HSD17B2* expression. No significant time or diet effects were observed for mRNA expression of estrogen metabolizing enzymes *CYP1A1*, *CYP1B1*, and *CYP3A4* (data not shown).

Prior to menarche, stromal immunoreactivity in breast biopsies for EST was diffuse, cytoplasmic, and of moderate intensity. Stromal immunoreactivity generally also was diffuse although weaker in intensity after menarche than before. Epithelial cells in ducts and acini exhibited heterogenous (10-50% of cells), cytoplasmic, moderate immunoreactivity for EST. Similar epithelial staining for EST was observed in post-menarchal tissues, but occasional nuclear staining was also observed at this later time point. For pre-menarchal STS, about half (8/15) animals displayed weak to moderate cytoplasmic staining in up to 75% of epithelial cells, whereas other animals were negative for STS immunoreactivity. Following menarche, only 3/15 animals had STS immunoreactivity in epithelial cells (ducts and acini), and it was generally weak. The stroma was negative for immunoreactivity in all animals at both time points. Immunoreactivity for HSD17B1 was cytoplasmic and most intense but heterogeneous in the myoepithelial cells and stroma immediately surrounding lobules and ducts and was absent in adipocytes. Generally <20% of epithelial cells in either lobules or ducts showed weak cytoplasmic and/or nuclear immunoreactivity for HSD17B1. Occasional vessel walls demonstrated weak to moderate cytoplasmic immunoreactivity for HSD17B1. There was no appreciable trend in HSD17B1 protein expression between pre- and post-menarche. Similar to HSD17B1, HSD17B2 protein expression between the two time points showed no appreciable trend. Immunoreactivity for HSD17B2 was cytoplasmic and diffusely intense in stromal cells and adipocytes. There was weak cytoplasmic staining in generally <10% of epithelial cells of either ducts or lobules. Endothelial cells exhibited moderately intense, heterogeneous cytoplasmic staining of HSD17B2.

**Discussion**

Here we evaluated the effects of dietary soy, which contains phytoestrogens, on ER-dependent activity in the pubertal breast. Our findings indicate that during the pubertal transition, estrogen responsiveness in the breast decreased with maturity independent of diet. Enzymes for estrogen synthesis and bioactivation also changed across the pubertal transition but were not influenced by diet. Dietary soy treatments resulted in downregulation of ER transcription after menarche, which appeared to be independent of ER promoter methylation at the sites examined; this change occurred alongside a decrease in GATA-3 expression. Further, GREB1, which is a classic estrogen-induced marker showed lower mRNA expression in the post-menarchal breast of soy-fed animals, suggesting a mild buffering effect of soy after menarche.

Estrogens are key regulators of mammary gland morphogenesis during development, and exposure to this hormone is a risk factor for breast cancer. Estrogen is mainly produced in the ovaries starting at puberty and, in combination with progestogenic and growth factor signals, results in a dramatic change of the mammary gland (25). In humans (26) and macaques (21), a large increase in mammary lobular differentiation can be seen during menarche. The biological effect of estrogen is mediated by ER, a ligand-activated transcription factor that belongs to the nuclear receptor superfamily. Classic ER signaling works through the binding of ligand to ER; the ligand-ER complexes will dimerize and bind to Estrogen Response Elements (ERE) in the DNA and recruit coregulators to activate gene transcription (2, 27). In the human breasts, ERs are present from the fetal stage onwards but little is known about the dynamics of the expression and functionality across childhood and pubertal development (28). These unique features of postnatal mammary gland development suggest that adolescence may be a critical period of susceptibility to potential hormonal disruption, particularly by environmental estrogens. This disruption may occur through both direct ER interactions and alterations of estrogen metabolism. Serum estradiol levels are correlated with palpable breast albeit a very low endogenous estrogen level detected in normal pre-pubertal children (29). Supporting this, an autopsy study showed that ER$\alpha$ mRNA

level was higher in the breast of pre-menarchal compared to peri-menarchal girls (30) with a similar finding also reported in monkeys (31). To our knowledge, our current report is the first to longitudinally illustrate the developmental profile of ER and ER-regulated markers in the breast across puberty. We found decreasing ERα but not ERβ across the menarchal transition. Further, the classic estrogen-regulated markers *TFF1, PGR* and *GREB1* also decreased over time, which supports that estrogen responsiveness in the breast is high before sexual maturity and decreases with adulthood.

The similar chemical structure between IF and estradiol allows IF to bind to ER although with weaker affinity (32). Depending on the estrogen environment, dose, target tissue and other factors, IFs can exert both estrogen-agonist and antagonist effects (33). In previous studies in premenopausal and postmenopausal monkeys, dietary IFs did not elicit clear estrogenic effects, while having modest ER modulatory effects when given with exogenous estrogen (34, 35). Here, we showed that pubertal soy intake resulted in transiently lower expression of mRNA for ER, which indicates that IFs may antagonize ER transcription mainly after menarche. Further, *GREB1* expression was also lower in the soy group after menarche, whereas other ER-regulated markers (i.e. *TFF1* and *PGR*) were not altered. While not experimentally confirmed, these findings suggest that soy intake during adolescence may result in a subtle decrease in estrogen responsiveness in adulthood. In line with this, exposure to IF-rich diet in rats during pre/peri-puberty has been shown to reduce estrogen-induced proliferative response in ovariectomized-adult breast (36).

Mechanisms for any such ER modulating effects of soy is unclear. The soy IF genistein has been shown to antagonize estrogen-induced transcriptional activity by competitive binding to ER (33). IFs also have more affinity to ERβ than ERα (37), and ERβ can reduce ERα-mediated transcription (2). More recently, soy has been proposed to also act via epigenetic mechanisms, including changes in DNA methylation. Methylation is primarily associated with transcriptional

silencing of genes, although there is a limited understanding of methylation-dependent regulation of ER and ER-regulated genes in physiological settings, particularly in normal breast development. DNA methylation is sensitive to modulation by lifestyle factors; the plasticity is high during childhood and decreases with age (38). Prior work has shown that DNA methylation is susceptible to IF (8, 9). There is no clear evidence linking early life soy exposure, DNA methylation, ER-mediated responses, and breast cancer risk. Nevertheless, there has been continued speculation on epigenetic modulation as a mechanism of soy action (39, 40, 41).

In this study, the lower ER mRNA found in the soy group did not appear to be mediated by an altered methylation within the CpG sites examined. The *ESR1* gene has multiple promoters (42); here we assessed CpG sites within promoter B, which is a CpG rich region. CpG island promoters are mainly unmethylated regardless of expression status of the gene, while low CpG promoters are methylated during active or inactive states (43). In breast tumors, however, promoter B is often found methylated (44). We found a generally low level of methylation within this region across pubertal development with a significant increase in 2 of 4 CpG sites with maturity. The increase, however, was very subtle (~1%) and is unclear whether this level of change is biologically relevant. For *ESR2*, we assessed methylation of the region that corresponds to the promoter ON described in humans (45, 46); this region is not methylated in normal human breast epithelial cells and is hypermethylated in most breast cancer cell lines (47). Here, we found that the macaque pubertal breast showed very low methylation in this region. The promoter for *PGR* is also CpG rich with a very low overall methylation level. *PGR* expression in cancer is epigenetically regulated via methylation of the alternative promoters A and B (48), but little is known regarding the regulation in normal breast. We found that specific CpG sites within promoter A of *PGR* also had a transient increase in methylation level after menarche regardless of diet. Whether a minute methylation change in certain sites of the promoter could contribute to the decrease in *ESR1* and *PGR* mRNA with maturity is unclear.

There was no change in the methylation of ERE within the promoter of *GREB1* despite the differential gene expression with diet and time. *GREB1* has three consensus EREs spanning ~20 kb upstream of TSS that are functional transcription enhancers. The region we assessed in this study is the closest to the TSS (1.6 kb upstream TSS), which has basal promoter activity, strongest ER recruitment, and showed repressed activity in the presence of ER antagonism (49). Our result suggests that the methylation status of CpG sites within this region may not regulate *GREB1* transcription. The *TFF1* promoter has high level of methylation, consistent with the fact that the region is low in CpG content. Although methylation may not be the main mechanism regulating transcriptional activity in low CpG promoters, DNA methylation status is important for tissue-specific regulation of certain genes including *TFF1* (43, 50). The change in methylation level around the ERE in the *TFF1* promoter across puberty was transient (~5-10%), however, CpG methylation showed a significant inverse correlation with mRNA expression. This finding suggests that cytosine methylation in the ERE may interfere with ER and/or other transcription factor binding and affect gene activity in puberty. It has been shown that a dramatic change in methylation is typically attained in cancerous conditions. Interestingly, a mild difference in methylation level (i.e. as transient as ~7%) can repress gene transcription, as shown in an in-vitro study using minichromosomes in 293/EBNA1 cells (a derivative of the 293 human embryonic kidney carcinoma cell line) (51). The biological relevance of our finding on the *TFF1* promoter methylation change across puberty warrants further investigation.

Mammary gland luminal and myoepithelial cells originate from multipotent stem cells. Luminal epithelial differentiation involves GATA-3, a transcription factor that interacts with ERα in a positive-feedback loop to regulate cell fate (52). GATA-3 is required for activity of the *ESR1* promoter and ER-mediated transcription (53). Here we showed that in the macaque breast, GATA-3 was expressed in ductal and alveolar luminal epithelial cells but not myoepithelial cells, similar to that in mice (54). Interestingly, overall GATA-3 expression in the SOY group was high around the time of menarche, indicating a high luminal differentiation at this stage as GATA-3

promotes the differentiation of lineage-restricted progenitor cells (55). This finding is consistent with the large increase of lobuloalveolar differentiation found in these animals at this stage (21). Although insignificant, there was also a trend of lower GATA-3 with soy following menarche, which was consistent with the pattern of ERα expression. In the mammary gland of ovariectomized mice, exogenous estrogen can induce downregulation of GATA-3 expression by ER modulation, which can be altered by some but not all SERMs (56). Our findings suggest that soy IF could potentially downregulate the ER-GATA-3 co-modulatory loop after menarche.

The mammary gland is capable of metabolizing and synthesizing estrogen locally through the conversion of circulating inactive estrogens by the interactive work of various enzymes. STS, HSD17B1 and aromatase produce the bioactive estrone and estradiol, whereas EST and HSD17B2 catalyze the conversion into the less active forms. Activity of these key enzymes affects estrogen action in the breast. In post-menopausal women, intra-mammary biosynthesis is the major source for estrogen and plays an important role in the pathogenesis of cancer (57). The dynamic of these enzymes in the breast during puberty, despite being a period highly influenced by estrogen, is not fully understood. Here we showed that independent of diet, sulfatase/sulfo-transferase pathways predominated before menarche and decreased with maturity. This pattern may indicate the essential role of STS-EST pathways for maintaining equilibrium of active/inactive estrone during the highly estrogen-responsive stage of breast development. Aromatase whose role is to convert androstenedione and testosterone to estrone and estradiol, respectively, showed a relatively weak but consistent mRNA level across pubertal development. After menarche, the 17βHSDs – which catalyze interconversion between estrone and estradiol showed elevated mRNA amid no change in protein expression. Taken together, these patterns are consistent with the fact that production of estrone and its precursors occurs early in puberty and is followed later by estradiol (58, 59). One of the mechanisms of IF action is thought to be by modulation of enzymes for estradiol biosynthesis and sulfatase/sulfo-transferase pathways, which is based heavily on in-vitro studies (reviewed in (60)). Our findings indicate that IF exposure

within a dietary matrix, even at a high physiologic dose, does not affect tissue expression of steroidogenic enzymes. Other enzymes such as CYP1A1, CYP1B1, and CYP3A4 are important for further hydroxylating estrogens into metabolites of different genotoxicity. Importantly, these enzymes are induced and regulated by estrogen substrates via ER (61). Although IFs can alter CYPs activity in-vitro, exposure to IF via dietary soy during adulthood (62) or puberty did not appear to affect their expression in the mammary gland. Overall, the immunoreactivity profile of the steroidogenic enzymes in the macaque breast is highly comparable to that in the normal human breast (63, 64), further highlighting the unique translational potential of the macaque breast model.

Estrogen signaling during adolescence is a key driver of mammary gland development. Here we show for the first time that ER expression and response markers in the breast are higher during the pubertal transition, supporting the idea that this life stage represents a critical period for phenotypic modulation by estrogens. We found minimal effects of dietary pubertal soy exposure containing a high dietary dose of IF phytoestrogens. Soy effects included modestly lower ERs and select downstream estrogen-regulated markers in the breast in adolescence, a change which did not appear to be mediated by differential CpG methylation within the promoter sites examined. Our findings suggest that adolescent intake of dietary soy may dampen estrogen-responsiveness in the breast in young adulthood. However, the observed effects might be too subtle to conclude ER pathway modulation as the main mechanism of soy action. Additional experimental evidence is needed to confirm this finding and examine whether any such effects may alter breast cancer risk. Since molecular signaling in breast development involves a complex orchestration between estrogen and various molecules such as progesterone and different growth factors (65), future studies are also needed to examine potential effects of soy IF on those molecular regulators, and to investigate whether changes in estrogen responsiveness established in puberty persist throughout life.

**Acknowledgments:**

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**Authors' Contributions:**

Conception and design: JMC, CEW, TCR, FND, CJW, LDM

Development of methodology: FND, JMC, CEW, CJW, TCR, LDM, TDH, SM, ZH

Acquisition of sample and data: FND, CJL, CJW, ZH

Analysis and interpretation of data: FND, CJW, JAT, JWC, JMC

## Figures

**Figure 1**

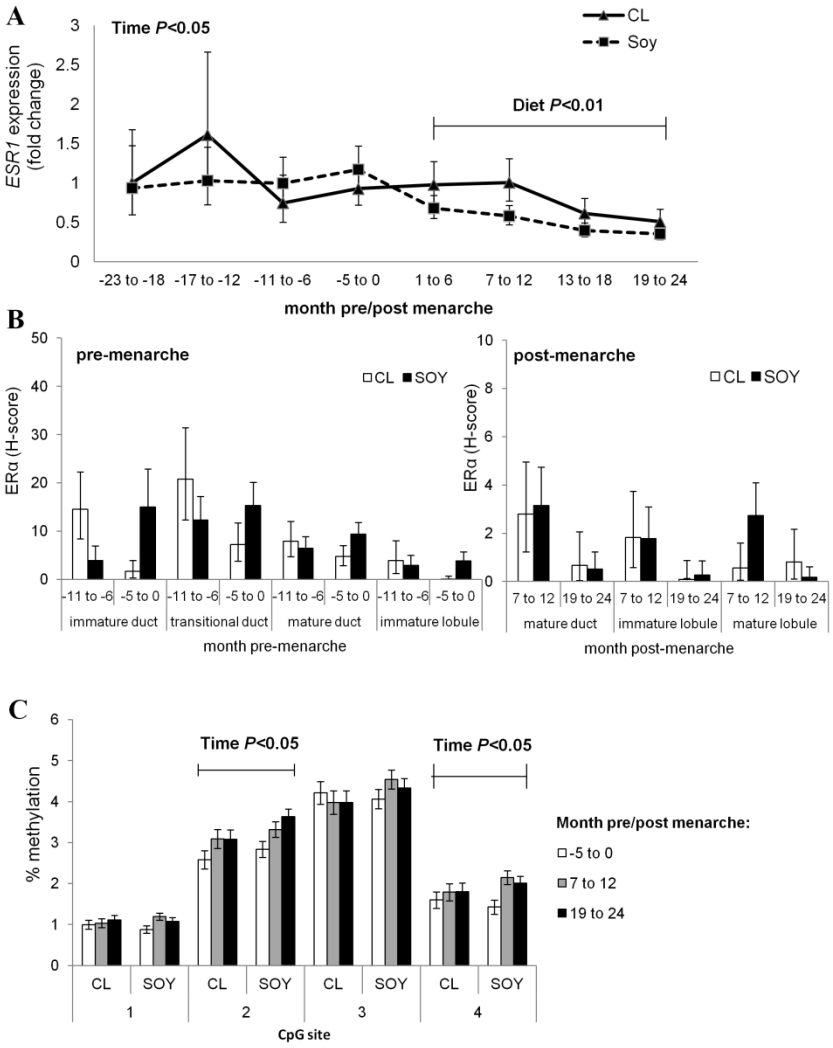



**Figure 1.** ERα expression in the macaque breast across pubertal transition. ESR1 mRNA level decreased with maturity, with a significant soy effect only after menarche (A). ERα protein did not differ by diet; the expression was lower in post-menarche (B). Methylation level on CpG sites within promoter B of ESR1 did not differ by diet (C). Values are LSM for n=11-17 monkeys/group (bars = SEM). Significant main effects are indicated in each panel.

**Figure 2**

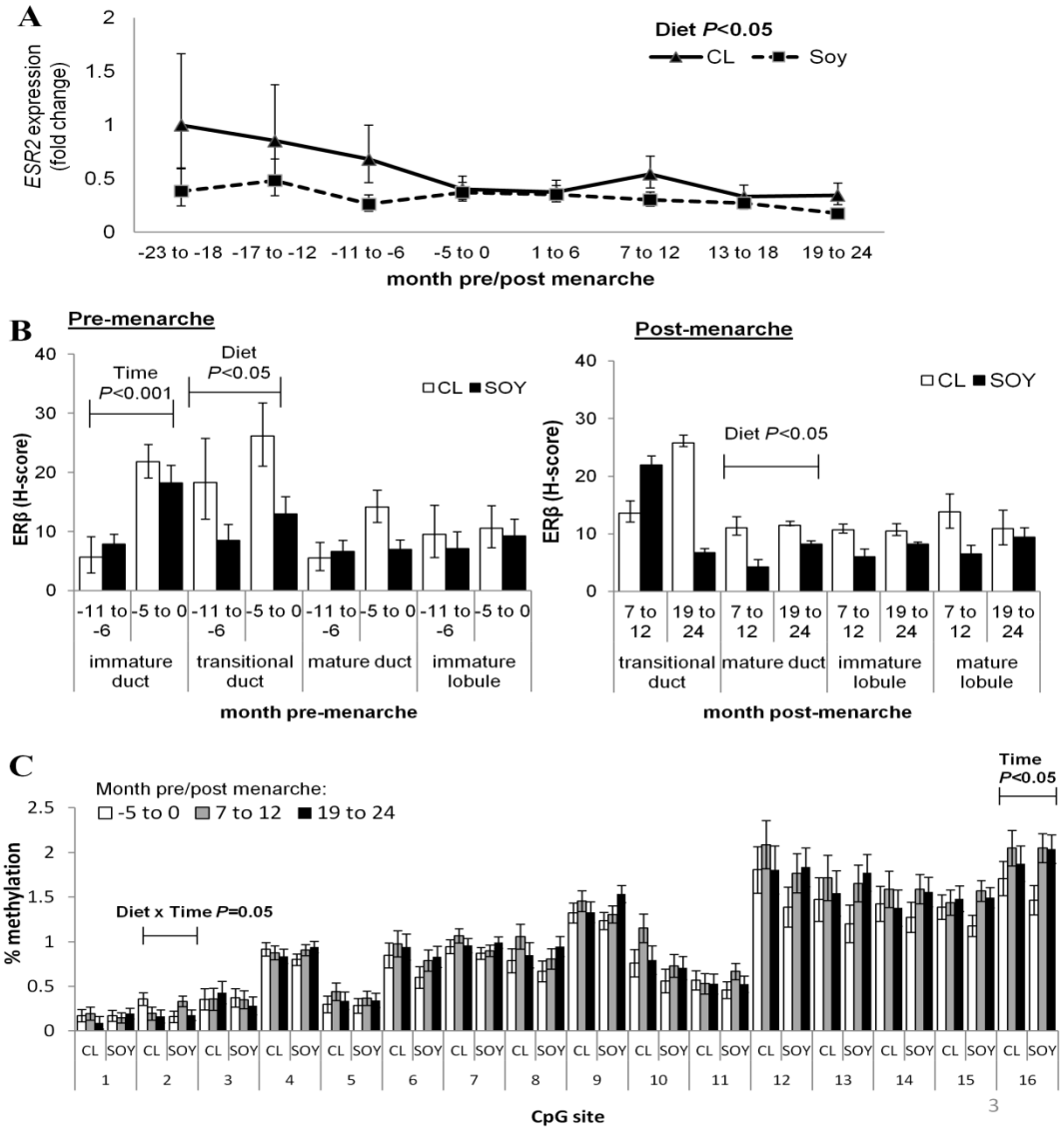



**Figure 2.** ERβ expression in the macaque breast across pubertal transition. ESR2 mRNA did not change over time; the overall expression was lower with soy (A). Protein expression was lower with soy only in transitional ducts (before menarche) and mature ducts (after menarche) (B). There was no effect of diet or time on methylation of CpG sites in the promoter upstream of ESR2 (C). Values are LSM for n=11-17 monkeys/group (bars = SEM). Significant main effects are indicated in each panel.

**Figure 3**

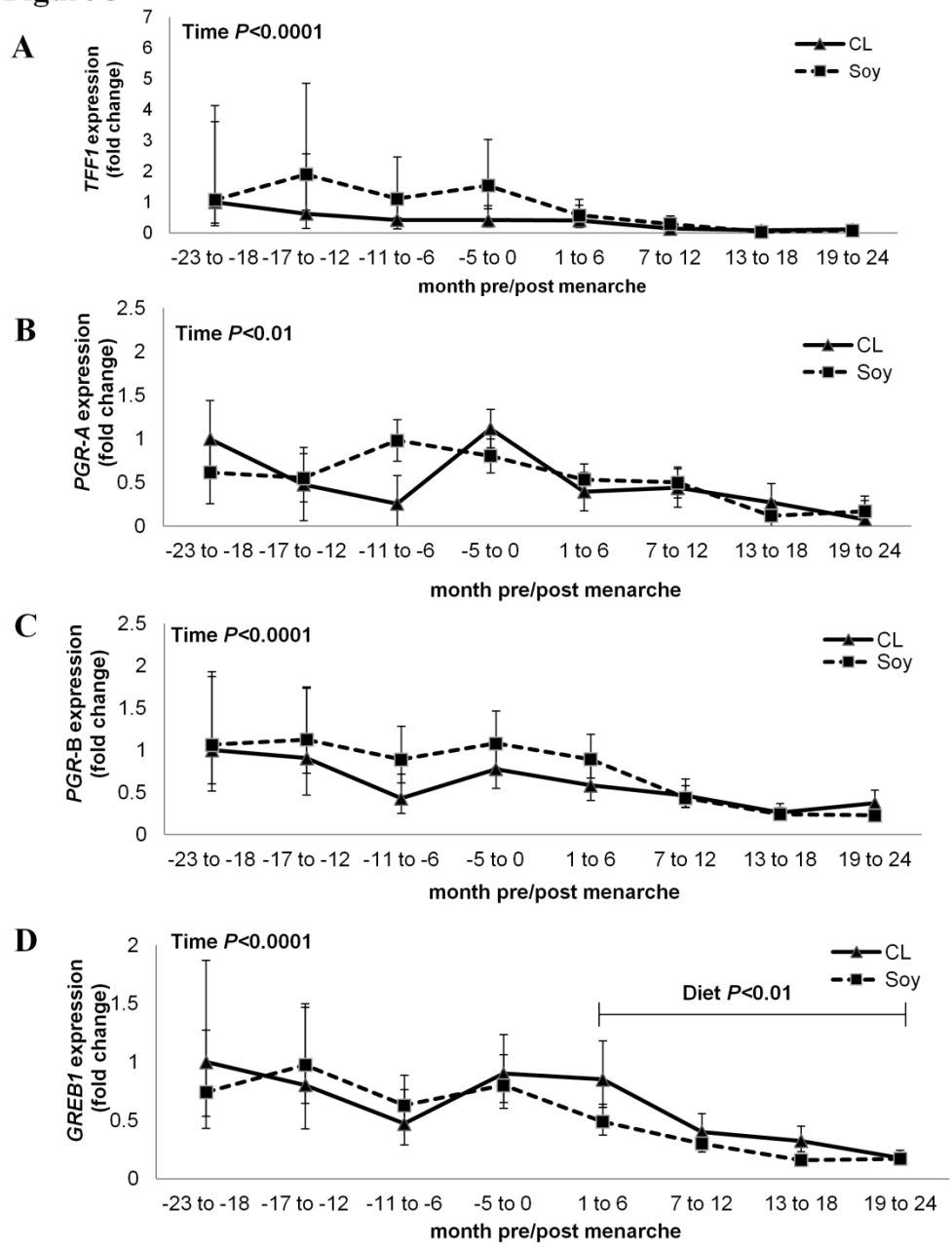



**Figure 3.** mRNA expression of ER-regulated markers decreased with maturity. TFF1 (A) and PGR (B,C) did not differ by diet. GREB1 (D) was lower in the soy group after menarche. Values are LSM for n=11-17 monkeys/group (bars = SEM). Significant main effects are indicated in each panel.

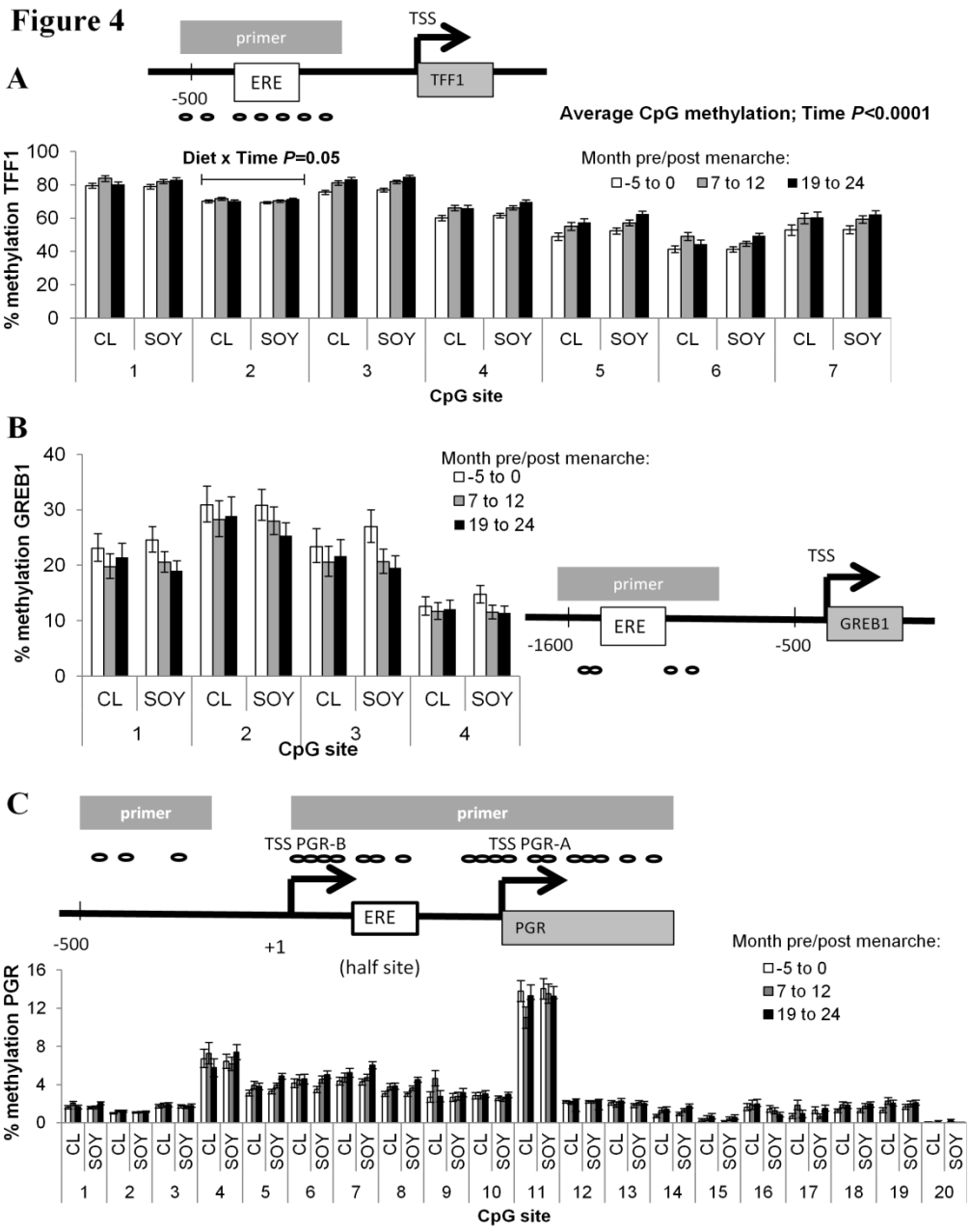



**Figure 4.** Promoter methylation of ER-regulated markers. Methylation of 7 CpG sites near the ERE within TFF1 promoter increased with time (A). We assessed methylation of 4 CpG sites near the ERE proximal of GREB1; there was no diet or time effect (B). Level of CpG methylation within PGR promoter A and B was low, and they did not differ by diet or time (C). Values are LSM for n=11-17 monkeys/group (bars = SEM). Significant main effects are indicated in each panel.

**Figure 5**

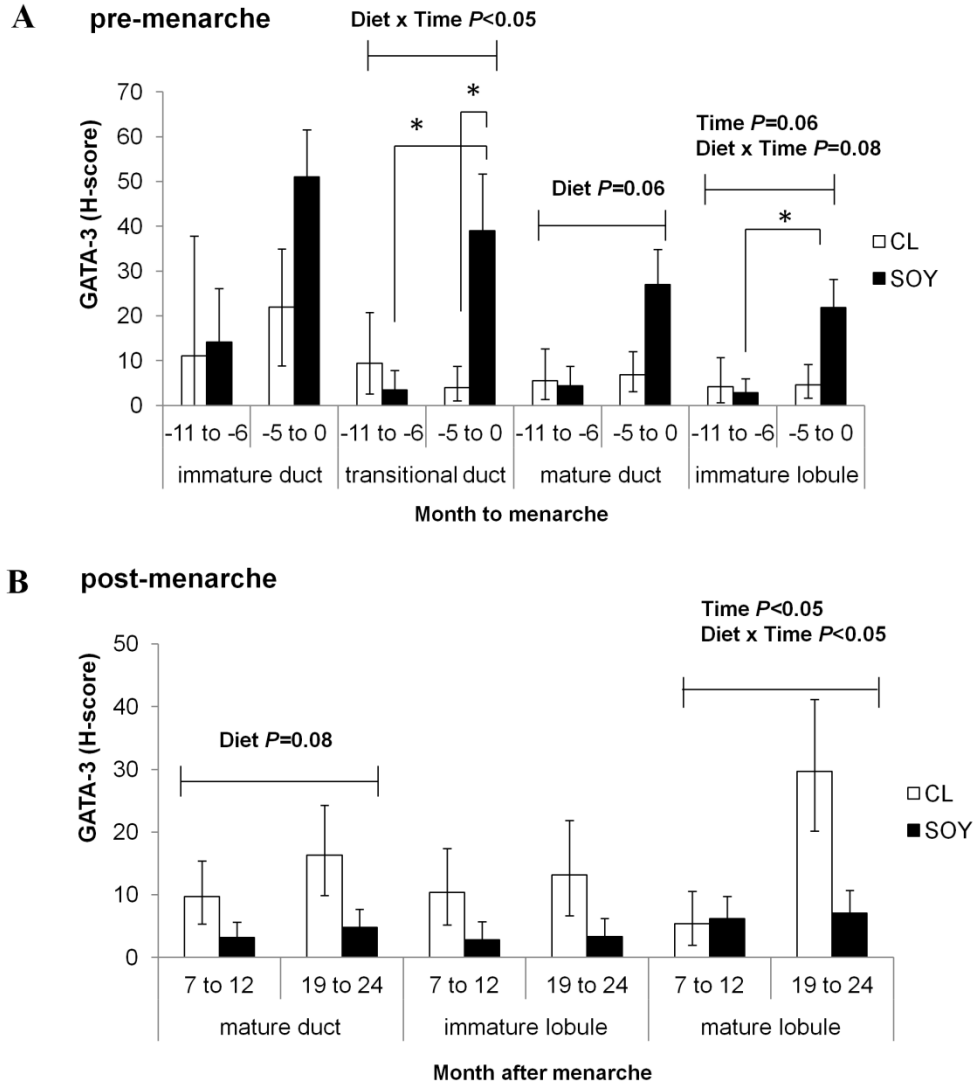



**Figure 5.** GATA-3 protein expression in the pubertal macaque breast. GATA-3 immunoreactivity appeared to differ by diet before (A) and after menarche (B). Values are LSM for n=11-17 monkeys/group (bars = SEM). Significant main effect and interactions are indicated in each panel. Asterisks (*) indicate significant difference ($P$<0.05) with LSM Tukey HSD test.

**Figure 6**

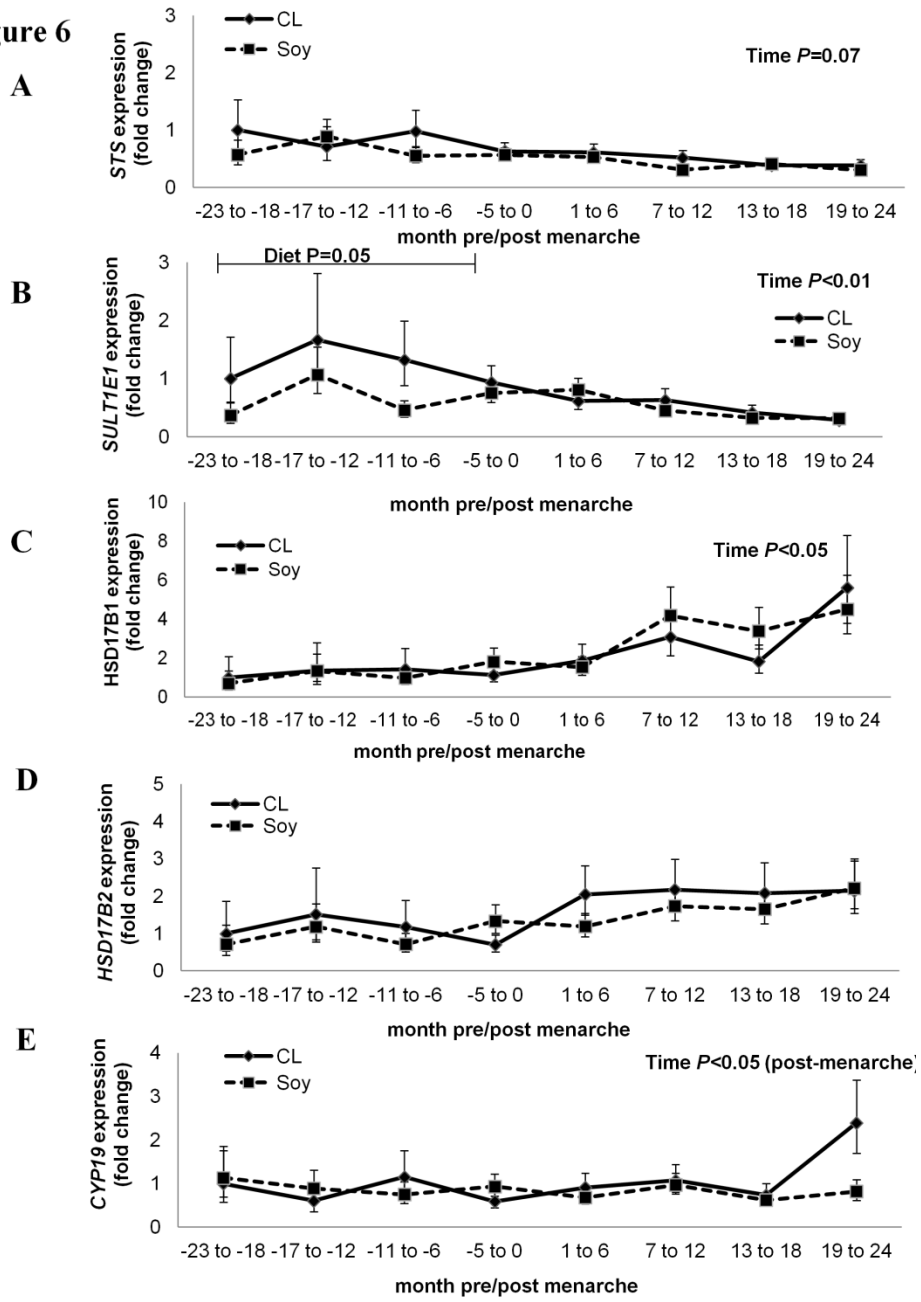
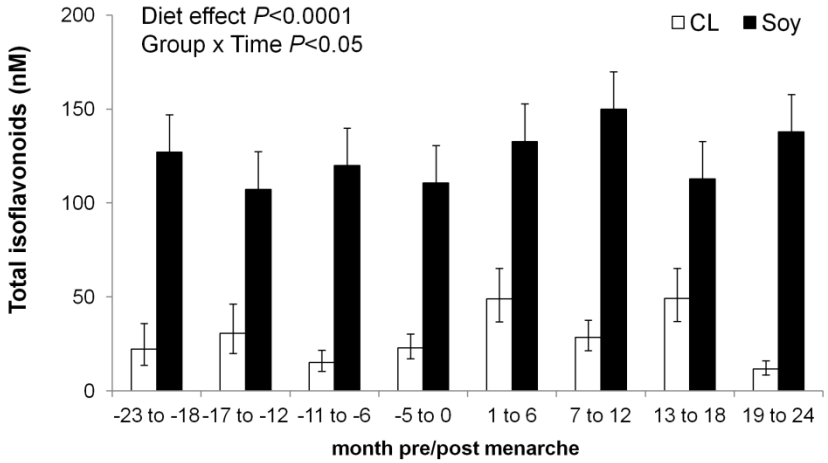



**Figure 6.** mRNA expression of enzymes for estrogen synthesis and metabolism was not affected by dietary treatment. STS (A) and SULT1E1 (B) decreased with maturity. HSD17B1 expression increased across pubertal transition (C), while HSD17B2 (D) did not differ with time. The expression of CYP19 increased after menarche (E). Values are LSM for n=11-17 monkeys/group (bars = SEM). Significant main effects are indicated in each panel.

## ONLINE SUPPLEMENTARY MATERIALS

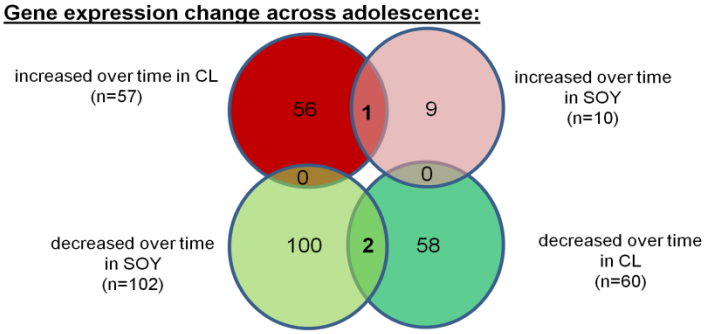
**Supplementary Figure 1**




**Supplementary Figure 1.** Serum isoflavonoid concentrations across pubertal transition. Level of circulating total isoflavonoids (genistein + daidzein + equol) was significantly higher in the soy-fed monkeys. Values are LSM for n=11-17 monkeys/group (bars = SEM). Significant main effects and interactions are indicated in the panel.

**Supplementary Figure 2**

**A** **Gene expression change across adolescence:**




***Venn diagram based on sets of gene filtered by Paired t-test P <0.05 (unadjusted); Fold-change >1.5 n=4 monkeys per diet group***

**B** **Top 10 differentially expressed genes:**

| | at 7 -12 months post-menarche (n=157 genes) | FC | *P*-value | at 19-24 months post-menarche (n=190 genes) | FC | *P*-value |
|---|---|---|---|---|---|---|
| upregulated in SOY (vs. CL) | NBEAL2-like | 6.74 | 0.041 | LEP | 2.62 | 0.041 |
| | TFF3-homolog | 3.11 | 0.028 | CA6-like | 2.62 | 0.042 |
| | ALDH9A1-like | 2.79 | 0.049 | MIR675 | 2.58 | 0.003 |
| | CPA3 | 2.73 | 0.015 | PPIA-like | 2.38 | 0.001 |
| | DPT | 2.40 | 0.034 | STX3-like | 2.27 | 0.017 |
| downregulated in SOY (vs. CL) | RPLP2-like | 1.99 | 0.040 | CXCL9 | 4.38 | 0.006 |
| | ANKRD62-like | 1.99 | 0.027 | CXCL10 | 2.88 | 0.034 |
| | CAPN3 | 1.94 | 0.021 | RTD1B | 2.41 | 0.006 |
| | FAM3B-like | 1.83 | 0.006 | DPPA2 | 2.33 | 0.019 |
| | ZNF850 | 1.83 | 0.017 | IGLV7-46 | 2.05 | 0.005 |

***n=4 monkeys per diet group***
***Filtered by FC>1.5; unadjusted P<0.05 (by empirical Bayes)***

**C** **Gene enrichment analysis:**

| | Top-10 significantly enriched gene sets[1] | | | | | |
|---|---|---|---|---|---|---|
| | CL | Size | FDR | SOY | Size | FDR |
| Enriched among genes that were upregulated over time | Olfactory transduction | 128 | 0.00 | Ribosome | 60 | 0.01 |
| | Neuroactive ligand receptor interaction | 207 | 0.00 | Olfactory transduction | 128 | 0.01 |
| | Autoimmune thyroid disease | | | | | |
| | Cytokine-cytokine receptor interaction | 26 | 0.00 | | | |
| | Intestinal immune network for IgA production | 212 | 0.00 | | | |
| | | 34 | 0.00 | | | |
| Enriched among genes that were down-regulated over time | Spliceosome | 93 | 0.00 | ECM receptor interaction | 70 | 0.00 |
| | Valine, leucine, isoleucine degradation | 28 | 0.00 | Focal adhesion | 159 | 0.00 |
| | Huntington disease | | | TGF-beta signaling pathway | 68 | 0.00 |
| | Insulin signaling pathway | 126 | 0.00 | Adherens junction | 59 | 0.01 |
| | Arrhythmogenic right ventricular cardiomyopathy | 107 | 0.00 | | | |
| | | 59 | 0.00 | | | |

**Supplementary Figure 2.** Gene expression profile in the post-menarchal macaque breast by microarray. (A) Venn diagram showing number of genes that changed with time (i.e. between 7-12 months to 18-24 months after menarche) in the control (CL) and soy group (SOY), based on unadjusted p-values from paired t-test in each diet group. (B) The list of top-10 genes that showed significant difference by dietary treatment using empirical Bayes statistics. (C) Result of enrichment analysis against KEGG gene sets, showing the top-10 significantly enriched KEGG pathways in CL and SOY groups across time. FC = fold-change; FDR=false discovery rate.

**Supplementary Table 1A.** Cynomolgus macaque and human primer probe sets used to generate custom Taqman-based assay for qRT-PCR.

| Gene | Accession No.[a] | Species [b] | | Primer/Probe Sequence (5' to 3')[c] |
|---|---|---|---|---|
| *ESR1* | DQ469336 | Mf | F | TCACATGATCAACTGGGC**A**AAGA |
| | | | R | AGAAGGTGGACCTGATCATGGA |
| | | | P | CACAAAG*cc*TGGCACCC |
| *ESR2* | HQ702565 | Mf | F | GTATCTCTGTGTCAAGGCCATGAT |
| | | | R | CATCCTGGGTCGCTGTGA |
| | | | P | CCTGCTCAATTCC*a**a***TATGT |
| *PGR-B** | - | Hs | F | GCCAGACCTCGGACACCTT |
| | | | R | CAGGGCCGAGGGAAGAGTAG |
| | | | P | CCTGAAGTTTCGGCCATACCTATCTCCCT |
| *TFF1* | DQ464113 | Mf | F | GTGCTTCCATCCTAATACCATCGA |
| | | | R | GCAGATCC**A**TGCAGAAGTGTCTAAA |
| | | | P | TCCCTCCAGAA*ga*GGAGTGT |
| *CYP19* | DQ529980 | Mf | F | CCCCAAACCCAATGAATTTACTCTTG |
| | | | R | GGCCCAAAGCCAAATGG**T**T |
| | | | P | AAAGTACCTATAAGGAA*ca*TTCTT |
| *HSD17B1* | DQ529983 | Mf | F | CAGGCCTGGGCCT**A**CTG |
| | | | R | C**G**TTCACGTCCAGCACAGA |
| | | | P | CCCA**C**CGCCTCCAGC |
| *HSD17B2* | DQ529984 | Mf | F | AAAGG**A**AGGCTGGTGAATGTCA |
| | | | R | ATGCCAGC**T**TT**G**CCATTGG |
| | | | P | CCCCT*cc*TCCCATGCTG |
| | | | P | ATGTT*cc*TGAGCTCTCG |
| *STS* | DQ529981 | Mf | F | GGGATGCTGTTGAGGAAATGGA |
| | | | R | CCAATCTCAGCTCATC**T**AGAAGGTT |
| | | | P | ATCTGC*cc*CACACTCC |
| *SULT1E1* | DQ529982 | Mf | F | GAGAGAAC**G**GG**C**CAGGTT |
| | | | R | CG**A**GAGGTGTCCTGGATCAG |
| | | | P | ATGTT*cc*TGAGCTCTCG |

| Gene | Accession No.[a] | Species [b] | | Primer/Probe Sequence (5' to 3')[c] |
|---|---|---|---|---|
| *CYP1A1* | D17575 | Mf | F | CCACAGCCACCTCCAAGATC |
| | | | R | TGGCCGACATGGAGATTCG |
| | | | P | CCTACACTGATCATGCTTTT |
| *CYP1B1* | AB179009 | Mf | F | CCGCGCTGCAGTGG |
| | | | R | CACTCGAGCCTGCACATCA |
| | | | P | CTCCTCTTCATCA*gg*TATCC |
| *CYP3A4* | S53047 | Mf | F | CACACCTTTGCCTTTATTGGGAAAT |
| | | | R | AGCCCCACACTTTTCCATACTTTT |
| | | | P | C*aa*ACGTCCAAAAGCC |
| *GAPDH* | DQ464111 | Mf | F | TGCCCTCAA**T**GACCACTTTGTC |
| | | | R | ACCCTGTTGCTGTAGCCAAAT |
| | | | P | TCGTTGTCATA*cc*AGGAAAT |
| *ACTB* | DQ464112 | Mf | F | ACCCCAAGGCCAACCG |
| | | | R | CCTGGAT**G**GC**C**ACGTACATG |
| | | | P | AAGATGACCCA*ga*TCATG |

[a] Genbank identification code or cited reference. [a]*Hs*, *Homo sapiens*; *Mf*, *Macaca fascicularis* (cynomolgus macaque). [c] Bold letters indicate differences between macaque and human sequences. Italized lowercase of base pairs indicate exon boundaries. Underlined sequence represents an 8-base deletion in the HSD17B1 pseudogene. F, forward primer; R, reverse primer; P, probe. Asterisk (*) indicates that the assay was based on that reported in Sakaguchi et al. Gynecol Oncol. 2004 May;93(2):394-9.

**Supplementary Table 1B.** Applied Biosystems (ABI) Taqman gene expression assays used in qRT-PCR

| Gene ID | NCBI Accession no. | Species[a] | ABI assay ID |
|---|---|---|---|
| *PGR*[b] | XM_001095317.1 | *Mm* | Rh02830094_m1 |
| *GREB1* | XM_001089374.1 | *Mm* | Rh02866842_m1 |

[a]*Mm*, *Macaca mulatta* (rhesus macaque). [b] PGR was used to generate PGR-A expression level; quantification of PGR-A was done by subtraction of PGR-B (custom assay) from PGR, as previously described in Sakaguchi et al. Gynecol Oncol. 2004 May;93(2):394-9.

**Supplementary Table 2.** Cynomolgus macaque primer sets (forward, F; reverse, R; and sequencing, S) for pyrosequencing.

| Gene | Identity[a] | | Sequence (5' to 3') | CpG site |
|---|---|---|---|---|
| ESR1 | 100% | F<br>R<br>S | GGGATTGTTGTTTTGTTTTGATATTG<br>CTTCCCTAAACCACCTTTAAC<br>ATTGGTTTAAATATTATTTTAGGT | 4 |
| ESR2 | 99% | F<br>R<br>S | GTAGAGGGGAGTAGTGTTTGA<br>TAAACCCTACTACAAACCTCTTAAATCT<br>GGGAGTAGTGTTTGAGTTTA | 10 |
| | 99% | F<br>R<br>S | GTAGAGGGGAGTAGTGTTTGA<br>ACCCTACTACAAACCTCTTAAATCTAA<br>GATTTTAAAGTGGGAGTATT | 6 |
| TFF1 | n/a | F<br>R<br>S | TTTTTGGGGATAGAAGGAAAGAGG<br>AAACTCATAAAACCCATTCCATCTACT<br>AGGAAAGAGGGAGGA | 2 |
| | 96.7% | F<br>R<br>S | AGATTAGAGTAGATGGAATGGGTTTTA<br>AACCCTCCCACCAAAATAAATACT<br>TTTTTTTTTTTTTTTTTGTAAGGTT | 3 |
| | 96.1% | F<br>R<br>S | ATTAGAGTAGATGGAATGGGTTTTA<br>AAATTCAAAAAATCCCTCTTATCC<br>GGTTTTTTAGATATGAGTAGG | 2 |
| PGR-A | 96.5% | F<br>R<br>S | GGGGTTTAGAATTGTTGTTTTTAGTATTT<br>AACCAAACCTCCAACACCTTACCT<br>GTAGTTTTAGTTTTGGAATATG | 7 |
| | 95.5% | F<br>R | AGAGTAGTTAGTTTAGGGAGATAGGTAT<br>CCCCTCCTCCTTCCCTTTT | 10 |

| | | | | |
|---|---|---|---|---|
| | | S | GTTTAGGGAGATAGGTATG | |
| PGR-B | 98.3% | F<br>R<br>S | AGTGATAGTTGTGGATTGGTTAGAT<br>CCATCCCAAAAAACCTACTAT<br>TGGATTGGTTAGATAGTTT | 3 |
| GREB1 | 97.3% | F<br>R<br>S | TAGGGTTTGTTTTTGAAGGGTAGA<br>ATAACCCAATTACCACACTTTTT<br>TGAAGGGTAGAGTTGATA | 2 |
| | 96.4% | F<br>R<br>S | TGGTAATTGGGTTATTTTGATTTAGAAGT<br>ACCCAAACATACTACTACAAATATTTCA<br>TTTGATTTAGAAGTAATTAAAA | 2 |

[a] Sequence identity between macaque and human DNA for each amplicon.

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# CHAPTER 3

## Endogenous and exogenous equol are antiestrogenic in reproductive tissues of apolipoprotein E-null mice

Fitriya N. Dewi, Charles E. Wood, Johanna W. Lampe, Meredith A. J. Hullar, Adrian A. Franke, Debbie L. Golden, Michael R. Adams, and J. Mark Cline

The following manuscript has been published in the Journal of Nutrition *[J.Nutr. (2012;142:1829-1835), American Society for Nutrition]*. Stylistic variations result from the demands of the journal. Fitriya N. Dewi performed the experiments, completed the statistical analyses and data interpretation, and prepared the manuscript. Charles E. Wood provided guidance on the experiment and manuscript preparation. Johanna W. Lampe and Meredith A. J. Hullar generated the serum isoflavonoid and fecal bacterial data. Adrian A. Franke provided significant guidance on data interpretation and manuscript preparation. Debbie L. Golden coordinated the parent study. Michael R. Adams was the principal investigator of the parent study. J. Mark Cline was the principal investigator of the study, performed histopathologic review, and provided signficant guidance throughout the project.

**Endogenous and Exogenous Equol are Anti-Estrogenic in Reproductive Tissues of Apolipoprotein E-null Mice**[1,2,3]

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RUNNING TITLE: Equol Effects on Reproductive Tissues of Mice

**ABSTRACT**

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[7]Abbreviations used: ASF (Altered Schaedler Flora), CL (Casein and lactalbumin), ER (Estrogen Receptor), HE (Hematoxylin and Eosin), HIF (High isoflavone), IF (Isoflavones), IHC (Immunohistochemistry), LIF (Low isoflavones); ODMA (O-desmethylangolensin), PGR (Progesterone Receptor), TR-FIA (Time-Resolved Fluorescence Immunoassay), SPI (Soy Protein Isolate).

Equol is an isoflavone metabolite produced by intestinal microbiota in a subset of people consuming dietary soy. Equol producers may show different responses to soy foods and phenotypes related to cancer risk. Here we assessed the effects of soy isoflavones (IF), endogenous microbial equol production, and dietary racemic equol in a 3x2x2 factorial experiment using gnotobiotic apoE-null mice ($n$=9-11/group/sex). At age 3-6 wk, equol-producing microbiota were introduced to half of the colony ($n$=122). At age 6 wk, mice were randomized to receive diet that contained one of three protein sources: casein and lactalbumin, alcohol-washed soy protein (low IF), and intact soy protein (high IF), with total IF amounts of 0, 42, and 566 mg/kg diet, respectively. Half of each diet group also received racemic equol (291 mg/kg diet). After 16 wk of dietary treatment, serum isoflavonoid profiles varied with sex, soy IF amount, and intestinal microbiota status. There were no treatment effects on tissues of male mice. In females, reproductive tissue phenotypes differed by equol-producing ability (i.e. microbiota status) but not dietary equol or IF content. Equol producers showed lower uterine weight, vaginal epithelial thickness, total uterine area, endometrial area, and endometrial luminal epithelial height compared to nonproducers ($P$<0.05 for all), with an association between microbiota status and estrous cycle ($P$ >Chi-square=0.03). Exogenous equol reduced expression of progesterone receptor (PGR) and the proliferation marker Ki67 ($P$<0.0001) in vaginal epithelium and endometrium; for endogenous equol, only PGR was reduced ($P$<0.0005). Our findings indicate that equol diminishes estrogen-dependent tissue responses in apoE-null mice.

## INTRODUCTION

Epidemiological evidence has shown that the incidence of chronic diseases such as cancer and cardiovascular disease is lower in Asia than in Western countries. Lifestyle factors, including diet, have been identified as potential determinants (1-2). Soy-based foods rich in phytoestrogen isoflavones[7] (IF) are a primary component of many Asian but not Western diets (3). Intake of soy and the primary soy IF genistein and daidzein has been widely studied in association with cancer and other chronic diseases, but the results have been mixed and inconclusive (4). The lack of consensus regarding health effects of soy IF interventions is due to a variety of potential factors, including variation in IF formulations across studies and interindividual differences in metabolism and response to soy diet (5).

Equol is a metabolite of daidzein produced by intestinal bacteria in about 30% of adult non-Asian and non-vegetarian populations consuming dietary soy protein (6-7). Approximately 10-30% are intermittent equol producers (8-9). Inter-individual variation in the ability to produce equol is a consequence of difference in gut microbial community. Several bacterial strains that can produce equol have been identified in-vitro (10); however, the nature of the bacteria and how a person harbors them is incompletely understood. Factors such as dietary habit may modulate the composition and activity of gut microbes, hence affecting equol production (7). Recent evidence suggests that the ability to metabolize daidzein into equol may influence the health-related responses to soy exposure (11). However, it is unclear whether such effects are driven by equol directly or if equol production simply serves as a marker for other intestinal microbiota-mediated effects. Equol has distinct biological activity compared to daidzein and genistein. It binds to estrogen receptors (ER) α and β with greater affinity than daidzein (12) and has antiandrogenic properties by binding to dihydrotestosterone (13). Equol producers and nonproducers may exhibit different phenotypes related to cancer risk and respond differently to IF, potentially accounting for some of the individual variation in response to soy foods and their related health effects. Still, direct effects of equol or equol production are poorly understood. The intestinal microbiota is

only capable of producing the S-(-) enantiomer of equol (S-equol), while the commercially available synthetic form of equol is a racemic R/S-(±) mixture. In healthy humans, racemic equol differs from the individual enantiomers with respect to bioavailability and pharmacokinetics (14), suggesting that endogenously-produced and exogenous equol may have distinct biological activities.

Equol-producer status has now become an important consideration in interpretation of soy study results. Furthermore, interest in identifying factors contributing to the equol-producer phenotypes and their association with human health has increased, alongside development of equol-based dietary supplements (15-16). The purpose of this study was to evaluate the effects of endogenously produced equol and exogenous racemic equol on reproductive tissues in mice fed a soy IF diet. The work was carried out in apoE-null mice, which are susceptible to atherosclerosis; the atherosclerosis-related findings will be reported elsewhere.

## MATERIALS AND METHODS

### *Animals and Diets*

ApoE-null mice were used in the study. All breeding and procedures involving mice were done at Taconic Biotechnology in their AAALAC (Association for the Assessment and Accreditation of Laboratory Animal Care)-accredited facility in Germantown and Rensselaer, NY and were approved by the Taconic institutional animal care and use committee. Tissue-related procedures were performed at Wake Forest School of Medicine. The gnotobiotic apoE-null mouse breeding colony was initially established by exposure of Altered Schaedler Flora (ASF) (17) to germ-free apoE-null mice at 7 wk of age via aqueous fecal suspension from ASF-maintained donor. Preliminary assessment of serum equol concentrations indicated that mice harboring ASF were not equol producers (equol nonproducers).

This study utilized the offspring ($n$=243) of apoE-null breeders harboring ASF. At age 3-6 wk, half of the colony ($n$=122) was exposed to intestinal microbiota from normal apoE-null

mice via fecal material to generate an equol-producing phenotype (equol producer). Throughout the study, mice were maintained under gnotobiotic conditions in plastic film isolators, and microbial status was monitored monthly by fecal culture. Microbial status of the nonproducers was verified under Taconic's Defined Flora microbiological health standard, whereas equol producers were maintained at the Murine Pathogen Free status (18).

At age 6 wk, both equol producers and nonproducers were randomly assigned to dietary treatment groups (see Supplemental Figure 1) with *n*=9-11 in each group, per sex. The diets (see Supplemental Table 1) used one of three protein sources; 1) casein and lactalbumin (CL), 2) alcohol-washed soy protein (low IF; LIF); or 3) intact soy protein (high IF; HIF). The total IF amounts in the diets were 0, 42, and 566 mg/kg diet, respectively. The ratio of daidzein:genistein:glycitein was 1:1.5:0.2. Additionally, the diets also contained 291 mg/kg racemic equol (i.e. CL+, LIF+, HIF+), or no equol (i.e. CL-, LIF-, HIF-). The exogenous equol was given as a synthetic product chemically synthesized from daidzein and comprised of a 50/50 racemic R/S (±) mixture. Diets were manufactured by Research Diets, Inc. (New Brunswick, NJ). Archer Daniels Midland Company (Decatur, IL) provided the isolated soy proteins and analyzed IF content in the isolates. Racemic R/S (±) equol was provided by Solae, LLC (St. Louis, MO). We used a dosing strategy for IF in mg/kJ to adjust for the metabolic rate difference between humans and mice, and to provide IF dose relevant to human exposure. Human daily energy consumption was considered to be 7531 kJ/d and our diet compositions were designed to create a dosing strategy of approximately 275 mg IF/7531 kJ for HIF, 20 mg IF/7531 kJ for LIF, and 140 mg equol/7531 kJ for exogenous equol. Additionally, we assessed the effect of soy IF on microbiome of the equol producers at 2 wk post dietary treatment (see Supplemental Methods).

Following 16 wk of dietary treatment, mice were humanely euthanized by $CO_2$ asphyxiation. Mice were weighed and blood collection was done by cardiac puncture. Tissues collected were ovaries, oviduct, uterus, vagina, testicles, epididymis, and accessory glands of male reproductive tract (prostate, seminal vesicles, and coagulating glands). Tissues were fixed in

10% neutral buffered formalin. After 24 hours, tissues were removed from formalin and stored in 70% ethanol. Uteri and testes were weighed as markers of estrogenic action on the reproductive tract. All tissues were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (HE). Histopathologic examinations were done by board-certified veterinary pathologists (C.E.W. and J.M.C.).

We identified the estrous cycle stage (proestrus, estrus, metestrus, and diestrus) of each mouse based on histology of the vagina, uterus, and ovaries (19-20). Mice that did not meet all the criteria of a cycle stage were noted as "undetermined stage" and excluded from cycle-related analyses.

*Serum Isoflavonoids*

Isoflavonoid concentrations were measured from serum samples using time-resolved fluorescence immunoassay (TR-FIA) at the Fred Hutchinson Cancer Research Center, as described previously (21). Serum equol, genistein, and daidzein concentrations were measured in subsets of mice. Equol was measured across all treatment groups ($n$=5-10 per group, per sex), genistein was measured in CL-, LIF-, and HIF- groups ($n$=5-10 per group, per sex), and daidzein was measured only in mice fed a HIF- diet ($n$=10 per group, per sex). The lowest levels of quantitation for equol, genistein, and daidzein were 0.66, 1.0 and, 0.5 nmol/L, respectively.

*Tissue Histomorphometry*

HE-stained slides were used for histomorphometric evaluation of reproductive tissues with computer-assisted technique as described previously (22) using Infinity 3 digital camera (Lumenera; North Andover, MA) and Image Pro-Plus 5.1 software (Media Cybernetics Inc.; Bethesda, MD). We measured cross-sectional uterine and endometrial areas, endometrial luminal epithelial height, and vaginal epithelial thickness as markers of estrogenic action on the reproductive tract. Cross-sectional areas were measured at 4x (total uterine area) and 10x

(endometrial area) magnifications. Endometrial luminal epithelial height and vaginal epithelial thickness were measured at 20x magnification in three randomly chosen sites. All measurements were made unaware of the treatment groups.

*Immunohistochemistry*

For immunohistochemistry (IHC), we used a biotin-streptavidin-alkaline phosphatase staining method modified for antigen retrieval from paraffin embedded tissue, as previously described (22). Staining was done for Ki67 and progesterone receptor (PGR) as markers for cell proliferation and ER activity, respectively. Primary antibodies were rabbit monoclonal anti-Ki67SP6 (Thermo Scientific; Fremont, CA) and rabbit polyclonal anti-PR sc-538 (Santa Cruz Biotechnology Inc.; Santa Cruz, CA). All measurements were made unaware of the treatment groups. Positively stained cells were given a score based on staining intensity to obtain a semi-quantitative measurement of intensity and distribution by H-score calculation (22-23).

*Data Analysis*

The experiment was a 3x2x2 factorial design to test the effects of soy IF, microbiota status, and exogenous equol. Measured outcomes were expressed as continuous variables; non-normally distributed data were log-transformed or square-rooted to improve distribution for analysis, and re-transformed to original scale for presentation of results. We performed 1-way, 2-way, or 3-way analysis of variance (ANOVA) by Fit Model procedure in JMP (version 9.0.2, SAS Institute; Cary, NC). Post-hoc comparisons were made using Least Square Means Student's T-test (between 2 treatment groups or 2 levels in a binary category) or Tukey's Honestly Significant Difference test (for pairwise comparisons involving more than 2 levels or 2 treatment groups). Estrous cycle stage was expressed as categorical variable. Contingency table and chi-square statistic tests (i.e. the Likelihood Ratio Chi-square test and the Pearson Chi-square test) were used to assess the association between estrous cycle and each fixed factor (i.e. soy IF,

microbiota status or exogenous equol). For female reproductive outcomes, we performed analyses both with and without estrous cycle as a covariate in the model. For serum isoflavonoid concentrations, we initially included sex as a fixed factor in the model, and further analyzed the data separately by sex.

Two out of 18 females fed a HIF+ diet showed histopathologic lesions considered to be unrelated to treatment (see results). These two mice were excluded from our dataset.

## RESULTS

*Serum Isoflavonoids*

Importantly, we succeeded in developing equol-producing and non equol-producing phenotypes in our mouse model (Figures 1A and 1D). Without exogenous equol treatment, mice that were defined as equol producers had significantly greater serum equol concentrations compared to nonproducers fed the same diet ($P<0.001$ for both sexes). There was also a main effect of soy IF on serum equol concentrations ($P<0.0001$ in both sexes) in which mice fed a HIF- diet showed greater equol concentrations relative to those fed LIF- and CL- diets. Among the equol producers, fecal microbial communities also differed between the mice fed HIF- and CL- diets ($P<0.001$; see Supplemental Figure 2), but not by sex.

When exogenous racemic equol was given, serum equol concentrations in both equol producer and nonproducer were significantly higher than those without racemic equol ($P<0.0001$ for main effect of exogenous equol) and notably higher (approximately 7-10 fold) than those produced endogenously by IF-fed mice with equol-producing intestinal bacteria (Figures 1B and 1E). With exogenous equol, male equol producers showed lower serum equol concentrations compared to nonproducers ($P<0.01$ for the main effect of microbiota). A similar pattern was observed in the females although the effect was not statistically significant (P=0.08).

Serum genistein and daidzein concentrations were higher in female mice compared to males ($P<0.05$ for both). Using a 2-way analysis, we found a main effect of dietary soy IF on

serum genistein concentration ($P$<0.0001 in both sexes) whereby genistein was higher in mice fed a HIF- diet compared to LIF- and CL- diets (Figures 1C and 1F). In the females, there was also a main effect of microbiota status ($P$<0.05) in which equol producers showed higher serum genistein concentration than nonproducers. Although daidzein showed a similar pattern, the difference by microbiota status was not statistically significant in either males ($P$=0.08) or females ($P$=0.05). The males had mean serum daidzein concentrations of 158 nmol/L (nonproducers) and 459 nmol/L (equol producers). In female mice, the mean concentrations were 513 nmol/L and 927 nmol/L in nonproducers and equol producers, respectively.

*Histopathology*

Histopathologic lesions were present in few mice and did not differ across treatments. In females, lesions included vaginal mucification, vaginitis, uterine atrophy, endometritis, uterine and ovarian cysts, uterine gland dilatation, ovarian interstitial cell hyperplasia, persistent estrus, and mesonephric duct remnants. Two mice (one from each microbiota type) in the group that received a HIF+ diet showed pyometra. Based on the fact that only a few mice had this lesion, we suspected that the finding was incidental. In males, lesions included prostatitis (in one mouse) and mild degenerative changes in the testes which were not related to treatment. No neoplasms were seen in this study.

*Body Weight*

In female mice, body weights were not different by microbiota status or dietary treatments. In males, there was a main effect of soy IF diet ($P$<0.05) in which average body weights were 7% lower in the HIF group relative to CL ($P$<0.05). There was also a main effect of microbiota ($P$<0.05) limited to mice not receiving exogenous equol, whereby equol producers had 11% lower body weights compared to nonproducers ($P$<0.005).

*Tissue Weight and Histomorphometry*

There was no significant effect of soy diet, microbiota status, or exogenous equol on testis weight. In the females, we found a main effect of microbiota status on reproductive tissues (Table 1). Uterine weight, total uterine area, endometrial area, endometrial luminal epithelial height, and vaginal epithelial thickness were lower in equol producers compared to nonproducers ($P<0.05$ for all). Except for vaginal epithelial thickness, these measurements were not significantly affected by either soy diet or exogenous equol, and there was no interaction between microbiota status and dietary treatments. There was a main effect of soy IF on vaginal epithelial thickness ($P<0.05$), which was 19% and 15% greater in HIF relative to LIF and CL groups, respectively. Interestingly, the significant main effects of microbiota on histomorphometry measurements disappeared when estrous cycle stage was added as a covariate. Furthermore, we found that estrous cycle stage was significantly associated with microbiota status ($P<0.05$) but not with soy diet or exogenous equol. At the time of necropsy, equol producers were found predominantly in metestrus, whereas cycle stage across nonproducers was more evenly distributed with the majority being in proestrus and estrus (Figure 3).

*Immunohistochemistry*

We next evaluated the expression of PGR as an estrogen-response marker in the vagina and uterus (Figures 3A-B). In a 3-way analysis, we found main effects of exogenous equol and microbiota status but not soy diet. Exogenous equol significantly lowered PGR expression in the vagina and uterus ($P<0.0001$ in both tissues). A main effect of microbiota ($P<0.0005$ in both tissues) was found whereby equol producers had lower PGR expression than nonproducers. For vaginal epithelium, there was a significant interactions among soy diet, equol and microbiota status ($P$-interaction$<0.005$), equol and microbiota status ($P$-interaction$<0.05$), and soy diet and microbiota ($P$-interaction$<0.005$). In the absence of racemic equol, PGR expression was lower in the vagina of equol producers compared to nonproducers in both LIF-($P<0.0001$) and HIF-

($P$<0.05). A similar pattern was observed in the uterus of HIF- group, although not statistically significant ($P$=0.07). The main effects of exogenous equol and microbiota status remained significant after estrous cycle stage was added as a covariate in the model.

Similar to PGR, we did not find a main effect of soy IF on the proliferation marker Ki67. There was a main effect of exogenous equol on Ki67 expression in the vagina and uterus ($P$<0.0001 in both tissues), and it remained significant after estrous cycle stage was included as a covariate. Ki67 labeling was lower in mice that received exogenous equol compared to those without equol (Figures 3C-D), regardless of soy IF dose. In uterine tissue, there were significant interactions between soy IF, microbiota status, and exogenous equol ($P$-interaction<0.05), and between soy IF and microbiota status ($P$-interaction<0.05).

**DISCUSSION**

In this study we evaluated the interactive effects of soy IF diet, endogenously-produced equol, and exogenous racemic equol on reproductive tract measures in adult mice. Treatment effects were seen only in female mice. Both endogenous and exogenous equol resulted in lower expression of markers of estrogenic action independent of soy IF dose, suggesting that equol may result in modest buffering effects on endogenous estrogen activity in the reproductive tract. A significant association was observed between microbiota status and estrous cycle stage, which may partially explain the observed effects.

This study utilized gnotobiotic apoE-null mice harboring ASF, a well-developed microbiota used for generating mice with standardized bacteria (17, 24). We showed that equol-producing capacity in ASF-treated mice was minimal (serum equol concentration was <35 nmol/L following a high IF diet). Importantly, we managed to induce equol-producing capacity in these mice after introduction of intestinal microbiota from normal apoE-null mice, thus providing genetically comparable equol producers. The serum equol concentration of these mice was low compared to normal apoE-null mice (25). However, this lower equol production was not

considered to be a major limitation since it is more comparable to that found in human equol producers. A person is categorized as "equol producer" if serum equol concentration is >83 nmol/L following a soy-based meal, although these levels are typically <20% of total serum IF (11, 26).

Studies investigating effects of soy IF and their metabolites on the reproductive system have reported equivocal or conflicting results. Most rodent studies have found that high doses of IF (>40 mg/kg body weight), genistein in particular, induced estrogen-like uterotropic effects (3). Similarly, genistein has been shown to increase vaginal epithelial height in rats (27). Studies of IF effects on the male rodent reproductive tract have been mixed, with some (25, 28-29) but not others (30-31) reporting estrogenic effects of IF. Studies of dietary soy IF or equol in male and female macaque monkeys have found no adverse effects on the reproductive tract, which is consistent with results from most human studies (32-37).

Our current results support the idea that soy IF do not have estrogen agonist effects in the reproductive tract of male or intact female mice when given in the diet, at doses relevant to human exposure, and within a soy protein matrix. This finding differed with our previous report that showed estrogenic effects of IF concentrates (100% aglycone) after 16 weeks of treatment (25), highlighting the importance of IF form in the diet as a determinant of absorption, metabolism, and downstream tissue-effects. Moreover, our results support the idea that equol, either racemic or naturally-produced, does not elicit estrogen agonist effects in mice at physiologic concentrations (<3000 nmol/L).

An interesting new finding of this study was that equol-producer phenotype, and not the soy diet itself, was associated with less estrogenic activity in the female reproductive system. We found lower uterine weight, uterine and endometrial size, endometrial luminal epithelial thickness, and vaginal epithelial thickness in mice with equol-producing intestinal microbiota. The relatively low serum equol concentrations (<300 nmol/L) associated with these effects suggest that equol-producing bacteria (rather than equol itself) altered endogenous estrogen

exposure in some way. Gut bacteria have been shown to produce enzymes that metabolize estrogens during enterohepatic circulation (38-40). In a small human trial, equol producers showed lower estrogen concentrations than nonproducers (41), which may be attributable to a higher fecal estrogen excretion (42). We did not measure circulating estrogen concentrations in our study, and therefore cannot distinguish between such indirect intestinal microbiota effects and potential direct effects of S-equol.

Another potential contributing factor is alteration in estrous cycle. While an equol-rich supplement (SE5-OH) did not alter estrous cycle in mice (16), others have reported that soy consumption in premenopausal women resulted in longer menstrual cycle length and suppression of gonadotropins (43), which may indicate an effect of soy IF on the hypothalamo-pituitary gonadal axis. This effect, however, was small and could not be confirmed by subsequent studies. Our results show that reproductive cycles differ by equol-producing ability. This supports the hypothesis that the equol-producing phenotype may have important health-related effects, irrespective of the amount of soy consumed (12). Several studies in women have associated soy-rich diet with reduced risk of endometrial cancer (44-46), whereas rodent studies have reported the opposite; increased risk with genistein (3, 47) and mild uterotropic effect of dietary soy (3, 48) and racemic equol (49-51). Our findings support the idea that equol-producing ability may reduce the endometrial response to dietary soy IF.

Exogenous equol used in this study was a racemic mixture containing 50% S-equol and 50% R-(+)-equol (R-equol). S-equol binds more strongly to ER-β whereas R-equol has more affinity to ER-α (52-53). A recent study showed that chronic feeding of S-equol had no effect on uterine weight, while R-equol did increase uterine weight in non-ovariectomized rats (54). In contrast, we observed the opposite effect in which mice treated with racemic equol mixture showed lower expression of PGR and proliferation marker Ki67 in the endometrium and vagina, suggesting less estrogenic exposure. Our finding may indicate that equol, like other isoflavonoids,

can elicit both estrogenic and anti-estrogenic responses depending on concentration of circulating estrogens and other factors.

Our results showed equol concentrations were greater in equol producers (relative to nonproducers) and in mice that received diet with exogenous equol supplementation. These data confirm the success of the microbiota and dietary interventions, and provide further support for the key role of gut microbiota in the equol-producing phenotype (55). We also found that soy IF had to be given in a high dose for our equol-producer model to produce endogenous equol. It should be noted that our LIF dose was equivalent to 20 mg IF/7531 kJ, which was only slightly lower than the typical daily IF consumption in Asian population (25-50 mg/d) (56), but it did not result in significantly higher serum equol concentrations relative to the CL control group. This may need to be considered for future studies using this model.

Levels of circulating genistein and daidzein were marginally higher in the equol producers compared to nonproducers, which may indicate a higher efficiency of the equol-producing bacteria not only to convert daidzein to equol but also to hydrolyze the non-bioavailable daidzein glycosides in the soy diet to the bioavailable aglycones. Interestingly, serum equol concentrations in equol producers were lower than nonproducers in mice fed an exogenous equol. This may indicate that equol-producing bacteria continue to reduce equol as substrate to further breakdown products, reducing uptake into the circulation. Alternatively, a higher equol intake with the exogenous equol may possibly inhibit the enzymes involved in production of equol from daidzein. Thus, daidzein may be shunted to O-desmethylangolensin (ODMA) and dihydrodaidzein, which we did not measure.

Although serum equol concentrations were the same across both sexes, we found a significant sex effect on genistein and daidzein metabolism. Females showed higher genistein and daidzein concentrations than males. This is consistent with previous reports that showed women had longer excretion half-life values of daidzein and genistein (57) and higher peak concentration with lower clearance rate of daidzein (6) compared to men. Our prior study using apoE-null mice

showed a similar pattern for genistein whereas equol tended to be higher in males (25). Overall, the results support the ideas that soy IF metabolism may be sex-specific and that caution is warranted when generalizing results from dietary soy intervention studies to the opposite sex.

Reports on the potential action of equol as a natural selective estrogen modulator have contributed to the recent development of equol-rich supplements for postmenopausal symptoms (14, 16, 58-59). It is not clearly understood, however, how equol affects the reproductive tract, particularly prior to menopause. Our results show that mice with equol-producing ability had less estrogenic reproductive tissue phenotypes relative to non-equol-producers. We speculate that the findings may be associated with endogenous equol effects on estrous cycle and other factors related to endogenous estrogen exposure, which require further investigation.

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Authors' contributions to the manuscript were as follows: M.R.A. and J.M.C. designed the study; F.N.D. and D.L.G. conducted the research; J.W.L and M.A.J.H generated serum isoflavonoid and fecal bacterial data; C.E.W. and J.M.C. performed histopathology evaluations; and F.N.D. analyzed the data and wrote the manuscript with significant contribution from C.E.W., A.A.F., and J.M.C. All authors read, contributed to, and approved the final manuscript. F.N.D. and J.M.C. had primary responsibility for final content.

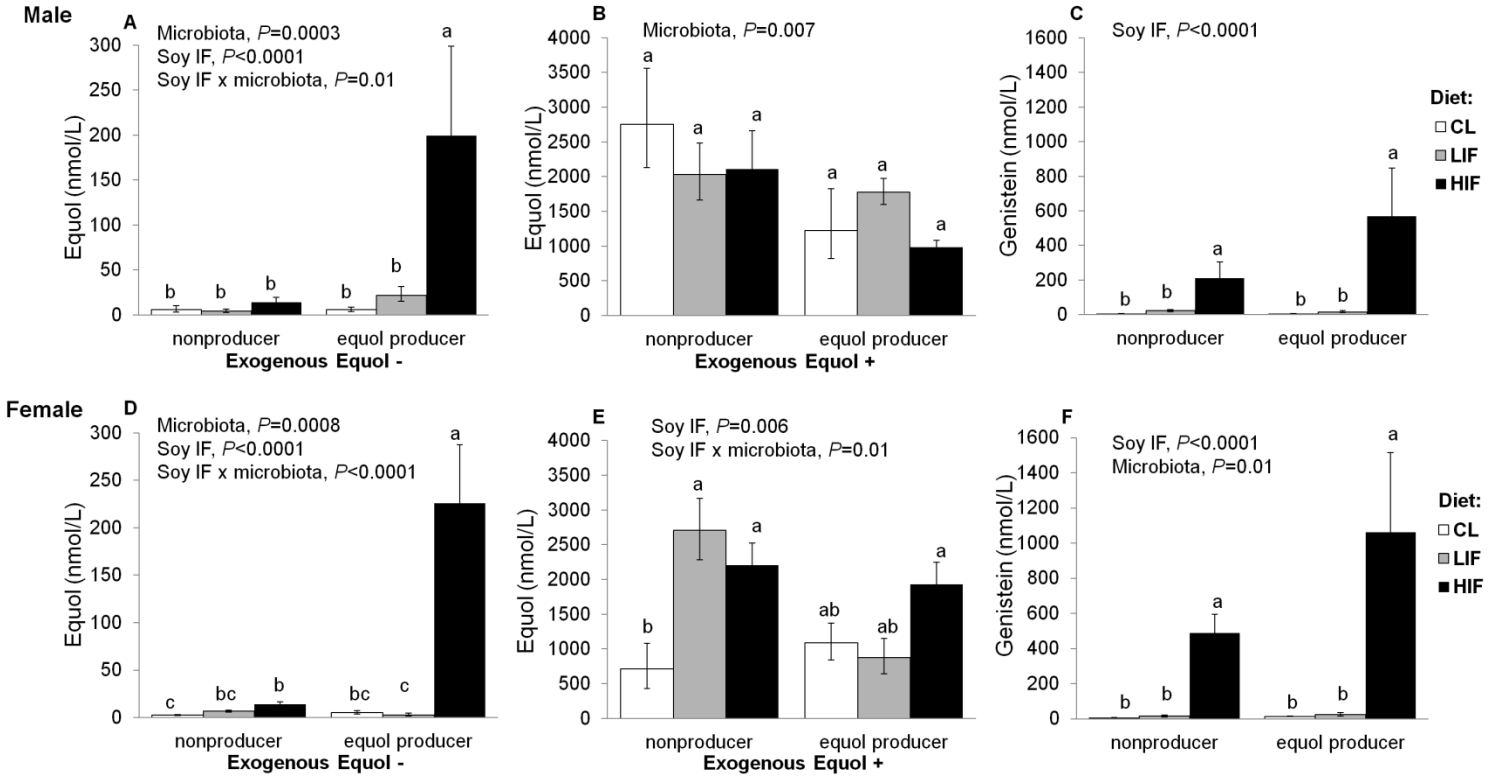


**Figure 1.** Serum equol (A,B,D,E) and genistein (C,F) concentrations in male (A-C) and female (D-F) apoE-null mice after 16 wk of dietary soy treatment with (B,E) or without equol (A,D) by microbiota status. Values are means ± SEM, $n$=5-10 or $n$=5 (B,E) per group. Significant main effects and interactions are shown. [a,b,c] Within each panel, labeled means without a common letter differ, $P$<0.01 or $P$<0.05 (E). CL, Casein and lactalbumin; HIF, High isoflavones; LIF, Low isoflavones.

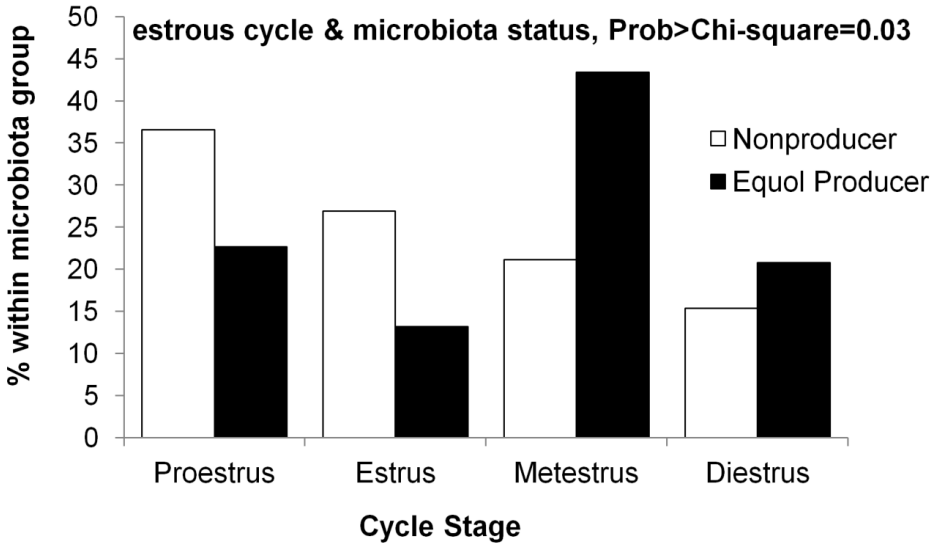


**Figure 2.** Distribution of estrous cycle stage by microbiota status in apoE-null mice after 16 wk of dietary soy and equol treatment. Values are percentage of mice within a microbiota group in a particular estrous cycle stage (proestrus, estrus, metestrus, diestrus) at the time of necropsy, *n*=52 (nonproducer) or *n*=53 (equol producer). Significant association is shown.

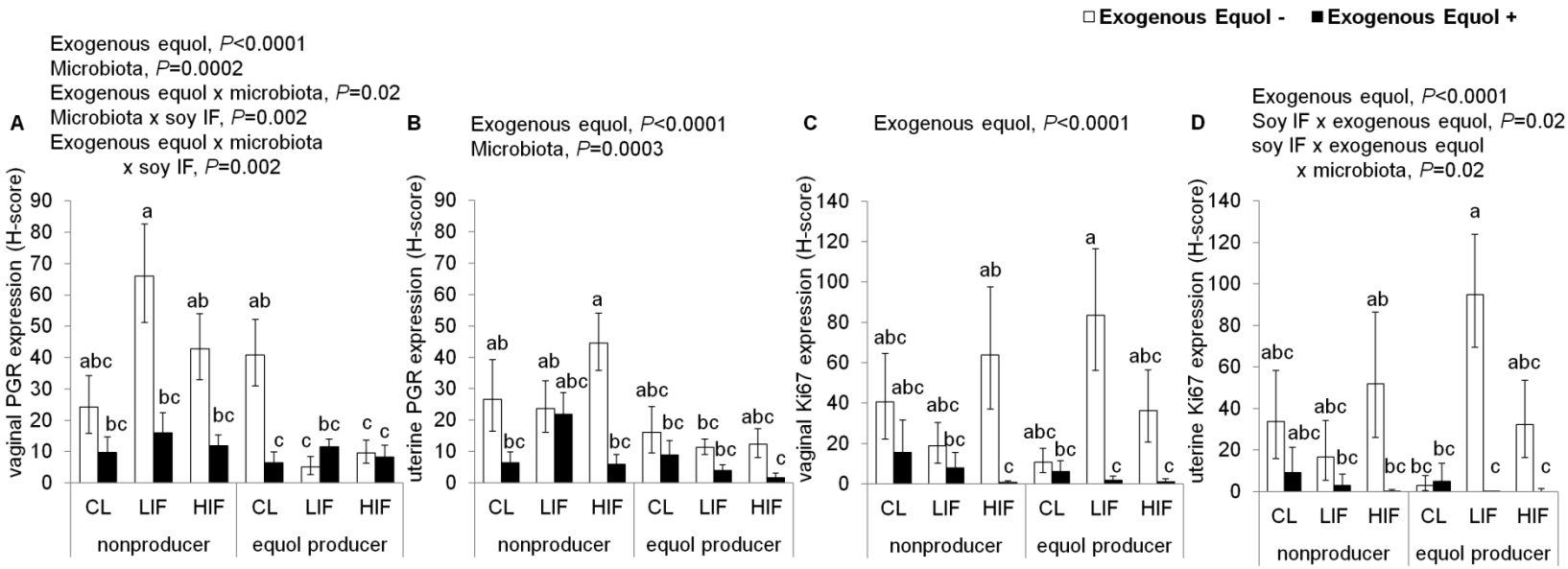


**Figure 3.** Expression of Progesterone Receptor (A,B) and the proliferation marker Ki67 (C,D) by immunostaining in the vagina (A,C) and uterus (B,D) of apoE-null mice after 16 wk of dietary soy treatment with or without exogenous equol by microbiota status. Values are means ± SEM, $n$=8-11 per group. Significant main effects and interactions are shown. [a,b,c] Within each panel, labeled means without a common letter differ, $P$<0.05. CL, Casein and lactalbumin; HIF, High isoflavones; LIF, Low isoflavones; PGR, Progesterone Receptor.

**Table 1.** Main effect of intestinal microbiota status on the reproductive tissue weight and histomorphometry of female apoE-null mice after 16 wk of treatment with dietary soy and equol[1].

| | Microbiota Status | | | |
|---|---|---|---|---|
| | Nonproducer | Equol Producer | *P*-value[2] | Adjusted *P*-value[3] |
| Uterine Weight, *mg* | 52.2 (49.6, 55.0)[a] | 45.8 (43.8, 47.9)[b] | 0.047 | 0.33 |
| Uterine Area, $mm^2$ | 1.58 (1.48, 1.68)[a] | 1.28 (1.21, 1.35)[b] | 0.015 | 0.30 |
| Endometrial Area, $mm^2$ | 0.87 (0.81, 0.94)[a] | 0.68 (0.64, 0.73)[b] | 0.014 | 0.30 |
| Endometrial Luminal Epithelial Height, $\mu m$ | 18.3 (17.9, 18.7)[a] | 17.0 (16.6, 17.4)[b] | 0.020 | 0.16 |
| Vaginal Epithelial Thickness, $\mu m$ | 101.3 (97.3, 105.3)[a] | 88.3 (84.7, 91.9)[b] | 0.013 | 0.25 |

[1] Values are mean (mean – SEM, mean + SEM), $n$=54-59 (nonproducer) or $n$=55-60 (equol producer). Labeled means (superscripts) in a row without a common letter differ, $P<0.05$.

[2] *P*-value for main effect of intestinal microbiota status.

[3] *P*-value for main effect of intestinal microbiota status after adjusting for estrous cycle stage as a covariate.

**Online Supporting Material**

**Supplemental Methods**

We assessed the effect of dietary soy IF on gut microbiota profile in equol-producing mice. Necropsy was done at 2 wk post dietary treatment on equol producers fed CL- or HIF- diets (*n*=5 per diet group, per sex). We extracted genomic DNA from fecal material (1). The fecal microbial community was measured on triplicate genomic DNA extractions using terminal restriction length polymorphism (T-RFLP), a 16S rRNA gene fingerprinting method (1-2). Peaks in the T-RFLP traces were analyzed as the arcsine transformed relative percent total peak area using multivariate analysis (Non-metric Multidimensional Scaling, NMS) (3) based on Sorenson's distance measure. Twenty-eight T-RFLP fragments, ranging from 56 to 478 bp, were identified using an internal standard of known size and the software package DAX (van Mierlo Inc.; the Netherlands). Data analysis was done using multi response permutation procedures (MRPP) (4).

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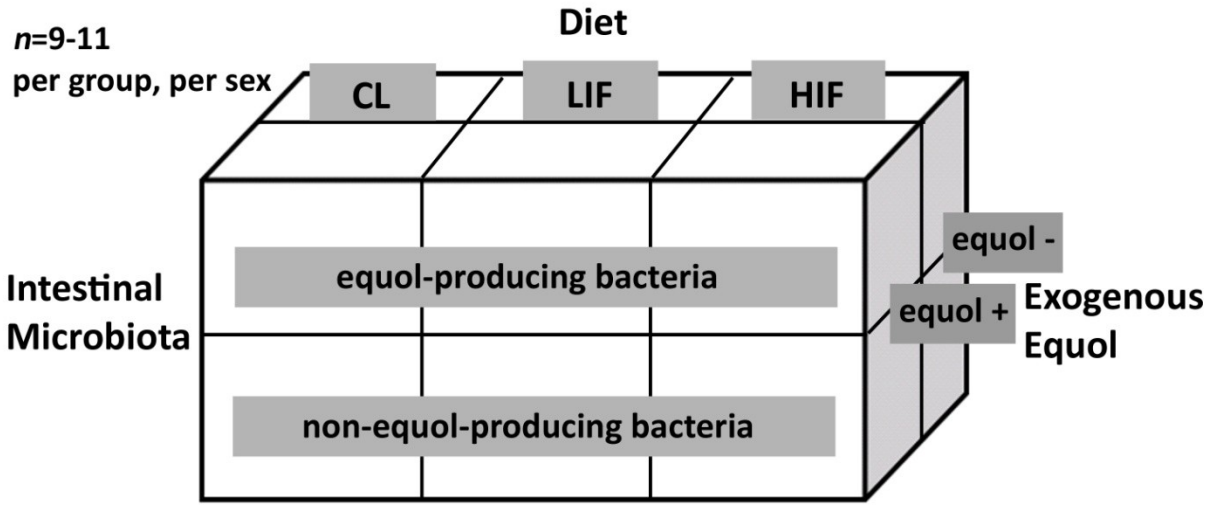
**Online Supporting Material**

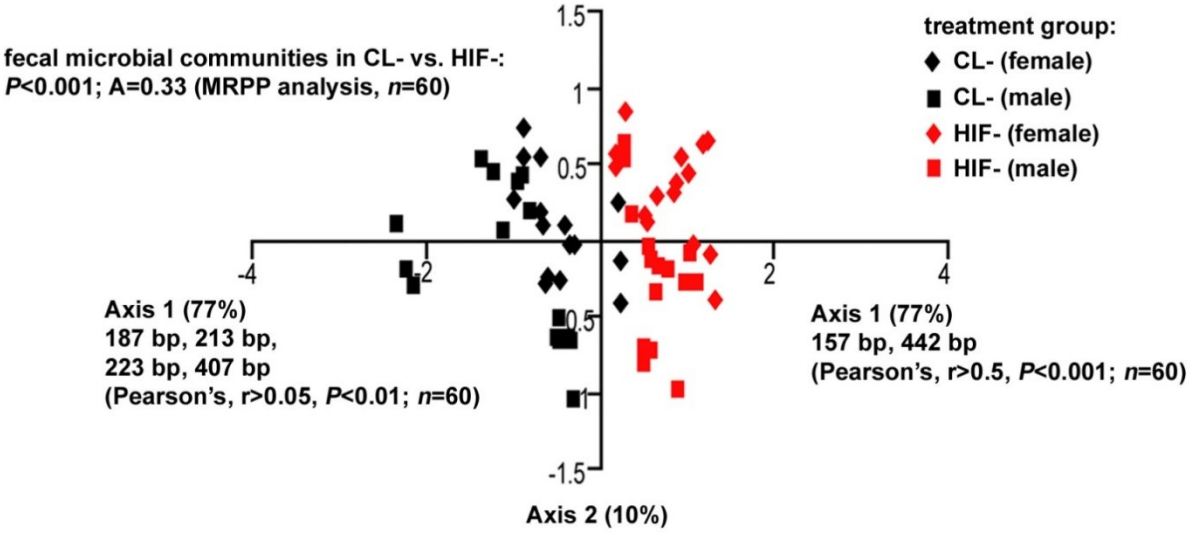
**Supplemental Figure 1**




**Supplemental Figure 1.** Three-way 3x2x2 factorial design to assess the effect of soy isoflavones diet, intestinal microbiota status, and dietary racemic equol on the reproductive tissues of apoE-null mice. CL, Casein and lactalbumin; HIF, High isoflavones; LIF, Low isoflavones.

**Online Supporting Material**

**Supplemental Figure 2**




**Supplemental Figure 2.** The fecal microbial communities from equol-producing apoE-null mice after 2 wk of treatment with CL- or HIF- diet. MRPP analysis showed a significant effect of diet on the gut microbiota profile. The T-RFLP peaks (in bp), a measure of the microbial composition, were significantly correlated with axis 1 and the diets, as indicated in the figure. Colors indicate diet groups; black is CL- and red is HIF-. Diamond and squares represent female and male mice, respectively. CL-, Casein and lactalbumin without exogenous equol; HIF-, High isoflavones without exogenous equol; MRPP, Multi Response Permutation Procedures.

**Online Supporting Material**

**Supplemental Table 1.** Compositions of the diet used to assess the effect of soy isoflavones and exogenous racemic equol on the reproductive tissues of apoE-null mice[1]

| Ingredient | CL- | CL+ | LIF- | LIF+ | HIF- | HIF+ |
|---|---|---|---|---|---|---|
| | | | *g/kg dry weight* | | | |
| Casein | 106.5 | 106.5 | | | | |
| Lactalbumin | 101.5 | 101.5 | | | | |
| Soy Protein Isolate (alcohol-washed)[2] | | | 201.5 | 201.5 | | |
| Soy Protein Isolate (intact)[3] | | | | | 201.5 | 201.5 |
| Total isoflavones | | | 0.04 | 0.04 | 0.57 | 0.57 |
| Racemic equol[4] | | 0.29 | | 0.29 | | 0.29 |
| DL-methionine | | | 3 | 3 | 3 | 3 |
| Corn starch | 231 | 231 | 231 | 231 | 231 | 231 |
| Maltodextrin | 75 | 75 | 75 | 75 | 75 | 75 |
| Sucrose | 280 | 280 | 280 | 280 | 280 | 280 |
| Cellulose | 100 | 100 | 100 | 100 | 100 | 100 |
| Lard | 52 | 52 | 48 | 48 | 48 | 48 |
| Safflower oil | 10 | 10 | 10 | 10 | 10 | 10 |
| Mineral Mix S10001[5] | 35 | 35 | 35 | 35 | 35 | 35 |
| Vitamin Mix V10001[6] | 10 | 10 | 10 | 10 | 10 | 10 |
| Choline Bitartrate | 2 | 2 | 2 | 2 | 2 | 2 |
| Yellow dye | 0.05 | 0.05 | 0.02 | 0.02 | | |
| Red dye | | | 0.02 | 0.02 | 0.02 | 0.02 |
| Blue dye | | | | | 0.02 | 0.02 |

[1] CL, Casein and lactalbumin; CL-, CL without exogenous equol; CL+, CL with exogenous equol; LIF, Low isoflavones; LIF-, LIF without exogenous equol; LIF+, LIF with exogenous equol; HIF, High isoflavones; HIF-, HIF without exogenous equol; HIF+, HIF with exogenous equol.

[2] Soy protein isolate (SPI) provided by Archer Daniels Midland Company (Decatur, IL). Total isoflavones (IF) contained in the isolate was 0.2 g/kg SPI, which consists of daidzin, genistin, glycitin, their malonyl and acetyl forms, and the aglycones (daidzein, genistein, glycitein). The ratio of daidzein:genistein:glycitein was 1:1.5:0.2.

[3] Soy protein isolate (SPI) provided by Archer Daniels Midland Company (Decatur, IL). Total isoflavones (IF) contained in the isolate was 2.8 g/kg SPI, which consists of daidzin, genistin, glycitin, their malonyl and acetyl forms, and the aglycones (daidzein, genistein, glycitein). The ratio of daidzein:genistein:glycitein was 1:1.5:0.2.

[4] Equol supplement used was a racemic mixture with 50% S-(-) and 50% R-(+) forms, provided by Solae, LLC (St. Louis, MO).

[5] Mineral mix S10001 composition (g/kg mixture): calcium phosphate, 500.0; magnesium oxide, 24.0; potassium citrate monohydrate, 220.0; potassium sulfate, 52.0; sodium chloride, 74.0; chromium potassium sulfate, 0.55; cupric carbonate, 0.3; potassium iodate, 0.01; ferric citrate, 6.0; manganous carbonate, 3.5; sodium selenite, 0.01; zinc carbonate, 1.6; sucrose, 118.03.

[6] Vitamin mix V10001 composition (in 10 g): retinyl palmitate, 4,000 IU; cholecalciferol, 1,000 IU; vitamin E acetate, 50 IU; menadione sodium bisulfite, 0.5 mg; biotin, 0.2 mg; cyanocobalamin, 10 µg; folic acid, 2 mg; nicotinic acid, 30 mg; calcium pantothenate, 16 mg; pyridoxine-HCl, 7 mg; riboflavin, 6 mg; thiamin-HCl, 6mg.

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# CHAPTER 4

## Effects of dietary soy and equol on the mammary gland of apolipoprotein E-null mice

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Fitriya N. Dewi performed the experiments, completed the statistical analyses and data interpretation, and prepared the manuscript. Cynthia J. Willson performed the histopathology review. Charles E. Wood provided advice on the overall study. Debbie L. Golden coordinated the parent study. Michael R. Adams was the principal investigator of the parent study. J. Mark Cline was the principal investigator of the study, and provided signficant guidance throughout the project.

**The effects of dietary soy and equol on the mammary gland of apolipoprotein E-null mice**[1]

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[7]Abbreviations used: ASF (Altered Schaedler Flora), CL (Casein and lactalbumin), ER (Estrogen Receptor), HE (Hematoxylin and Eosin), HIF (High isoflavone), IF (Isoflavones), IHC (Immunohistochemistry), LIF (Low isoflavones); ODMA (O-desmethylangolensin), PGR (Progesterone Receptor), SPI (Soy Protein Isolate).

SUPPLEMENTARY MATERIAL: none

**Abstract**

The capability to metabolize soy isoflavones (IFs) into equol may have a key role in determining the health effect of dietary soy. Soy intake is inversely associated with breast cancer risk, which has been thought to involve IF effects on breast development. In this study, we evaluated the interactive effects of soy IF diet, equol-producing phenotype, and exogenous racemic equol on the mammary glands of apoE-null mice (n=9-11/group/sex). We used gnotobiotic non-equol-producing mice (n=243); half of the colony were introduced to equol-producing microbiota at age 3-6 wk. Dietary treatment started at age 6 wk whereby mice received diet with either casein and lactalbumin (CL), alcohol-washed soy protein (low IF) or intact soy protein (high IF) with human equivalent dose of 0, 20 or 275 mg IF/d, respectively, with or without additional racemic equol (equivalent to human dose of 140 mg equol/d). After 16 wk of dietary treatment, we assessed total epithelial area and progesterone receptor (PGR) expression. There was no evidence for gynecomastia in the males. In females, total mammary epithelial area was not affected by IF dose or dietary equol; they did differ between equol producers and nonproducers that received low IF diet ($P<0.05$). Nonproducers fed only a racemic equol without soy IF showed higher PGR expression than equol producers fed the same diet ($P<0.05$). PGR expression was also higher than nonproducers that received racemic equol with low IF ($P<0.05$) or high IF ($P<0.0001$). The findings indicate a subtle interactive effect of soy IF and equol on the breast. Our results suggest that long-term dietary soy IF intake *per se* has little effect on the mammary gland. If anything, there could be a modest effect of endogenously-produced equol and/or the equol-producing microbiota on mammary growth, limited to those exposed to low soy IF diet. Exogenous racemic equol may potentially elicit steroid receptor agonist/antagonist effect on the mammary gland, depending on the microbiota status and the co-presence of other isoflavonoids.

**Introduction**

Epidemiological studies suggest that dietary soy intake is inversely associated with breast cancer risk, most consistently when the exposure occurs earlier in life (1). The effect has been attributed to isoflavones (IFs), the phytoestrogenic compounds in soybeans that can bind to estrogen receptor (ER) and elicit estrogen agonist/antagonist action. The major IFs contained in soy foods are genistein, daidzein and glycitein. In most animals, daidzein is further metabolized into equol by the intestinal microbiota (2), and equol becomes the predominating circulating isoflavonoid. Interestingly in humans, only 30% of the Western population and 60% of Asians and vegetarians have the capability to produce equol (3).

Equol is present as two enantiomers: the naturally produced S-(-) equol (s-equol) or the chemical R-(+) equol (r-equol). These enantiomers can elicit different biological effects (2, 4). Equol has greater binding affinity to ER compared to its precursor daidzein, more comparable to genistein (5). Similar to those IFs, equol preferentially binds to ERβ and is suggested to share the characteristics of a natural selective ER modulator (4). Importantly, the ability to produce equol has been shown to affect an individual's response to soy consumption (6-7). Despite the biological effect of equol, daidzein-metabolizing capability is rarely taken into account in epidemiological studies that assessed the health effect of dietary soy. This paradigm has started to shift in the recent years. Although still limited, more human studies have included the identification of equol-producing phenotype in their subjects (8). The overall association between this phenotype and breast cancer risk remains somewhat mixed; some showed differential effect of soy treatment on mammographic density in equol producers and nonproducers (9-10) while others reported no effect of equol-producing ability (11-12). Although it remains unclear whether equol is in fact the major contributor of the response to soy consumption or merely a marker for a certain gut microbiota profile, the interest on equol-based dietary supplement for various health conditions has been increasing (13-14).

We have previously reported the anti-estrogenic effect of equol on the reproductive tissue of female mice (15). In the current study, we extended the use of tissues collected from the same experiment to assess the interactive effect of IF-containing diet, and endogenous and exogenous equol on the mammary gland of adult intact apoE-null mice.

## Materials and methods

### *Mice and Diets*

The current study used mammary tissues (i.e. inguinal mammary gland) collected from adult male and female apoE-null mice of different microbiota status and dietary treatments. The study design has been described previously (15). Briefly, the study started with apoE-null mice harboring the specific microbiota Altered Schaedler Flora (16) ($n$=243), which were non-equol-producers (nonproducers). Half of the colony was exposed to intestinal microbiota from normal apoE-null mice at the age of 3-6 wk to gain equol-producing capacity (equol producers). At age 6 wk, both equol producers and nonproducers were randomly assigned to dietary treatment groups with $n$=9-11/group, per sex. The diets given to each group were casein-lactalbumin diet (CL), alcohol-washed soy protein diet (low IF), or intact soy protein diet (high IF) to mimic human equivalent doses of 0, 20 or 275 mg IF/7530 kJ, respectively. Each diet contains no exogenous equol (CL-, LIF-, HIF-) or a R/S ($\pm$) racemic equol (CL+, LIF+, HIF+) with an equivalent dose of 140 mg equol/7530 kJ. The levels of circulating isoflavonoids after consuming these diets have been previously reported (15). Archer Daniels Midland Company (Decatur, IL) provided the isolated soy proteins, Solae, LLC (St. Louis, MO) provided the racemic equol, and all diets were manufactured by Research Diets (New Brunswick, NJ). All breeding and procedures involving mice were done at Taconic Biotechnology in their AAALAC accredited facilities in Germantown and Rensselaer, NY and were approved by the Taconic institutional animal care and use committee.

Mice were euthanized after 16 wk of dietary treatment. Mammary gland was collected and fixed in 10% neutral buffered formalin. After 24 h, tissues were removed from formalin and stored in 70% ethanol. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). Histopathology examination of the mammary gland tissues was done by veterinary pathologist (C.J.W.) in consultation with board-certified veterinary pathologist (J.M.C.). Estrous cycle stage was determined by evaluating the histology of the reproductive tissues as previously reported (15). Mice that did not meet all the criteria of a cycle stage (17), or those that showed persistent estrus were excluded from estrous cycle-related analyses.

***Tissue histomorphometry***

HE-stained slides were evaluated for the presence of mammary glandular structures. The mammary tissue of male mice did not show appreciable epithelial structures and were excluded from further evaluation. The slides were used for histomorphometric assessment of total mammary gland epithelial area (18); we utilized Infinity 3 digital camera (Lumenera) and Image Pro-Plus 5.1 software (Media Cybernetics). Using 2X magnification, we manually traced any glandular epithelial structures (i.e. ductal and lobular) within the tissue to derive percentage of total epithelial area relative to the total area examined. Lymph nodes and muscle (when present) were excluded from the measurement.

***Immunohistochemistry***

We used a biotin-streptavidin-alkaline phosphatase staining method as previously described (15) to assess the immunolocalization of progesterone receptor (PGR) in the mammary tissue of the female mice, as a marker for ER-regulated activity. The antibody used was anti-PR sc-538 (Santa Cruz Biotechnology). All measurements were made blind to treatment groups. Positively stained cells were given a score based on staining intensity to obtain a semi-quantitative measurement of intensity and distribution by H-score (19-20).

### *Data analysis*

Non-normally distributed data were log-transformed or square-rooted for analysis, and retransformed to original scale for presentation of results. We performed a 1-way, 2-way, or 3-way ANOVA by Fit Model procedure in JMP (version 10.0.0, SAS Institute) to model the main and interactive effects of dietary soy, microbiota status and exogenous equol to compare total epithelial area and the immunolocalization of PGR in the female mammary tissues. All nonsignificant fixed effects and interactions ($P \geq 0.1$) were excluded from the final model. Post-hoc comparisons were made using least square means Tukey honestly significant difference (HSD) test. Estrous cycle stage was expressed as a categorical variable and included in the model as a covariate.

## Results

### *Histopathology*

No neoplasms were seen in this study. Across all males ($n$=122), there were 7 mice that showed appreciable ductal structures in the mammary gland; these mice were of different diet groups and microbiota status. Only 1 out of the 7 had relatively dense glands, which was a nonproducer fed a HIF- diet. The finding was not treatment-related and considered as incidental. Other incidental lesions were present in few mice: xanthomatous dermatitis within the subcutis adjacent to the mammary gland found in 2 females, panniculitis (multifocal, mild to moderate, pleocellular) in 3 males, and lymphoplasmacytic infiltrates in the mammary gland of 1 female.

### *Mammary epithelial area*

Regardless of treatment, the adult virgin mouse mammary gland consisted of <5% epithelium. There was no main effect of microbiota status, diet or exogenous equol on total mammary epithelial area. As shown in Figure 1, we found interactions of soy diet x exogenous equol x microbiota ($P<0.05$), soy diet x microbiota ($P<0.05$), and exogenous equol x microbiota

($P$=0.05), with a marginally significant effect of estrous cycle stage (P=0.06). The proportion of epithelial area was lower in the equol producers than nonproducers, limited to those receiving LIF- ($P$<0.05). Total epithelial area was higher in mice at diestrus (2.4 $\pm$ 0.2 %) compared to those in proestrus (1.7 $\pm$ 0.1 %) ($P$=0.05).

***Immunohistochemistry***

We found a main effect of diet on PGR expression on the epithelial structure of the mammary gland ($P$<0.0005) along with significant interactions between soy diet x exogenous equol ($P$<0.01) and soy diet x exogenous equol x microbiota ($P$<0.005) (Figure 2). There was a higher PGR expression with CL+ compared to LIF+ ($P$<0.05) and HIF+ ($P$<0.0001) limited in nonproducers. PGR expression in nonproducers fed CL+ was also significantly higher than CL- ($P$<0.005), and compared to equol producers fed a same diet ($P$<0.05). A difference by microbiota status was found only in mice fed CL- whereby equol producers showed higher PGR expression than nonproducers ($P$<0.05).

**Discussion**

We investigated the effects of dietary soy IF, endogenously-produced equol and exogenous equol on the mammary gland of adult apoE-null mice. There was no evidence of treatment-related gynecomastia observed in the males, and we did not find lesions indicative of adverse estrogenic effect or neoplasia in the mammary gland of adult female mice. Total mammary epithelial area did not seem to differ by IF dose or dietary racemic equol. There was a difference between equol producers and nonproducers limited to those fed a low IF diet whereby the total epithelial area was significantly lower in the equol producers. This difference did not appear to be associated with PGR expression. Immunolocalization of PGR was high in nonproducers fed a racemic equol diet without soy IF, and low when equol was given with IF. Our results suggest that dietary exposure to soy IF has negligible effect on mammary gland

development in apoE-null mice. If anything, there could be a microbiota effect with a low IF dose. On the other hand, exogenous racemic equol may potentially elicit a subtle steroid receptor antagonist/agonist effect on the mammary gland, depending on the microbiota status and the co-presence of other isoflavonoids.

Although the general notion is that soy does not have feminizing effects on men (21), there has been a case report of gynecomastia in a 60-year old male who consumed a soy product (22). The subject was reported to have consumed a high dose of IF reaching >300 mg IF/d and showed a 4-fold increase in circulating estrogen; the gynecomastia resolved after discontinuation of drinking soy milk. An extremely high dose of IF is possible to cause a dysregulation of estrogen-androgen metabolism, which we did not measure in the present study. The IF dose used in our study was high, although still lower than the reported human case; we found very few males (7 out of 122) had some degree of ductal development, whereby one male (a nonproducer) in the high IF group had appreciable ductal structures. Based on the fact that only a few mice had this lesion, which also did not show a treatment-related pattern, we suspected that the finding was incidental. The lack of estrogenic effect of soy exposure in the male mammary gland is consistent with prior reports in macaques (23) and humans (24). We previously reported no effect of diet, microbiota status or exogenous equol on the testis weight of these male mice. Importantly, there was no histopathological lesion found in the testicles, epididymis and accessory sex glands, which indicated the lack of estrogenic effects on the male reproductive tract (15). In contrary, however, a previous work in our lab found that consumption of soy aglycone IF concentrates (i.e. as 100% aglycones) elicited estrogenic effects on the reproductive tissues of male mice, which was shown by reduced plasma testosterone levels, atrophy of seminiferous epithelium, atrophy of accessory sex glands, and squamous metaplasia of seminal vesicles; mammary gland was not assessed in the study (25). The opposite findings highlight the importance of IFs form in the diet (i.e. aglycone vs. conjugated) to determine the tissue effects.

In this study, mice were started on a soy IF diet around the peripubertal age, the stage where the breast undergoes the most rapid ductal growth in females (26). We did not find a significant difference in the total epithelial area of the mammary gland by adulthood, which may suggest that the growth of mammary gland were not overtly affected by exposure to dietary soy and/or exogenous racemic equol in this model. Nulliparous mouse mammary development is mainly a process of ductal branching. As reviewed by Richert et al. (26), prior to adulthood the development process consists of terminal end buds (TEB) elongation, primary duct branching and sprouting of the secondary branches. TEBs are usually regressed by the age of 10-12 wk. In the adult breast, lateral and alveolar buds develop to give rise to more ductal branching, and eventually rudimentary alveolar structures. Short tertiary side-branching occurs in response to cycling ovarian hormones. Consistent with this, we showed that mammary gland of adult virgin mice consists mainly of ducts with scant formation of lobuloalveolar structures. There was a marginal effect of estrous cycle where mice in proestrus and diestrus appeared to show the lowest and greatest proportion of epithelium, respectively. This is in line with studies that showed the highest proliferation of mammary epithelial cells during metestrus which leads to prominent lobular development by metestrus-diestrus (27-28).

Nulliparity is a risk factor for breast cancer, which is thought to be partially explained by the lack of terminal differentiation of the mammary gland before pregnancy. In the adult rodent breast, the immature glandular structures undergo mild proliferation, some level of differentiation and involution during phases of the estrous cycle (28). These actively dividing cells are more prone to carcinogenesis and DNA damage. The breast of adult virgin mouse also consists of undifferentiated stem cells that are highly responsive to hormones such as estrogen and progesterone for their cell-fate regulation (29-30). While dietary soy and racemic equol did not appear to affect the proportion of epithelial structures, we found that equol producers and nonproducers respond differently to IF. There appeared to be less proportion of epithelial cells within the breast of equol producers fed a low but not high IF. It is unclear whether the smaller

proportion of epithelial area indicates a phenotype of a lower cancer risk (i.e. lower amount of highly proliferative ductal structures) or a sign of adverse inhibition of glandular development. S-equol has been shown to enhance terminal duct differentiation into lobular structures in the mammary of a rat model (31). However, we were not able to distinguish between ductal and lobuloalveolar compartments in our study. Further, the equol producing capability in our mouse model was lower than that of a wild type mouse whereby the serum equol concentration did not differ between nonproducers and equol producers when fed a low dose IF (15). Therefore, the differential mammary gland growth did not appear to be mediated by endogenous equol. It is also possible that the interaction between gut bacteria and diet affects certain metabolic pathways (e.g. estrogen, progesterone) that could potentially have an impact on the breast (32-33).

To evaluate the effect of soy on estrogen action, PGR expression was assessed. PGR is one of the classic markers of ER-mediated activity; the gene is regulated by the binding of estrogen-occupied ER to the estrogen responsive element half site within the promoter (34). Here, we showed a differential PGR expression with racemic equol particularly among nonproducers. This finding might suggest an estrogen-agonist effect of racemic equol in the adult virgin mammary gland. Our finding was consistent with a study in rats that showed higher cell proliferation and PGR expression following a high dose of dietary racemic equol (35). Interestingly, the co-presence of IFs (i.e. via dietary IF consumption) appeared to drive a contradictive effect; there was a lower PGR expression following intake of racemic equol with a high IF diet. Although equol was the predominating isoflavonoid in the circulation of these mice, the presence of genistein might affect ER-binding and subsequent ER activity. Genistein has a higher affinity to ER compared to equol (5); genistein may antagonize the effect of estradiol by competitive binding to ER (36). Collectively, the observed effects were less clear compared to our previous findings in the reproductive tissues that showed anti-estrogenic effect of equol (15). This observation may suggest that equol action is likely to be tissue-specific. It was initially thought that the importance of PGR in the breast was mainly pertaining to estrogen regulation.

However, more studies have shed the critical role of progesterone and its receptor for mammary gland development, proliferation, and cell-fate determination (37). While adolescent branching requires estrogen and ER, adult tertiary side branching requires progesterone and PGR (38). It is unclear whether the effect of racemic equol on PGR expression found in this study was indicative of an altered ER activity or progesterone/PGR-mediated signaling.

The mechanism behind nutritional modification for mitigating disease risk involves a complex interaction between the hormonal condition, biological compound of the diet, as well as gut microbiota. Our results suggest that dietary soy *per se* does not have a strong effect on mammary gland development, whereas microbiota status may modestly affect mammary tissue response to the soy treatment. Exogenous racemic equol may have steroid receptor-modulatory properties, and the biological action of equol may be affected by co-presence of other IFs.

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**Figure 1**

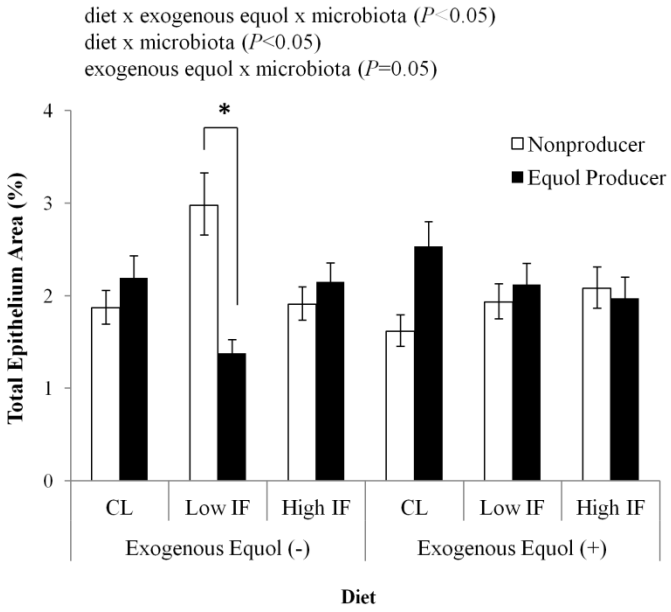


**Figure 1.** Total epithelial area in the mammary gland of equol-producing and nonproducer female apoE-null mice after 16 wk of treatment with dietary soy isoflavones and racemic equol (*n*=8-11 per group). Values are least square means (LSM) and error bars represent SEM. The significant main effects and interactions are indicated in the graph. Asterisk (*) indicates significant difference with *P*<0.05 by LSM Tukey HSD test.CL, Casein and lactalbumin; IF, isoflavones.

**Figure 2**

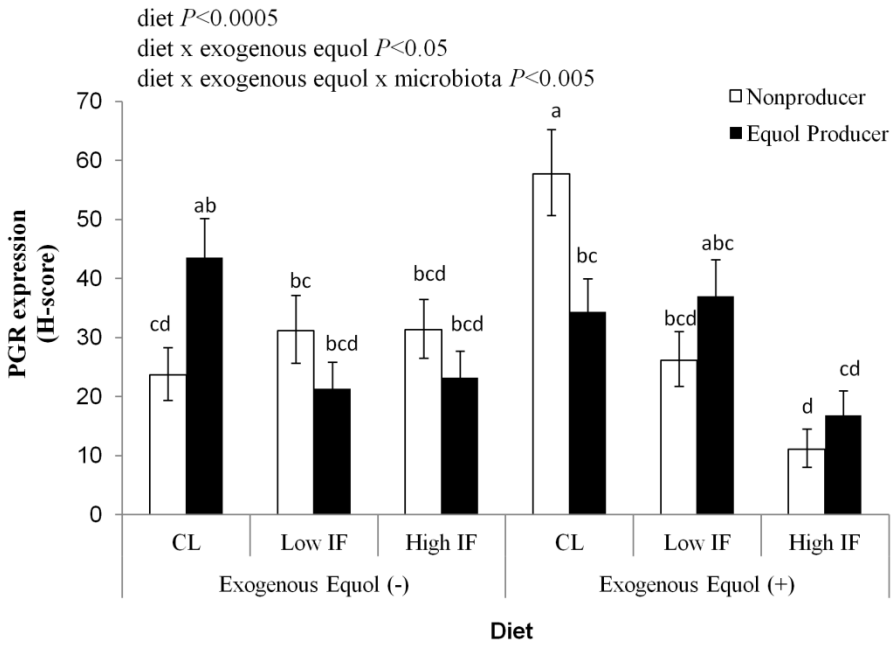


**Figure 2.** Progesterone receptor expression by immunostaining in the mammary gland of equol-producing and nonproducer female apoE-null mice after 16 wk of treatment with dietary soy isoflavones and racemic equol ($n$=8-11 per group). Values are least square means (LSM) and error bars represent SEM. The significant main effects and interactions are indicated in the graph. [a,b,c,d] Within each panel, labeled means without a common letter differ, $P$<0.05 by LSM Tukey HSD test. PGR, Progesterone receptor; CL, Casein and lactalbumin; IF, isoflavones.

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## SUMMARY AND DISCUSSION

The primary aim of this project was to investigate the effect of soy exposure beginning pre-adulthood on the breast and uterine tissues in relation to estrogen pathways. We utilized two animal models: cynomolgus macaques and apoE-null mice. The macaque study was specifically designed to longitudinally assess tissue changes across pubertal development to model young/adolescent girls consuming a North American diet with or without soy IF exposure. Our data showed that soy intake did not alter pubertal growth and onset of menarche. Importantly, soy exposure initiated at puberty appeared to enhance mammary gland lobular differentiation which resulted in breast composed of more abundant mature glandular structures in adulthood. This phenotype was observed along with modestly lower expression of ERs and ER activity markers, suggesting that early soy exposure could possibly result in lower estrogen responsiveness in the adult breast. These changes were not explained by differential CpG methylation within the promoter regions examined, level of circulating estradiol and progesterone, or expression of enzymes for intra-mammary estrogen biosynthesis and metabolism. In the mouse study, animals' gut microbiota were modified to attain equol-producing vs. non-equol-producing capability, a discrepancy in IF-metabolizing profile observed in human populations but not in macaques. Mice were exposed to soy IF diet with or without dietary equol starting at peripubertal age. Our results showed that adult female equol producers had lower estrogenic reproductive tissue phenotypes compared to non-producers. We also found that exogenous equol, and not soy IF diet *per se,* can modulate estrogen-dependent tissue responses, which was also influenced by microbiota status; the effect was less clear in the mammary gland. Our findings indicate the importance of endogenously-produced equol and/or the associated gut microbiota to affect estrogen-dependent response.

**Pubertal development**

Puberty is a complex event that involves orchestration between gonadotropin-releasing hormone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and steroid hormones. Exposure to circulating endogenous estradiol during puberty leads to mammary gland and uterine morphogenesis and growth along with bone growth. In the final phase of puberty, menarche occurs as a result of the long exposure to estradiol which exerts negative feedback on the hypothalamic-pituitary-gonadal (HPG) axis to induce cyclical estrogen production, ovulation, and uterine bleeding. Ovulatory cycles then follow after the mechanism of estradiol-induced LH surge is established (1).

*a. Mammary gland*

Unlike most tissues that are largely developed during fetal life, the mammary gland remains in a primitive form after birth. Critical morphogenesis occurs under the effect of hormones and growth factors during important post-natal events such as puberty, pregnancy, lactation and menopause (2). These reproductive stages and their hormonal regulation affect gene transcription within the mammary gland; gene families and pathways that change across different life stages include those related to estrogen, androgen, prolactin, insulin-like growth factor signaling, extracellular matrix, and differentiation (3). Puberty is the first turning point for mammary gland differentiation. The duration of pubertal breast development (i.e. from breast growth spurt to menarche) is approximately 2-3 years in humans (4); the biology of the process has been described based on schematic impressions and more recently based on studies utilizing limited numbers of autopsy samples (2, 5). Our current study demonstrates the unique value of macaque model to longitudinally illustrate the morphological pattern of pubertal ductal branching and lobuloalveolar differentiation spanning ~2 years before and after menarche transition. During the early phase of pubertal breast development, ductal structures elongate and divide, followed by formation of terminal end buds (TEBs) that subsequently give rise to new branches and alveolar buds or small ductules. The ductules cluster around a terminal duct and form lobuloalveolar units.

This structure is the functional unit that undergoes further differentiation throughout life whereby the ductules increase in number and size. Using the nomenclature developed by Russo et al. (6), lobuloalveolar or lobule units in the macaque breast can also be categorized into types 1, 2 and 3 which consists up to 11, 47 and 80 ductules per unit, respectively. As in humans, the lobular differentiation profile in the macaque breast highly varies between individuals. While the immature lobule type 1 can be formed as early as 2 years before menarche, the more differentiated lobule type 2 is typically formed closer to menarche. The biggest change in glandular differentiation can be found around the menarchal transition, and the complexity of the lobuloalveolar unit further increases with recurrent menstrual cycles. By early adulthood, the macaque breast is composed mainly of type 1 and type 2 lobules. Type 3 lobules, which typically predominate only when pregnant and lactating, can also be found; this is similar to the breast in nulliparous women (7). This marked lobular development is lacking in rodent models. As shown in the present mouse study and others, the adult nulliparous mammary gland of rodents consists mainly of ducts, with scant lobuloalveolar structures. The remarkably comparable developmental profile of macaque breast to humans highlights the importance of this species as a valuable translational model system.

*b. Uterus*

Like the mammary gland, the uterus also develops very slowly prior to puberty. The development of endometrial glands involves multiple factors which include estrogen, progesterone, pituitary hormones and growth factors (8). In macaques and humans, the endometrium is inactive between birth and onset of puberty, consisting of rudimentary glands (9, 10). Any developmental process occuring at this stage has been thought to be an ovary- and steroid-independent process. During puberty, the uterine and endometrial size increase progressively due to the marked increase in estrogen and progesterone. Following the onset of menarche, the endometrium starts to undergo cyclical proliferative (follicular), secretory (luteal), and menstrual phases under the regulation of ovarian steroids and their receptors, resulting in

cyclical change in the thickness and size of the endometrium. At each menstrual phase, the superficial part of the endometrium regresses and is shed, along with blood loss from the stromal vessels, a feature unique to some primate species including macaques and humans.

In mice and rats, the uterus also lacks endometrial glands at birth, consisting of very simple epithelium supported by undifferentiated mesenchyme. Early post-natal uterine development is minimal and ovary-independent. Endometrial glands are formed across 1-2 weeks of age under the effect of systemic estrogen that is thought to begin at this age. The morphogenesis ends in very simple tubular glands (8). After puberty, the uterus in adult nulliparous animals also undergoes cyclical changes (i.e. estrous cycle) whereby uterine size, endometrium thickness, and endometrium luminal epithelium height change in concert with the hormonal fluctuation across the estrous cycle (11). Unlike humans and macaques, however, there is no endometrial sloughing (i.e. menstruation) in rodents.

**Mammary gland differentiation and the susceptibility to later-life cancer risk**

The concept of breast cancer risk protection by mammary gland differentiation initiated from the fact that early-age parity and nulliparity are associated with breast cancer risk in contradictive ways, which may be contributed in part by terminal differentiation of the gland that can only be attained during pregnancy (12). It was hypothesized that induction of breast differentiation by either pregnancy or hormonal treatment rids the population of cells that are susceptible to carcinogenesis (13). The theory has evolved from merely a hormonally-induced promotion of differentiation to "altered cell fate" hypothesis, whereby a hormonal milieu during key reproductive events (e.g. puberty, pregnancy) is thought to induce a molecular switch in stem cells to result in persistent changes in the intracellular regulation that governs cell proliferation and response to DNA damage (14).

All epithelial structures of the mammary gland (i.e. ductal, lobular and myoepithelial) initially originate from a common multipotent stem cell (15). Puberty is a critical stage for

determination of cell lineage commitment (16); it has recently been suggested that the expansion and maintenance of each cellular lineage during puberty is governed by multiple types of lineage-restricted stem cell (17). The adolescent breast is thought to have the highest number of stem cells that are likely to be dispersed across the entire gland with potent but limited self-renewal capacity, and highly susceptible for initiation of breast cancer development (18, 19, 20). After menarche, mammary epithelium undergoes cyclical proliferation and regression during each luteal phase, which indicates that the self-regenerative potential of progenitor cells remains in adulthood (20, 21, 22). We have previously showed that the mRNA expression of stem cell markers is high in peripubertal macaque breast or during pregnancy, indicating the high activity of stem cells during the important stages of mammary gland development (3). Breast cancer has also been shown to mainly originate in the undifferentiated cells within the terminal duct of a lobular unit which are vulnerable to chemical-induced carcinogenesis (23, 24), further highlighting the association between breast differentiation state and cancer risk.

**Estrogen responsiveness in the pubertal breast**

Pubertal mammary gland development involves various hormones and complex signaling factors (25). Pathways activated during pubertal breast development have also been implicated in cancer development and metastasis, indicating the potential of studying developmental markers during this stage as biomarkers for breast cancer risk (3). The focus of our current study is mainly on estrogen and ER-regulated genes. In the macaque breast, the expression of ERα and estrogen-regulated markers are the highest in pubertal animals compared to those of other reproductive stages (3). In the current study, we further demonstrated that the expression was high in early puberty and decreased with maturity, suggesting high estrogen responsiveness in the breast during peri-puberty. This finding supports the notion that a low estrogen milieu is likely to induce breast morphogenesis at puberty. In pre-pubertal children, estradiol level is correlated with palpable breast albeit a very low endogenous estrogen level is detected (26).

The lower level of ERα in adulthood could be attributed to the high endogenous estradiol after menarche; we observed a significant inverse correlation between estradiol and ERα expression. Studies have reported that estradiol can induce suppression of ERα mRNA via ligand-receptor interactions such as by recruitment of protein complex to the promoter that affects histone modifications (27), and via ER-mediated post-transcriptional suppression mediated by other gene products (28). ER protein suppression by estradiol may be achieved via ubiquitin-proteasome pathway (29). Additionally, the high level of progesterone post-menarche may also contribute to the reduction of ER; in this study we also found an inverse correlation between the circulating progesterone and ER expression. Progesterone may downregulate ER by enhancement of ER turnover and inhibition of estradiol-induced ER replenishment (30, 31). Unlike ERα, the role of ERβ in the normal and cancerous breast is not well established. In the present study we showed that overall ERβ expression was consistent across pubertal development.

Epigenetics play a role in normal mammary gland development and differentiation. It was shown that epigenetic regulation by histone modification or DNA methylation is involved in stem cell renewal, pluripotency and differentiation (32). Epigenetics is dynamic, especially during periods where cells and tissues are highly developing (33). An inverse relationship between DNA methylation and gene expression in the normal breast has been reported (32, 34, 35), although data are fairly limited. ERα gene expression in breast cancer cell lines can be modulated by promoter methylation (36), but our study suggests that the decreasing level of ERα mRNA with maturity in the mammary tissue is not accompanied by a change in the methylation level within *ESR1* promoter B, at least on the several CpG sites examined herein. Whether other *ESR1* promoter regions behave otherwise during pubertal development remains to be investigated in future studies.

Among the classic estrogen-regulated markers (i.e. *TFF1, PGR* and *GREB1*), *TFF1* appeared to be the only marker that is epigenetically regulated over time, supporting the importance of promoter methylation for tissue-specific expression of *TFF1* (37). Despite a higher

circulating estradiol after menarche, *TFF1* expression decreased with maturity. Although this transcriptional pattern could be related to the reduced number of ER present in the adolescent breast, it is also likely that the lower transcription is in part regulated by the change in CpG methylation within the promoter region. The promoter region of *TFF1* contains ERE to which the ER can bind and activate transcription. Here, we showed that the methylation level of CpG sites flanking the ERE showed >7% increase after menarche regardless of diet treatment. In the *in-vitro* setting, such a differential is enough to suppress gene expression (38). Methylation can repress transcriptional activity by at least two ways: directly by extension of methyl group to the major groove of DNA which can inhibit transcription factor binding, or indirectly by recruiting methyl-binding protein which will code for histone modifications to change the chromatin structure (39, 40).

**The effect of pubertal soy exposure on the breast**

***1. Lobular differentiation***

Pubertal breast can be a model system for studying epithelial cell morphogenesis and cancer susceptibility (41), and modulating breast differentiation during this life stage may have a long term impact on breast cancer risk. Interestingly, the association between soy intake and reduced risk of breast cancer is most consistently observed when the exposure occurs starting periods preceding puberty (42). Several rodent studies indicated that exposure to soy during pre/peri-puberty may reduce chemically-induced tumorigenesis by increasing mammary gland differentiation. Genistein exposure by injection or dietary treatment has been shown to decrease TEBs, increase lobuloalveolar numbers, and reduce tumor multiplicity or incidence (43, 44, 45). This present study is the first to show that in the primate breast, pubertal exposure to dietary soy may promote mammary gland differentiation after menarche, by enhancing maturation of type 1 lobule into types 2 and 3. This phenotype may be potentially protective of later life cancer susceptibility as the lobule type 1 is sensitive to neoplastic transformation due to its high rate of

cell proliferation, and rapidly dividing cells are more prone to DNA damage (19, 23). Importantly, this immature lobular structure has been identified as the site of origin of preneoplastic lesions such as ductal hyperplasia, which can evolve into the most common cancer ductal carcinoma (13).

Other than the morphological differences, we found that some markers for mammary gland differentiation were altered with pubertal soy exposure. GATA-3 is a transcription factor critical for mammary gland luminal epithelium differentiation (46); it is highly enriched in the mammary epithelium of pubertal mice (47). Here we found a higher expression of GATA-3 with soy approaching menarche, suggesting a soy promotional effect on breast differentiation during pubertal transition. GATA-3 is expressed throughout embryonic stage, puberty and pregnancy, although the dynamics between different reproductive stages has not been clearly described (48). A complete loss of GATA-3 in adult mouse mammary gland does not cause trans-differentiation of luminal cell into other cell types, but it results in proliferation of undifferentiated luminal cells and basement membrane detachment (49). We found that pubertal soy exposure transiently downregulated GATA-3 expression after menarche in the macaque breast. Importantly, however, the differential GATA-3 expression was not followed with an altered proliferation of the ductal or lobular epithelium cells. With the important role of GATA-3 to maintain cell fate in adulthood, future studies are needed to investigate the differentiation ability of the adult stem cells in the breast of soy-fed animals. Other transcription factors such as STAT5A/B and ELF5 are also important regulators of mammary gland differentiation. Transplantation of *Stat5a/5b*-null mammary stem cells from transgenic mice into cleared fat pads of athymic nude mice greatly reduced luminal progenitor cell population and inhibited alveoli formation during pregnancy, despite no effect on ductal outgrowth. Importantly, generation of luminal progenitor cells and alveoli formation were rescued by transgenic expression of *Stat5a*, suggesting the critical role of this molecule for stem cells differentiation into luminal progenitor cells (50). ELF5 plays a role downstream of STAT5; ELF5 specifies progenitor cells' differentiation into alveolar cells mainly

in pregnancy. *Elf5*-null transplants showed luminal progenitor cells accumulation and prevented alveoli formation, whereas *Elf5* overexpression in a transgenic model caused alveolar differentiation and milk secretion in virgin mice (51, 52). Interestingly, in contrary to GATA-3 pattern, we found that *STAT5A/B* and *ELF5* expression increased with maturity and the change was more prominent in the soy-fed animals. This finding supports the notion that these various transcription factors may function at distinct stages of mammary gland development. It remains unclear if they function within the same cells and how they, if at all, physically interact within the transcriptional network (48, 53).

### *2. ER-mediated transcription*

Estrogen, along with progesterone, is known to play a role in the switch of mammary gland developmental fate (14, 54). Thus, it is possible that the phytoestrogenic component of soy may contribute to modifying estrogen regulation during pubertal development to affect mammary gland differentiation. In MCF-7 breast cancer cell cells, long term treatment to daidzein and equol can downregulate ERα expression (55). Here we found that pubertal soy exposure downregulated post-menarchal transcription of the *ESR1* gene, which may be related to the lower GATA-3 found in the soy-fed animals post-menarche. GATA-3 can directly regulate the expression of ERα by binding to cis-regulatory elements located within the *ESR1* gene to allow for recruitment of transcriptional machinery to *ESR1* promoters, and vice-versa, ERα stimulates the transcription of GATA-3 (56). GATA-3, ERα and FOXA1 form a transcriptional circuit required for hormonal-dependent growth and differentiation of luminal epithelial cells (57). It remains unclear whether soy IF has direct effect on ER to affect this transcriptional path.

We also found lower ERβ protein expression in the ductal structures with soy exposure. Unlike ERα, not much is known regarding the degradation mechanism of ERβ. ERβ has been shown to induce proteasome-mediated degradation of ERα (58) and thus, one would assume degradation of ERβ can be achieved via the same ligand-mediated proteasome ubiquitin pathway.

Whether the presence of IF could affect ERβ conformation and enhance this degradation process is also yet to be answered.

Further, the expression of a classic ER-regulated gene, *GREB1*, was also downregulated in the soy-fed animals after menarche. The change might be related to the lower ER expression with soy at this stage. Alternatively, it might be attributed to some level of competitive binding to ER between estrogen and soy IF to result in the downregulation of estrogen action (59); the presence of IF may also elicit differential conformation on the ER and affect transcriptional activity (60). Another speculation may be that soy IF upregulates ERβ activity; this relates to the classic notion that ERβ can modulate ERα transcriptional activity (61, 62). In breast cancer cell lines, the two ER subtypes exhibit distinct as well as overlapping regions for DNA binding, possibly accounting for some of the differences in gene expression profile and physiological effects elicited by these two ERs (63, 64).

Collectively, the reduction in estrogen-associated gene expression indicates a modest downregulation of estrogen responsiveness in the adult breast of soy–fed animals. This phenotype may be important for estrogen-induced carcinogenesis. In line with our finding, a study in rats showed that exposure to soy diet only during puberty was sufficient to result in a less proliferative response to post-ovariectomy estradiol challenge (65). Another study showed that prepubertal exposure to genistein in mice upregulated *BRCA1*, a tumor supressor gene that also interacts with ER to downregulate ER and PGR expression, and result in a reduced DMBA-induced tumorigenesis later in life (66). Thus, pubertal soy effects on ER and estrogen responsiveness may have significant importance for modulating later life risk to carcinogenesis.

### *3. DNA methylation*

Epigenetic codes, including DNA methylation, are involved in cancer development. DNA methylation profiles are tissue- and cell-specific (34, 35); the patterns are generated during development, with higher plasticity in early life (33). Importantly, dietary components have been

shown to modulate DNA methylation of cancer-related genes (67). The landmark study by Dolinoy et al. (68) identified soy IFs as potential epigenetic modulators; they showed that maternal exposure to genistein in a mouse model increased methylation of a coat color gene, shifted the coat color and altered an obesity phenotype associated with the particular gene. In the macaque model, dietary soy was shown to affect tissue-specific DNA methylation patterns (69). Various studies have reported the potential role of soy and/or IF, mainly genistein, for cancer prevention by DNA methylation modulation (reviewed in (70)). It has been proposed that IF can alter transcription and/or the activity of DNA methyltransferase (DNMT), which in turn modulate DNA methylation level.

Numerous genes have been found to be hyper- or hypomethylated in breast cancer, which includes tumor supressor genes, oncogenes and ERs. Genistein can partially demethylate the promoter of a tumor supressor gene *GTSP1* in MDA-MB-468 breast cancer cells (71). Genistein and daidzein can also demethylate exons nearby to the promoter of *BRCA1* and *BRCA2* in several breast cancer cell lines (72). Also in-vitro, genistein was found to downregulate *DNMT* expression and demethylate the promoter of the gene encoding telomerase (73). In humans, dietary IF treatment did not result in significant change in methylation level of *RARβ2* and *CCND2, ER, p16,* and *RASSF1A* in the intraductal specimens of healthy premenopausal women. The study, however, reported a positive correlation between post-treatment circulating genistein concentration with methylation level for *RARβ2* and *CCND2* (74). It was not clearly described which region of the ER promoter was assessed in the particular study.

Among the seven known promoters for *ESR1*, promoters A, B and C are the most studied in regards to transcriptional regulation of the gene. Methylation level of *ESR1* promoter C in the normal mouse liver was shown to be reduced with in-utero exposure to arsenic (75), but not altered with soy IF diet (76). Promoter region B, which is the promoter we assessed in the current study, is typically methylated in invasive breast cancer (36). Despite the differential *ESR1* expression found in our study, there was no significant change in the methylation level of

representative CpG sites within promoter region B of *ESR1*. In breast cancer tissue and cell lines, ERα expression is also associated with hypermethylation of *PGR* (36, 77) but here we showed that in the normal breast, the differential expression of *ESR1* by soy was not associated with a methylation change in the CpG islands of the *PGR* promoter. ERβ expression is also regulated via methylation of the CpG islands within the promoter (78). Although promoter hypermethylation has been shown to associate with a decrease of the mRNA expression in breast cancer tissue and cell lines (58), our results suggest that the CpG islands up to 300 bp upstream of *ESR2* transcription start site (TSS) were not affected by soy. For *GREB1*, the gene has three perfect EREs spanning 20 kb upstream of TSS that bound to ER in the presence of estrogen, and enhance transcriptional activity of this gene. The ERE motif for *GREB1* has been reported to be conserved between species (79) and indeed, the ERE sequence in macaques is identical to that in humans. The region we assessed flanked the ERE closest to TSS (~1.6 kb upstream); this ERE has basal promoter activity, showed the strongest ER recruitment compared to the other two EREs, and has repressed activity in the presence of ER antagonist (80). Despite the lower mRNA expression of *GREB1* in the breast of soy-fed animals, we did not detect any difference in the CpG methylation around that particular ERE. Future studies are needed to explain whether other regions of ER promoters and the more distal EREs of *GREB1* are affected by the dietary treatment.

An interesting observation that we found during pyrosequencing assessment was that some macaques appeared to show some variation in the DNA sequence of *TFF1* and *PGR* promoter region that we examined, independent of dietary treatment. At this point, it is not yet determined whether the variability is due to base insertion/deletion, single nucleotide polymorphism or other reasons. This finding supports the notion that various kinds of polymorphism are commonly found in nonhuman primates, more than in humans (81).

### *4. Estrogen synthesis and metabolism*

Soy is also thought to modulate production and/or inter-conversion of estrogen, which has been postulated to be mediated by the effects on pituitary gonadotropin, ovary and/or the enzymes involved for the estrogen synthesis and metabolism (82). Although epidemiological studies showed variable results, it is suggested that soy is able to alter circulating ovarian hormones, serum hormone binding globulin (SHBG), and serum ratios of different estrogen metabolites in premenopausal women (reviewed in (82, 83)). A recent meta-analysis of 47 studies, however, showed that soy or IF consumption did not affect estradiol, estrone or SHBG levels in premenopausal women as well as postmenopausal women (84). A similar finding has been reported in monkeys (85).

To this date, limited data is available regarding soy effects on estrogen level in childhood and adolescence. In humans, a small pilot study done in pre/peripubertal girls reported no change in sex hormone excretion with short-term dietary soy intake (86). A more recent study conducted in Japanese children aged 3-6 years showed that soy intake was inversely associated with urine level of estrone and estradiol in boys, and positively related to testosterone in girls (87), which the authors speculated is a consequence of an altered aromatase action. A possible explanation to the different effects in those two studies might be the timing and/or duration of soy exposure; Japanese children are more likely to be exposed to soy since early age and even maternally. In a monkey model, a short-term cross-over study with soy diet in peripubertal rhesus macaques showed no effect of on SHBG and estradiol (88), which is consistent with our present study. The results suggest that pubertal soy intake up to a high IF dose of 120 mg/day does not appear to alter systemic estrogen before and after menarche.

Estrogen can be synthesized peripherally in various tissues such as breast as a result of interaction of various enzymes within the tissue; the biosynthesis pathway is described in Figure 1. In postmenopausal women, the intra-mammary path is the major source for estradiol; this enzyme interaction regulates estrogenic action, affecting pathogenesis and development of

hormone-dependent breast cancer (89). How this local steroidogenic pathway functions during pubertal breast development, however, is not known. Utilizing the macaque model, we were able to demonstrate the expression pattern of aromatase, steroid sulfatase, sulfotransferase, and 17β-hydroxysteroidegenases (17βHSDs) across the menarchal transition. Notably, the immunoreactivity profile of these enzymes in the macaque breast is comparable to that in the normal human breast (90, 91), further highlighting the unique translational potential of macaque model.

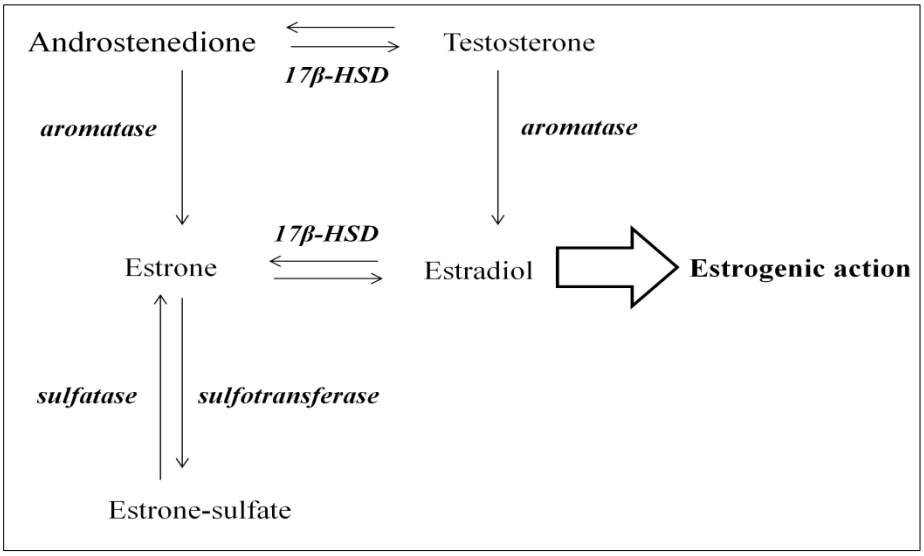


Figure 1. Estrogen biosynthesis pathways

Independent of diet, sulfatase/sulfotransferase pathways predominated before menarche and decreased with maturity. This pattern may indicate the essential role of local sulfatase/sulfotransferase pathways for maintaining equilibrium of active/inactive estrone during the highly estrogen-responsive stage of breast development. On the other hand, 17βHSD types 1 and 2 which are critical for estradiol interconversion appeared to increase with maturity whereas aromatase expression was consistently weak across pubertal development. Taken together, these patterns are consistent with the fact that production of estrone and its precursors occurs early in puberty and then is followed by estradiol (92, 93). The enzymes CYP1A1, CYP1B1, and

CYP3A4 are important for further hydroxylating estrogens into metabolites of different genotoxicity. Importantly, these enzymes are induced and regulated by estrogen substrates via ER (94). Here we showed that the mRNA expression of these enzymes was relatively consistent across puberty. Our study is the first to assess soy effects on this pathway in the adolescent breast. The findings suggest that pubertal exposure to dietary soy does not seem to affect enzyme activity within the mammary gland. Although IFs can alter CYPs activity *in-vitro*, exposure to IF via dietary soy during adulthood (95) or puberty is not likely to affect their expression in the mammary gland.

### *5. Other pathways*

Given the complex signaling interplay in mammary gland development (25), potential soy effects on other pathways and hormones other than estrogen has to be taken into consideration when investigating the breast differentiation effect in our study. We performed gene expression microarray analysis on a subset of post-menarchal breast samples to screen for potential markers and their associated pathways (i.e. at ~1 and 2 years post-menarche). Although we did not detect a strong difference by diet or time across this one year interval, there was an indication that the developmental regulation of the adolescent breast in the two dietary groups may involve different genes; further measures are required to validate these markers. Gene enrichment analysis was conducted to identify which biological/functional pathways show over-representation in our microarray dataset. We showed that genes that changed over time in the CL and SOY groups are likely to be associated with different KEGG pathways. We also performed analyses against *a priori* gene sets corresponding to a previously identified pubertal mammary profile (96, 97) and estradiol-regulated markers (98, 99). In our dataset, these markers appeared to be downregulated with maturity, as expected. We did not detect a significant enrichment of ER pathway markers with soy treatment, possibly indicating that ER-modulation may not be the

predominating mechanism behind the morphological difference between the two diet groups. The results are presented in Appendix 2.

**Potential adverse estrogenic effects of soy on the breast**

In contrast to the potentially protective effect of early life exposure, it is important to note that genistein has been shown to stimulate growth of existing mammary tumors. *In-vitro* studies showed that genistein, in the absence of estradiol, can stimulate the growth of estrogen-dependent MCF-7 breast cancer cells. The effect was found to be biphasic: a low concentration was growth-stimulatory whereas a high concentration (> 25,000 nmol/L) showed anti-proliferative effect which is presumably independent of ER (59, 100). Consistent with the *in-vitro* findings, genistein treatment in ovariectomized Sprague Dawley rats pre-induced with MNU caused larger and more proliferative estrogen-dependent tumors (101). In ovariectomized athymic nude mice bearing MCF-7 human breast tumors, dietary genistein also enhanced tumor growth (100, 102). The increase in tumor size was accompanied with higher cell proliferation and *TFF1* expression, indicating the estrogen-agonist action of genistein (102). With low levels of estradiol implant (i.e. comparable to that observed in postmenopausal women), genistein was found to act in additive manner to stimulate growth of MCF-7 cells in the same mouse model (103). These findings highlight the potential adverse effects of soy on breast cancer when exposed to older (i.e. postmenopausal) breast cancer patients. It is uncertain, however, if the clear estrogen-agonist effect is specific to genistein or all IFs, and whether IFs given as mixture within a protein matrix would elicit a similar effect. Nevertheless, the concern on soy estrogenic effect to promote cancer growth and recurrence has been raised and needs to be addressed.

In a study of breast cancer survivors in China, post-diagnosis soy intake was inversely associated with mortality and recurrence for both pre- and post-menopausal women, regardless of tamoxifen use (104). Notably, the studied population represents individuals with relatively "healthy lifestyle" and were of Chinese population, which were likely to have been exposed to

soy at some point early in their life. This issue was partially addressed in a study by Nechuta et al. (105) that found an association between post-diagnosis soy consumption and reduced recurrence (but not mortality) in breast cancer survivors from both the US and China, although the subjects with higher IF intakes tended to have healthier lifestyles. When stratified by tamoxifen use, the significant inverse association between soy and recurrence was only found among tamoxifen users, indicating some degree of interaction between soy and tamoxifen to elicit the protective effect. While the protective effect of soy might be biased by other factors, these studies indicate that post-diagnosis soy intake does not result in poorer breast cancer outcome. In contrary, there was a study that showed increase in nipple aspirate TFF1 level after short-term treatment with a diet containing 45 mg IFs in premenopausal women diagnosed with breast diseases (e.g. benign and cancer) (106). This finding, however, was not accompanied with greater proliferation, although the early stage of this study (i.e. with fewer patients) did show higher epithelial proliferation and PGR expression after dietary soy treatment, indicating estrogenic response to soy (107).

Our results in the present study indicate a lack of adverse estrogen-like effects of pubertal soy exposure on breast development. More importantly, the morphologic difference and the subtle estrogen-antagonist effects found with soy treatment are consistent with other studies that showed reduced tumorigenesis (44, 66, 108). We support the notion that timing of soy exposure could be key to benefit from soy.

**Timing of soy exposure in pre-adulthood**

Epidemiological findings have emphasized on the differential impact between soy exposure in adulthood and pre-adulthood. Specifically during pre-adulthood, it has been suggested that maternal soy exposure may elicit a different effect from childhood/adolescent exposure. Studies in rat models indicate that exposure to genistein only during in-utero/perinatal increased mammary tumorigenesis, as opposed to when exposure extends to puberty onset (i.e.

pre-puberty), from peripuberty onwards, or throughout life (109, 110). In contrary to our current findings in the macaque breast, a study in rats showed elevated ERβ with a lower ERα by soy exposure starting in-utero up to young adulthood; the change was followed by less response to estradiol challenge post-ovariectomy (111). The likely explanation to the discrepancy found with the different timing may be related to the extensive programming during embryonic stage; this period is the most susceptible for epigenetic modulation by dietary factors (33). In relation to this timing issue, we conducted a pilot study to assess mammary gland profile (morphology, transcriptomics, and DNA methylation) of peripubertal female monkeys that were exposed to dietary soy since before birth; the results are presented in Appendix 3. Our preliminary results indicate a possible weak effect of soy. The study, however, had various limitations which made it difficult to interpret further (e.g. small sample size, age variability during sampling, and the inability to determine menarche onset due to the large colony setting).

**Estrogen responsiveness in the uterus**

Uterus is considered to be an ERα predominant tissue. Previous studies have shown that both ER subtypes are expressed in almost all cell types within this tissue. These receptors appeared to be non-essential for the development and differentiation of the uterus before puberty whereas beginning puberty, uterine growth and endometrial development is highly responsive to estrogen via ER-mediated action (8). After puberty, the ERs are expressed with patterns specific to menstrual or estrous cycle stage. It is important to note that the regulatory role of ERs for uterine and endometrial development is species- and developmental-stage specific.

Our data demonstrated the expected increase of uterine size across peri-puberty in the macaques. We also showed an increase of the endometrium area and thickness during the early follicular phase of a menstrual cycle, which may be attributed to the increasing proliferation in response to estradiol. An increase in endometrial thickness by ultrasonography at this phase is known to correlate well with the increase in plasma estradiol (10). While ERα role in mediating

estradiol uterotrophic effects is well established (8), ERβ biological function is less understood. In the immature mouse uterus, the loss of ERβ increased proliferation and enhanced responsiveness to estradiol, indicating an anti-uterotrophic role of ERβ. It was also suggested that *PGR* induction is an ERα-mediated event whereas the repression of *PGR* is mediated by ERβ (112).

**Dietary soy and the uterus**

Soy IF is often considered as a natural selective ER modulator (SERM)-like compound due to its ability to elicit both estrogenic and anti-estrogenic effects depending on the target tissue, estrogen environment, and the ERs (113). Chemical SERMs (e.g. tamoxifen, raloxifene and bazedoxifene) can elicit tissue-specific mixed estrogen-agonist/antagonist effects, when used alone or in combination with exogenous estrogen. Tamoxifen and raloxifene are ER-antagonists in the breast, which can reduce the risk of ER-positive breast cancer (114, 115). Bazedoxifene also showed ER-antagonist properties in the breast and the uterus when given together with conjugated equine estrogens in a postmenopausal primate model, and showed no estrogenic effects when given alone (116, 117). Importantly, while raloxifene does not stimulate the endometrium (118), tamoxifen has partial estrogen-agonist effect on the uterus (119) and is associated with increased risk of endometrial cancer (114). While our study and others point to the potential health benefit of soy on the breast, the soy IF effect on the uterus is more controversial. There have been concerns on whether IF effects are contradictive in these two tissue types.

**Potential adverse estrogenic effects of soy on the reproductive tissues**

In human endometrial carcinoma (HEC-1) cells, all IF aglycones can act as agonist on both ERs; they showed lower potency but comparable transcriptional efficacy to that of estradiol. Genistein in particular, has greater transcriptional efficacy on ERα than estradiol (120). Utilizing

endometrial stromal and Ishikawa cells (i.e. a well-differentiated endometrial adenocarcinoma cell line), genistein, daidzein, and their conjugated forms genistin and daidzin were able to induce proliferation of these cells. The estrogenic activity was ~70% weaker than that of estradiol, and interestingly IFs can elicit estrogen-antagonizing effect when administered together with estradiol (121). The estrogenic effect of IF has also been demonstrated in animal models. Study in immature mice showed dose-dependent uterotropic effect of various phytoestrogens; genistein injection at concentrations 1,000 times greater than estradiol and 50,000 times greater than DES increased uterine weight (122). In pre-pubertal mice, transcriptional response of the uterus after injection with estradiol or genistein (i.e. with ~600-times greater dose than estradiol) were very similar although notably, the expression magnitude and timing of some ER-responsive genes varied between the two compounds (123). The study suggests that estrogen and genistein affect the same genes and biological pathways in a similar manner, further highlighting the potential estrogen-like effect of soy in the uterus. As reviewed by Wood et al. (83), genistein treatment using a dose of >50 mg/kg bodyweight in rats also induce uterotrophic effects, more consistently when genistein is administered via injection or oral gavage rather than within a dietary compound. Unlike in the *in-vitro* setting, however, genistein effect in the presence of estradiol is less clear; some showed no effect (124, 125), while others reported the estrogen-antagonizing effect (126), or that genistein and estradiol works in an additive manner (127).

Utilizing intact apolipoprotein E-null mice model, which is a similar model to the present study, dietary soy IFs given as aglycone concentrates at dose <50 mg/kg bodyweight was shown to elicit adverse estrogenic effects on the uterus. Lesions found were endometritis, increased endometrial epithelial height, uterine enlargement, and uterine squamous metaplasia. In males, adverse estrogenic effects were also observed such as reduced plasma testosterone levels, squamous metaplasia of seminal vesicles, and atrophy of seminiferous epithelium and accessory sex glands (128). In the present study, soy IFs dose were higher but they were given within a soy protein matrix; no estrogenic effect was observed. The findings may indicate that IFs' effect is

influenced by dietary exposure method (i.e. purified concentrate vs. within a protein matrix). In the macaque model, previous works in our laboratory have consistently showed a negligible effect of dietary soy on the uterus in adult pre-menopausal and post-menopausal monkey model without exogenous estrogen (85, 129). In a high estradiol environment, high IF diet appeared to block the uterotrophic effect of estradiol (130). In humans, some observational studies showed inverse association between soy intake and endometrial cancer risk whereas the majority of interventional studies in postmenopausal women reported no uterotrophic effect of soy supplement. However, there was one study that reported higher occurrence of endometrial hyperplasia in postmenopausal women treated with daily supplementation of 150 mg IF tablet for 5 years (131). Collectively, these findings suggest that soy IFs have potential estrogenic effect on the uterus, which can be influenced by the dosage, form of exposure, estrogen environment and the timing of exposure.

**The effect of pubertal soy exposure on the uterus**

With the preferential binding of soy IF to ERβ, we initially hypothesized that dietary soy, would elicit an estrogen-antagonist effect on the uterus starting peripubertally and thereafter. The less estrogen-responsive uterus, if observed, would in part explain the inverse association between soy intake and endometrial cancer risk (132, 133, 134, 135, 136). Our current findings, however, suggest that dietary soy did not have an impact on the pubertal uterine development in our macaque model. If anything, there appeared to be greater endometrial area with soy only during menarche transition; this difference by diet was observed only at the particular timepoint and disappeared with maturity. Based on ultrasonography alone, it is difficult to interpret whether the finding reflects a true estrogen-agonist effect during the early menarchal transition, which is a stage of major estradiol leap. Furthermore, there was no effect of soy on post-menarchal uterine area and endometrial thickness although a similar pattern was seen. Using a mouse model, a high soy diet *per se* showed no effect on the adult nulliparous uterus. Taken together, we suggest that

soy diet is not likely to elicit a strong estrogen-modulatory effect on the uterus by adolescence and later in adulthood.

Only few human studies have assessed the impact of early life soy intake on the uterus. One study longitudinally assessed tissue development by ultrasound in infants following feeding with soy formula (vs. breast milk and cow's milk formula), and reported no difference in organ size by soy treatment (137). A retrospective study also showed no association between exposure to soy during infancy and pregnancy- and menstruation-related outcomes (138). Numerous rodent studies have assessed the effect of in-utero/neo-natal exposure to soy diet or genistein. Although the results were inconclusive, there is an overall indication that perinatal exposure to genistein but not soy foods elicited uterotropic effects, which was associated with a higher uterine carcinogenesis in adult mice (139, 140). Among the very limited animal studies on pubertal soy exposure, the results thus far indicate a less profound uterotrophic effect compared to when soy and/or genistein is exposed earlier in life. In rhesus macaques, there was no effect of short-term dietary soy treatment on the uterine weight at adolescence (88). In a rat model, dietary genistein with dose approximating those achieved in infants receiving soy formula also did not cause uterotrophic response or altered steroid receptor (141). When given via injection, however, genistein showed an estrogenic effect on the uterus via alteration of ERα. Notably, bioavailability of genistein attained by this method is much higher than those via oral administration (139). Collectively, the timing of exposure and the form of IF treatment compound are important factors that determine the estrogen-modulatory effect of soy IF. Since developmental regulation during the fetal/neo-natal stage is likely to be non-ER-mediated, any modification during early life intervention may not involve estrogen and/or ER alteration. The mechanism underlying the persistent altered uterine phenotypes into adulthood has been speculated to be by permanent epigenetic changes. As for the compound form, purified genistein can be absorbed faster than a mixture of IFs given within a soy food matrix. Estrogen is critical for proliferation of the endometrium, and genistein has been thought to mimic the unopposed estrogen effect. When

given as soy IFs mixture, the bioavailability of the IFs is different and importantly, the presence of daidzein allows for the production of equol in animals. This metabolite predominates and is likely to be the main player driving the biological activity.

**Isoflavone metabolism and equol**

IF metabolism has been systematically reviewed by Larkin and colleagues, showing the key importance of gut microbiota in this process (142). When ingested, the β-glycosidic forms are hydrolyzed into aglycones by enzymes bound at the brush border of small intestine mucosa or produced by the gut bacteria. The malonyl- or acetyl forms, however, are poorly hydrolyzed in the small intestine; they directly enter the large intestine for further hydrolysis and reductive modification. Aglycone forms can be rapidly absorbed in the upper small intestine by passive diffusion and enter the circulation to reach target tissues. Aglycones can also undergo a classical enterohepatic circulation, conjugated in the liver and then transported to various tissues in their conjugated form via the systemic circulation. Conjugated IFs will serve as a source of tissue aglycones such as by in-situ glucuronidase activity, and they can also bind to ERs in the mammary gland. Notably, however, conjugated IF would need $10^6$ times higher intracellular concentration than endogenous estradiol to compete for ER binding (143). In the liver, these conjugates can also be secreted in the bile and transported back to the intestine for deconjugation to aglycones again. It has also been suggested that conjugation primarily occurs in the upper intestinal wall, surpassing that of the liver. Intestinal conjugation enables enteric recycling of IFs; conjugated IFs are secreted to both the apical (i.e. the intestinal lumen) and basolateral (i.e. blood) sides of the enterocyte mediated by multidrug-resistance related protein (142, 144). This recycling, in combination with the enterohepatic path, prolongs the systemic exposure to IF. It is important to note that IF metabolism profile varies between species. In addition to the differential equol-producing ability, the proportion of circulating isoflavonoids (i.e. conjugated vs. aglycones,

genistein vs. equol) also varies in different animals (145). This issue further complicates extrapolation of results of dietary soy treatment from one species to another.

Although aglycones only represent a small proportion of plasma IFs, the volume of distribution of aglycones has been found to be greater than their conjugated forms. Further, aglycones are also cleared from plasma much more rapidly suggesting a greater ability to enter the tissues including breast and the reproductive tract. Importantly, aglycones bind much more strongly to ERs and elicit greater biological activity than their conjugates (146, 147). In the colon, aglycones can also be processed by gut bacteria into intermediate metabolites such as dihydrodaidzein, dihydrogenistein, and further to o-desmethylangolensin (O-DMA) and equol. Interestingly, not all people have the ability to produce equol. Only 30-40% of non-vegetarian Caucasian has this ability (148), whereas up to 60-70% of vegetarians (149) and Asians (150, 151) are equol producers. However, whether the equol-producing capability is a stable phenomenon has also been a widely debated topic. Equol production was found to be relatively consistent in most individuals at least over a 1-3 year period (152, 153). More recently, studies showed that in addition to the ~30% of women that were consistent equol producers, there were others (~10-30%) that were intermittent equol producers (154, 155). Among those intermittent producers, some showed crossings of equol-producing status with the change of diet from low to high soy or vice versa, indicating that dietary soy intake and/or other unknown factors may affect one's equol producing ability.

**Bioavailability of isoflavonoids**

In the present work and other macaque studies, up to 70% of the circulating isoflavonoids are equol. This finding confirms the predominance of equol in the system of the macaque model, which is known to be consistent from 4 to 24 hours post-feeding (85, 129, 130). The previous work in our lab has shown that 99% of serum isoflavonoids were in the conjugated forms, although it was not specified if they were glucuronides or sulfates (129). Equol is also the

predominating isoflavonoid in the rodent models, and 98% of their serum equol is glucuronides which is the least potent compared to aglycone or sulfate-conjugated form (145).

Other than in the circulation, previous studies have confirmed the presence of IFs in target tissues. Endocrine-responsive tissues including mammary and uterus showed dose-dependent increase in total genistein concentration following a lifetime exposure to dietary genistein in Sprague Dawley rats (156). In the human breast, distribution of soy IFs and their metabolites is more dominant in the glandular fraction than the stroma (157). Utilizing liquid chromatography-mass spectrometry on breast tissues collected from healthy women undergoing esthetic breast reduction, an exposure to 20 mg of IF supplement or soy milk resulted in a breast tissue exposure to total IFs in the amount estimated to be ~40 times greater than estradiol-to-ERβ equivalent, suggesting the potential of IF to elicit biological effects in the tissue (143). The clearance of equol is slower than daidzein and genistein (158). Unlike in animals, human equol producers typically show a greater amount of circulating genistein and daidzein compared to equol (159, 160). Importantly, however, almost 50% of equol circulates in its free-bound form, higher than that of daidzein (161). Further, there was a human study (i.e. utilizing breast tissue collected from women undergoing breast reductions) that showed higher equol concentrations in the breast tissue than in the serum after soy supplement intake, and the tissue concentration was also ~10-fold higher compared to those of daidzein and genistein (162).

Our study in mice further confirms the importance of gut bacteria profile to affect metabolism of equol, daidzein, and genistein. Unlike regular apoE-null mice that are good equol producers, the predominating isoflavonoid in our equol producers were genistein which resembles the metabolic profile seen in humans. Importantly, in the present study we also showed that genistein and daidzein metabolism differ by sex, suggesting the warrant for caution when interpreting result of dietary soy intervention studies to the opposite sex.

**The equol effect**

Most of the mechanistic actions of soy compound have been explained based on the numerous studies done with genistein. Equol and genistein have different biological properties (120, 163). Among aglycone IFs, genistein has the strongest binding affinity to ER (preferentially to ERβ) with affinity less than one tenth of estradiol. Although still lower than genistein, equol affinity is higher than its precursor daidzein (120, 164). Genistein and equol can activate ER-mediated transcription, but genistein appeared to be a superagonist on ERα. The differential effects between S- and R-equol enantiomers on ERs have also been shown to be quite significant. S-equol has high affinity and preference to ERβ but its transcriptional potency is the same for both ER subtypes, whereas R-equol binds similarly to both subtypes but is a more potent transactivator with ERα. Additionally, S-equol is known to antagonize dihydrotestosterone.

In our study, the exogenous equol comprised of both enantiomers was anti-estrogenic in the reproductive tissues, which is likely due to ER-mediated action. We did not find a clear effect of racemic equol on the mammary gland. If anything, the effect was more complex whereby racemic equol was estrogenic when given without soy IF diet, and only in the nonproducers. In contrast, when equol was given along with a high IF diet, the effect appeared to be anti-estrogenic. In general, our result is contrary to a study in ovariectomized rats which reported strong estrogenic effects in the mammary gland and uterus after long-term equol exposure (165, 166). The discrepancy observed may indicate the importance of estrogen environment to affect ER-modulatory action. Our observation also suggests that microbiota status and co-presence of other isoflavonoids affect tissue response to the racemic equol. Unlike the mouse studies, however, a previous work by our group showed negligible uterotrophic or mammotrophic effect of racemic equol in the postmenopausal monkey model despite the high dosage used (129). The isoflavonoid metabolism and bioavailability profile differs between species, and this could also determine equol effect on the tissue.

While macaques can only model for equol producers, the results from our mouse study suggest that tissue response to dietary soy may differ by equol-producing status. Equol producers fed a soy diet showed less estrogenic response (i.e. lower cell proliferation and PGR expression), suggesting the potential anti-estrogenic role of endogenously-produced S-equol. It is unclear, however, if the effect was a direct equol effect on ER or other estrogen-associated regulation in the tissue, or merely a marker of microbiota effect on estrogen metabolism. Gut microbiota has a complex role in estrogen metabolism, mainly in the enterohepatic path by producing enzymes to hydrolyze the conjugated estrogens from the liver; the hydrolyzed estrogens can then be excreted or reabsorbed into circulation (167). Soy diet, being the substrate for microbiota, may affect the enzymatic conversion activity and thus modulate the excretion and the level of circulating estrogen. Alternatively, the observed effect might be driven by the differential microbiota profile. We showed that when animals were fed different diets, they harbor different microbiota, which may possibly lead to different enzymatic activity within the gut. Collectively, our findings support the notion that diet, microbiota and the host are involved in metabolic interplay. Diet/nutritional condition influences composition and activity of the microbiota; microbiota can act to achieve metabolic communication with the host to affect disease risk (168).

Interestingly, we found that equol producers had a less estrogenic reproductive tissue phenotype compared to nonproducers, regardless of soy IF dose. This was consistent with a study by Duncan et al. that reported hormonal differences between equol producers and nonproducers independent of IF amount (169), which suggest a certain estrogen-metabolizing profile elicited by the equol-producing gut microbiota. Our observed effect was partially mediated by an association between microbiota status and estrous cycle distribution. Estrous cycle is typically 4-5 days, with approximately one day for each stage and 1-2 days for diestrus. In our study, we found the majority of equol producers were at metestrus at necropsy, which could possibly indicate a longer metestrus in these animals. We speculate this finding as a potential effect of microbiota on the cycle regulation and/or the HPG axis. Soy effects on gonadotropins and menstrual cycle have

been reported inconsistently. A recent meta-analysis showed soy IF significantly reduced circulating FSH and LH, and increased cycle length in premenopausal women despite no change in estradiol and progesterone (84). They noted, however, that the results were no longer significant when the analysis only include studies that were at low risk of bias. Unfortunately, equol-producing ability was not considered in their analysis.

Several studies in humans indicate the differential effects of soy treatment with equol-producing phenotype. Given a soy IF diet, pre-menopausal equol producers have been found to generally have lower circulating estrogen, androgen and androstenedione, along with higher SHBG and mid-luteal progesterone, a hormonal pattern consistent with a less estrogenicity (169). Estrogen metabolism into 2-hydroxyestrogens (2OHE) and 16α-hydroxyestrone ($16OHE_1$), which are metabolites associated with breast cancer risk, was also shown to be affected by soy consumption only in the equol producers whereby soy intake increased urinary excretion of 2OHE and the $2{:}16OHE_1$ ratio (170). This finding, however, was not found with subsequent studies (171). Not much is known whether equol-producing phenotype determines the developmental effect of soy. Among the limited number of epidemiological studies that assessed soy effect on pubertal breast development and/or menarche onset, equol was either not measured (172, 173) or not assessed as a factor (174). There was only one study that reported higher equol found in precocious puberty patients relative to control (175). In regards to breast cancer, equol-producing status may determine soy association with mammographic density in postmenopausal (176, 177) but not premenopausal women (178). One case-control study showed an inverse association between equol excretion and breast cancer risk (179), whereas two recent studies showed no association (180, 181). It is not known if equol status modifies the association between soy intake and endometrial cancer risk.

More recent publications on equol effect have been related to the equol supplement called SE5-OH (Otsuka Pharmaceutical Co., Ltd., Japan), which is an S-equol based supplement that contains isoflavonoids approximately 5 mg S-equol, 1.2 mg daidzein, 1.4 mg genistein, 3.1 mg

glycitein, and other compounds (protein, fat, etc.) (182). From their reproductive and developmental toxicity assessment in rats, the supplement did not appear to elicit a significant effect on the uterus (183). It is unclear if this lack of effect also applies in humans. In a pilot trial with postmenopausal women in the US, small number of subjects (3/102) reported endometrial hyperplasia (184), whereas a study conducted with postmenopausal nonproducers in Japan reported no change in endometrial thickness and mammography (185).

**Conclusion**

In the present study, pubertal exposure to dietary soy promoted breast differentiation in a primate model. The expression of ERs and ER-regulated markers in the breast was modestly downregulated in the soy-fed animals after menarche, which may indicate a potentially lower estrogen-responsiveness of the breast in young adulthood following soy exposure. The effect did not appear to be mediated by alteration of CpG methylation within the specific promoter sites examined herein, or by modulation of local estrogen synthesis and metabolism. Future studies are needed to determine whether such a phenotype may influence breast cancer risk later in life. We also showed that menarche onset, circulating sex hormones, and uterine development did not differ by diet, which suggests that pubertal exposure to a high soy diet does not have an overt effect on pubertal development. Using a mouse model with altered gut microbiota profile, our results indicate that endogenous equol and/or equol-producing microbiota may influence estrogen-dependent tissue phenotype and response to soy diet.

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## APPENDIX 1

### Composition of diets

**Table 1.** Composition of diets in the nonhuman primate study

| INGREDIENT | Diet | |
|---|---|---|
| | CL[a] | SOY |
| | *g/100 g diet* | |
| Casein, USP | 8.50 | 1.30 |
| Lactalbumin | 8.50 | 1.30 |
| Soy Protein Isolate[b] | | 14.00 |
| DL-methionine | | 0.30 |
| Wheat Flour, self-rising | 35.76 | 35.76 |
| Dextrin | 9.00 | 8.90 |
| Sucrose | 7.00 | 7.00 |
| Alphacel | 8.03 | 8.72 |
| Lard | 5.00 | 5.00 |
| Beef Tallow | 4.00 | 4.00 |
| Butter, lightly salted | 3.10 | 3.10 |
| Safflower Oil (linoleic) | 3.00 | 2.56 |
| Crystalline Cholesterol | 0.061 | 0.061 |
| Vitamin Mixture[c] | 2.50 | 2.50 |
| Mineral mixture[d] | 5.00 | 5.00 |
| Calcium Carbonate | 0.400 | 0.357 |
| Calcium Phosphate, Monobasic | 0.150 | 0.145 |

[a] CL, Casein and lactalbumin-based diet (as control)

[b] Isolated soy protein with isoflavones (provided by Solae, LLC) containing 1.11 mg genistein, 0.48 mg daidzein, and 0.08 mg glycitein per gram of isolate (as aglycones).

[c] Complete vitamin mixture (made by Harlan Laboratories) consisted of (g/kg diet): 0.045 retinyl palmitate; 0.0625 *dl*-α-tocopheryl acetate; 0.125 iso-inositol; 0.025 riboflavin; 0.05625 menadione sodium bisulfite complex; 0.125 *para*-aminobenzoic acid; 0.1125 niacin; 0.025 pyridoxine hydrochloride; 0.025 thiamine hydrochloride; 0.075 calcium pantothenate; 0.03375 vitamin B-12 in manitol; 0.0005 biotin; 0.00225 folic acid; 2.25 ascorbic acid; 1.875 choline chloride; 0.005 cholecalciferol; and 20.15725 dextrose monohydrate.

[d] Mineral mixture without Ca and P (made by Harlan Laboratories) provided (g/kg diet): 15.696 $K_2CO_3$; 8.119 NaCl; 7.195 $MgSO_4(H_2O)_7$; 0.3934 $FeSO_4$; 0.0676 $MnSO_4(H_2O)$; 0.0455 $ZnCl_2$; 0.0145 $CuSO_4$; 0.0039 KI; 0.0024 $(CH_3CO_2)_7Cr_3(OH)_2$; 0.0012 NaF; 0.0002 $Na_2SeO_3$; and 18.463 dextrin.

**Table 2.** Composition of diets in the mouse study[1]

| Ingredient (g/kg dry weight) | CL | CL + Equol | Low IF | Low IF + Equol | High IF | High IF+ Equol |
|---|---|---|---|---|---|---|
| Casein | 106.5 | 106.5 | | | | |
| Lactalbumin | 101.5 | 101.5 | | | | |
| Soy Protein Isolate (alcohol-washed) [a] | | | 201.5 | 201.5 | | |
| Soy Protein Isolate (intact) [b] | | | | | 201.5 | 201.5 |
| Total Isoflavones | | | 0.042 | 0.042 | 0.566 | 0.566 |
| Racemic equol [c] | | 0.29 | | 0.29 | | 0.29 |
| DL-methionine | | | 3 | 3 | 3 | 3 |
| Corn starch | 231 | 231 | 231 | 231 | 231 | 231 |
| Maltodextrin | 75 | 75 | 75 | 75 | 75 | 75 |
| Sucrose | 280 | 280 | 280 | 280 | 280 | 280 |
| Cellulose | 100 | 100 | 100 | 100 | 100 | 100 |
| Lard | 52 | 52 | 48 | 48 | 48 | 48 |
| Safflower oil | 10 | 10 | 10 | 10 | 10 | 10 |
| Mineral Mix | 35 | 35 | 35 | 35 | 35 | 35 |
| Vitamin Mix | 10 | 10 | 10 | 10 | 10 | 10 |
| Choline Bitartrate | 2 | 2 | 2 | 2 | 2 | 2 |
| Yellow dye | 0.05 | 0.05 | 0.025 | 0.025 | | |
| Red dye | | | 0.025 | 0.025 | 0.025 | 0.025 |
| Blue dye | | | | | 0.025 | 0.025 |

[1] CL, Casein and Lactalbumin; IF, Isoflavones

[a] Soy protein isolate (SPI) provided by Archer Daniels Midland Company (Decatur, IL). Total isoflavones (IF) contained in the isolate was 0.21 g/kg SPI, which consists of daidzin, genistin, glycitin, their malonyl and acetyl forms, and the aglycones (daidzein, genistein, glycitein). The ratio of daidzein:genistein:glycitein was 1:1.5:0.2.

[b] Soy protein isolate (SPI) provided by Archer Daniels Midland Company (Decatur, IL). Total isoflavones (IF) contained in the isolate was 2.81 g/kg SPI, which consists of daidzin, genistin, glycitin, their malonyl and acetyl forms, and the aglycones (daidzein, genistein, glycitein). The ratio of daidzein:genistein:glycitein was 1:1.5:0.2.

[c] Equol supplement used was a racemic mixture with 50% S-(-) and 50% R-(+) forms, provided by Solae, LLC (St. Louis, MO).

# APPENDIX 2

**Enrichment analysis on pubertal gene expression dataset**

To determine the potential biological meaning of the gene expression profile from our microarray experiment, we assessed whether gene sets of certain biological/functional pathways show significant over-representation. Gene Set Enrichment Analysis (GSEA) was performed using GSEA-preranked method (http://www.broadinstitute.org/gsea/) in the GSEA software version 2.0.13 with default parameters (1). As described in Chapter 2, microarray analysis was done on the subset of mammary samples collected at 7-12 months (Time A) and 19-24 months (Time B) after menarche utilizing Affymetrix Rhesus Gene 1.1 ST WT Array Strips. We generated four gene-lists from pair-wise comparisons of RMA-normalized data by time (i.e. of Soy A vs. Soy B, and CL A vs. CL B) or by diet at each timepoint (i.e. Soy A vs. CL A, and Soy B vs. CL B). For GSEA, each gene list was ranked based on fold-change; the search was performed against hypothesis-driven gene sets (i.e. puberty-, estradiol- and ER-related) as well as Gene Ontology molecular function collection, available from molecular signature database (MsigDB) v4.0 (2). Enriched gene sets with false discovery rate (FDR) < 5% were considered significant and listed below.

Table 1. Result of enrichment analyses against puberty-related gene sets

| Gene set | Ref. | Size | Enrichment (FDR)[1] | |
|---|---|---|---|---|
| | | | CL A vs. B | Soy A vs. B |
| Genes up-regulated in pubertal mammary glands compared to mammary glands from other developmental stages | (3) | 47 | Enriched among genes that were downregulated over time (0.02) | Enriched among genes that were downregulated over time (0.00) |
| Genes up-regulated during pubertal mammary gland development between week 4 and 5 (in mice) | (4) | 214 | Enriched among genes that were downregulated over time (0.00) | Enriched among genes that were downregulated over time (0.00) |
| Genes down-regulated during pubertal mammary gland development between week 4 and 5 (in mice) | (4) | 132 | Enriched among genes that were downregulated over time (0.00) | Enriched among genes that were downregulated over time (0.00) |
| Genes up-regulated during pubertal mammary gland development between week 6 and 7 (in mice) | (4) | 142 | Enriched among genes that were downregulated over time (0.00) | Enriched among genes that were downregulated over time (0.00) |
| Genes down-regulated during pubertal mammary gland development between week 6 and 7 (in mice) | (4) | 58 | Enriched among genes that were downregulated over time (0.02) | Not significantly enriched among genes that were downregulated over time (0.06) |

[T]FDR=False discovery rate. Timepoint A= 7-12 months post-menarche; B=19-24 months post-menarche

Table 2. Result of enrichment analyses against estradiol-regulated markers or ER pathway gene sets.

| Gene set | Ref. | Size | Enrichment (FDR)[1] | |
|---|---|---|---|---|
| | | | CL A vs. B | Soy A vs. B |
| Genes rapidly up-regulated in breast cancer cell cultures by estradiol | (5) | 43 | Not significantly enriched among genes that were downregulated over time | Enriched among genes that were downregulated over time (0.00) |
| Genes up-regulated in MCF-7 cells (breast cancer) by estradiol (E2) | (6) | 28 | Not significantly enriched among genes that were downregulated over time | Enriched among genes that were downregulated over time (0.00) |
| Genes down-regulated in MCF-7 cells (breast cancer) by estradiol (E2) | (6) | 56 | Enriched among genes that were downregulated over time (0.00) | Enriched among genes that were downregulated over time (0.00) |
| Validated nuclear ERα network | (7) | 46 | Enriched among genes that were downregulated over time (0.02) | Enriched among genes that were downregulated over time (0.01) |
| Validated nuclear ERβ network | (7) | 10 | Not significantly enriched | Not significantly enriched |

[T]FDR=False discovery rate. Timepoint A= 7-12 months post-menarche; B=19-24 months post-menarche

Table 3. Enrichment analyses against 500 gene sets derived from the Molecular Function Ontology.

| | **Top-10 significantly enriched gene sets[1]** | | | | | |
|---|---|---|---|---|---|---|
| | CL A vs. B | Size | FDR | Soy A vs. B | Size | FDR |
| Enriched among genes that increased over time | Rhodopsin-like receptor activity | 97 | 0.00 | Structural constituent of ribosome | 55 | 0.01 |
| | Chemokine activity | 33 | 0.00 | Hematopoietin interferon classD-200 | 22 | 0.01 |
| | Chemokine receptor binding | 34 | 0.00 | domain cytokine receptor binding | | |
| | Cytokine activity | 85 | 0.00 | | | |
| | G-protein coupled receptor binding | 41 | 0.00 | | | |
| Enriched among genes that decreased over time | Structural constituent of muscle | 27 | 0.00 | Protease inhibitor activity | 37 | 0.00 |
| | Isomerase activity | 23 | 0.00 | ECM structural constituent | 22 | 0.01 |
| | UDP glycosyltransferase activity | 34 | 0.00 | Transmembrane receptor protein | 43 | 0.02 |
| | Cytoskeletal protein binding | 120 | 0.00 | kinase activity | | |
| | Translation regulator activity | 30 | 0.00 | Transmembrane receptor protein | 35 | 0.02 |
| | | | | tyrosine kinase activity | | |
| | | | | Lipid transported activity | 18 | 0.02 |

[T]Timepoint A= 7-12 months post-menarche; B=19-24 months post-menarche

Table 4. Enrichment analyses of gene expression by diet against estradiol-regulated markers or ER pathway gene sets.

| Gene set | Ref. | Size | Enrichment (FDR)[1] | |
|---|---|---|---|---|
| | | | CL vs. SOY at 7-12 months post menarche | CL vs. SOY at 19-24 months post menarche |
| Genes rapidly up-regulated in breast cancer cell cultures by estradiol | (5) | 43 | Enriched among genes that were upregulated with soy (0.03) | Not significantly enriched |
| Genes up-regulated in MCF-7 cells (breast cancer) by estradiol (E2) | (6) | 28 | Not significantly enriched | Not significantly enriched |
| Genes down-regulated in MCF-7 cells (breast cancer) by estradiol (E2) | (6) | 56 | Not significantly enriched | Not significantly enriched |
| Validated nuclear ERα network | (7) | 46 | Not significantly enriched | Not significantly enriched |
| Validated nuclear ERβ network | (7) | 10 | Not significantly enriched | Not significantly enriched |
| Plasma membrane ER signaling | (7) | 34 | Not significantly enriched | Not significantly enriched |

[T]FDR=False discovery rate.

Table 5. Enrichment analyses of gene expression by diet against 500 gene sets derived from the Molecular Function Ontology.

| | **Top-10 significantly enriched gene sets[1]** | | | | | |
|---|---|---|---|---|---|---|
| | CL vs. SOY<br>at 7-12 months post menarche | Size | FDR | CL vs. SOY<br>at 19-24 months post menarche | Size | FDR |
| Enriched among genes that were higher in SOY | Protease inhibitor activity | 37 | 0.00 | Structural constituent of muscle | 27 | 0.02 |
| | ECM structural constituent | 22 | 0.00 | | | |
| | Antigen binding | 15 | 0.01 | | | |
| | Enzyme inhibitor activity | 92 | 0.01 | | | |
| | G-protein-coupled receptor activity | 140 | 0.01 | | | |
| Enriched among genes that were lower in SOY | Structure-specific DNA binding | 42 | 0.00 | Chemokine activity | 33 | 0.00 |
| | Translation regulator activity | 30 | 0.00 | Chemokine receptor binding | 34 | 0.00 |
| | Single-stranded DNA binding | 25 | 0.00 | Cytokine activity | 85 | 0.01 |
| | Translation factor activity nucleic acid binding | 28 | 0.00 | G-protein-coupled receptor activity | 41 | 0.02 |
| | | | | Rhodopsin-like receptor activity | 97 | 0.03 |
| | Structural constituent of muscle | 27 | 0.00 | | | |

[T]FDR=False discovery rate.

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# APPENDIX 3

**The effect of exposure to dietary soy since in-utero on pubertal breast: a pilot study**

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**Summary of Methods**

We used mammary tissues collected from peripubertal females (age <3.5 years) that were bred in the WFU Primate Center Cynomolgus Macaque breeding colony supported by National Center for Research Resources (P40-RR021380). The animals were born from mothers fed a typical American diet with soy (Soy; n=13) or with casein and lactalbumin (CL; n=10) as the protein source, and they were continuously maintained under the same diet as their mothers. The diets mimicked macronutrient content of the typical American diet with 35% of calories from fat. Soy-fed animals receive IF at the amount equivalent (on a caloric basis) to 180 mg/person/day dose. We assessed nipple length, mammary gland morphology, markers of mammary gland differentiation, proliferation, estrogen receptors (ERs), and ER activity markers (PGR, TFF1, GREB1), and enzymes for estrogen synthesis and metabolism (STS, SULT1E1, CYP19, HSD17B1, HSD17B2, CYP1A1, CYP1B1, CYP3A4). Using a subset of samples, we also assessed global transcriptional profile with Affymetrix Rhesus GeneChip microarray, and genome-wide DNA methylation profile with Illumina Human Methyl27 BeadChip Array. For statistical analysis (JMP version 10.0.0, SAS Institute, Cary, NC), non-normally distributed data

were log-transformed or square rooted to improve normality; data were tested for homogeneity of variance using Levene's test. We used ANCOVA with age at sampling as a covariate. The covariate was excluded from the final model when found not significant, and one-way ANOVA was performed. Non-parametric data was analyzed using Wilcoxon test. Nipple length, a marker for pubertal progression, was repeated measures data analyzed using a mixed model with a random effect on animal to model diet and time main and interactive effects.

## Results

### *Serum isoflavonoids*

Measurement was performed utilizing liquid chromatography electrospray ionization mass spectrometry at the laboratory of Dr. Adrian Franke (University of Hawaii Cancer Center). To confirm that perinatal exposure can in fact occur via maternal factors, we measured total isoflavonoids from amniotic fluid (n=7 in Soy and n=13 in CL) and milk (pooled sample) from pregnant and lactating females in the breeding colony, respectively. Total isoflavonoids were 255.5 $\pm$ 93.3 nM (amniotic fluid) and 266.3 nM (milk), which confirmed that soy exposure occurred in-utero and neo-natally.

Circulating isoflavonoids during the time of breast biopsy (i.e. peripubertal age) were 1418.1 nM (1150.7 nM, 1747.5 nM) in Soy group compared to 16.7 nM (11.4 nM, 24.1 nM) in the CL group. Equol was the predominating isoflavonoid (>80%).

***Subjects***

**Table 1.** Subject characteristics. Values are LSM ± s.d.

| | CL | Soy |
|---|---|---|
| Sample size | 13 | 10 |
| Age at biopsy (years) | 2.96 ± 0.40 | 2.98 ± 0.39 |
| Body weight, neonatal (kg) | 0.34 ± 0.04 | 0.33 ± 0.06 |
| Body weight, peri-puberty (kg) | 2.50 ± 0.49 | 2.50 ± 0.40 |

***Pubertal development***

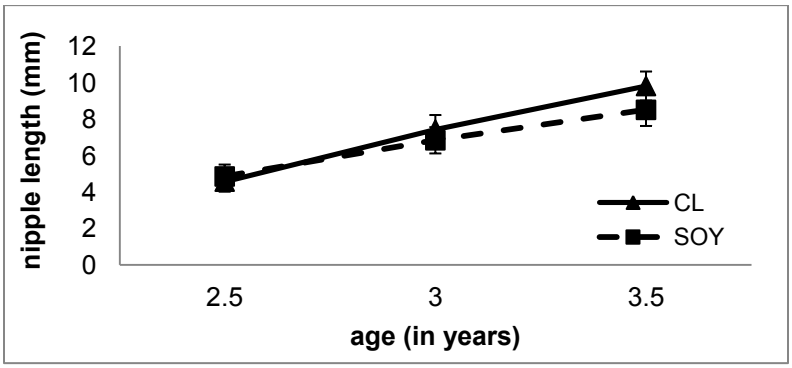



**Figure 1.** Nipple length measured periodically as marker for pubertal progression. Nipple length measurement across peri-puberty showed no difference between animals fed a soy (SOY) or casein lactalbumin (CL) diet. The length increased significantly with age ($P$<0.0001). Values are LSM. Error bars = SEM.

***Peri-pubertal breast phenotype***

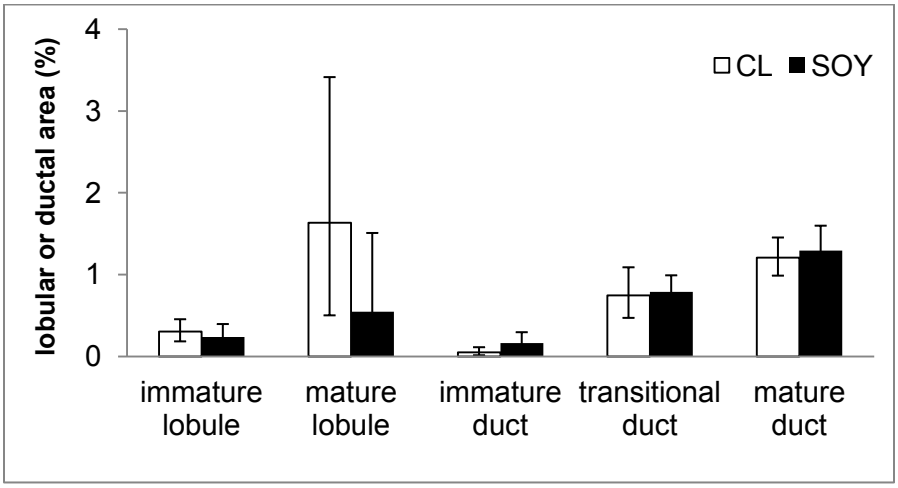



Figure 2. Mammary gland morphometry at peri-puberty as measured from digitized H&E-stained slides. The proportion of ductal and lobular structures did not differ between Soy group and Casein Lactalbumin (CL) group. Values are means, bars = SEM.

Table 2. Proportion of lobular structures as quantified from digitized images of whole mounts stained with toluidine blue. Values are means $\pm$ SEM.

| | CL (n=5) | Soy (n=3) | P-value |
|---|---|---|---|
| Terminal end buds/cm$^2$ | 11.97 $\pm$ 4.35 | 6.74 $\pm$ 0.59 | 0.53 |
| Small-sized lobules/cm$^2$ | 39.30 $\pm$ 11.00 | 50.85 $\pm$ 11.41 | 0.73 |
| Large-sized lobules/cm$^2$ | 0.00 $\pm$ 0.00 | 0.58 $\pm$ 0.36 | 0.05 |

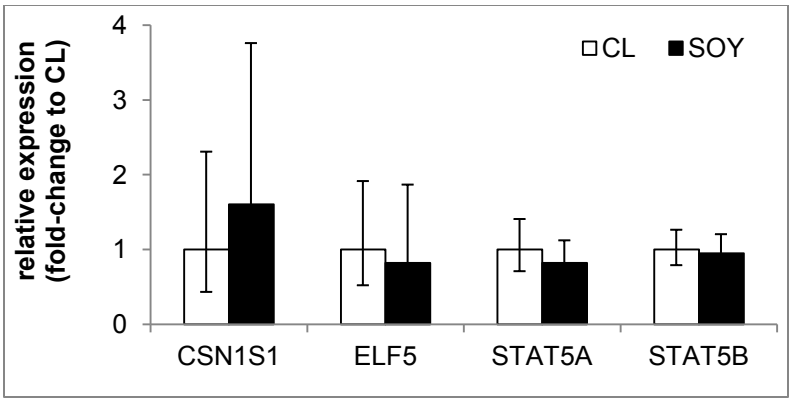



Figure 3. Differentiation markers assessed by qRT-PCR. The expression did not differ by diet groups. Values are means, bars = SEM.

***Mammary gland proliferation***

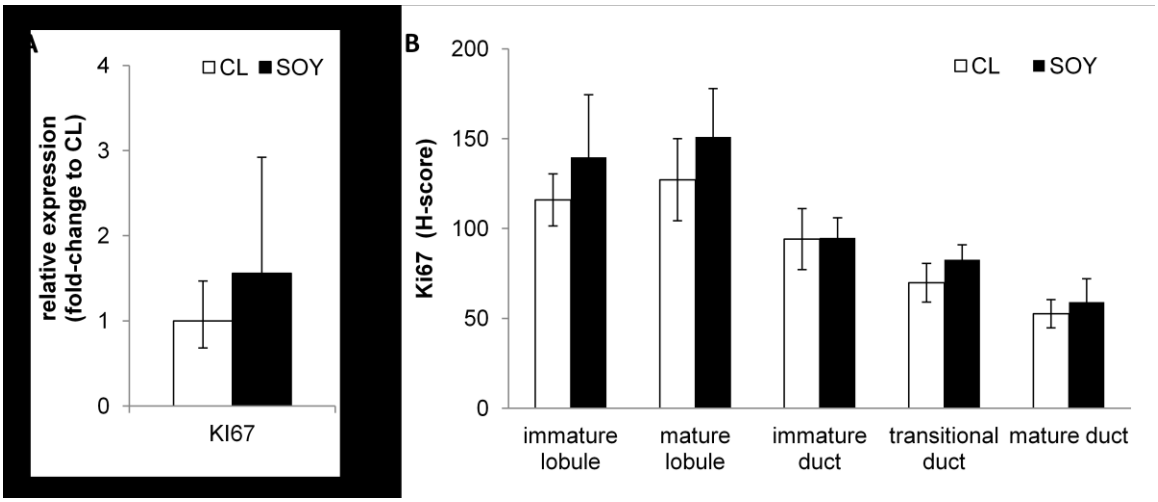



Figure 4. Expression of proliferation marker Ki67. There was no effect of dietary treatment on mRNA (A) or protein (B) expression. Values are means, bars = SEM.

***ER and ER activity markers***

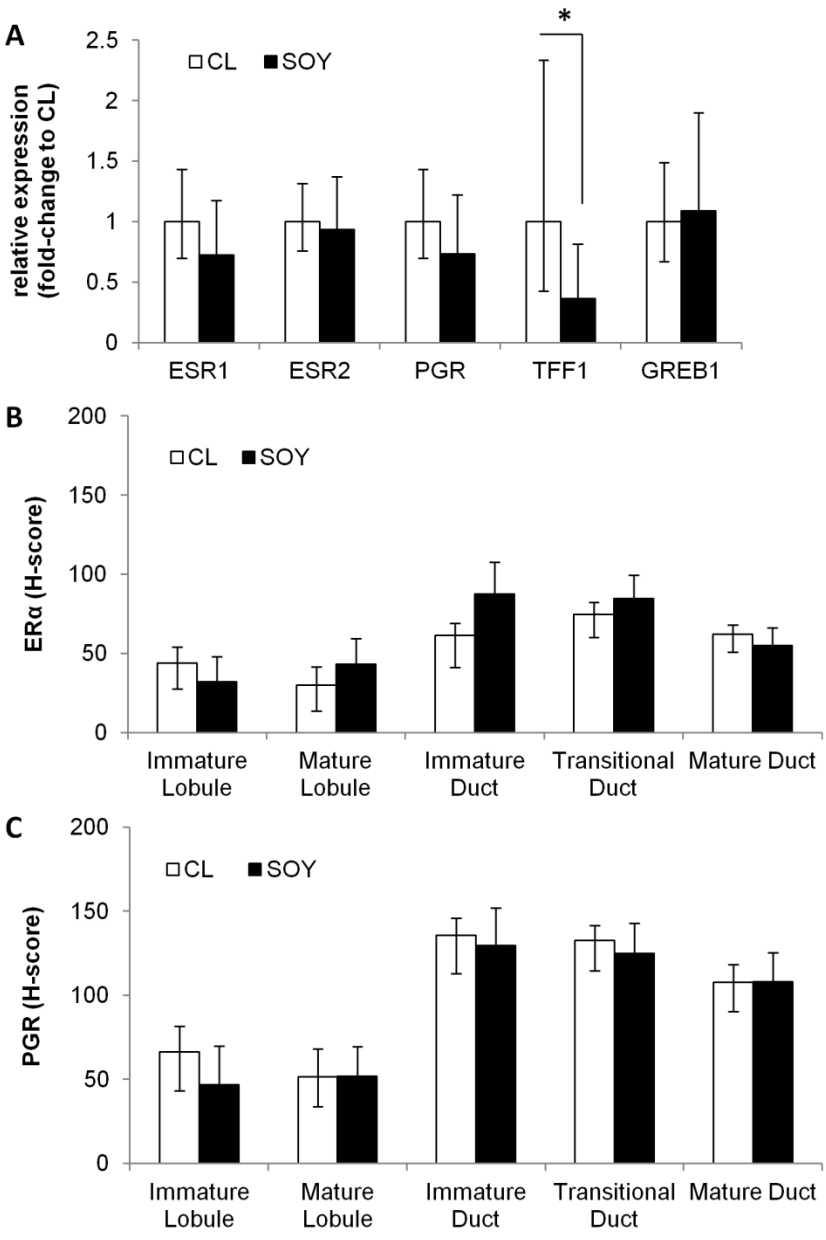



Figure 5. Expression of ERs and ER-activity markers. Except for *TFF1*, mRNA expression (A) was not affected by diet. Immunolocalization of ERα (A) and a classic ER-regulated marker PGR (B) was also not significantly affected by soy. Asterisk (*) indicates $P<0.05$ compared to CL. Values are means, bars = SEM.

***Enzymes for estrogen synthesis and metabolism***

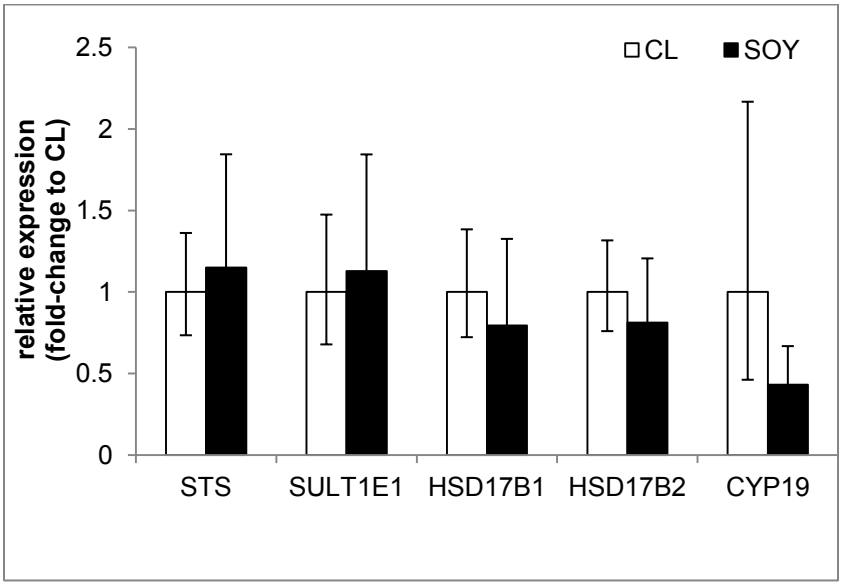



Figure 6. Expression of mRNA of steroidogenic enzymes showing no effect of diet. Values are means, bars = SEM.

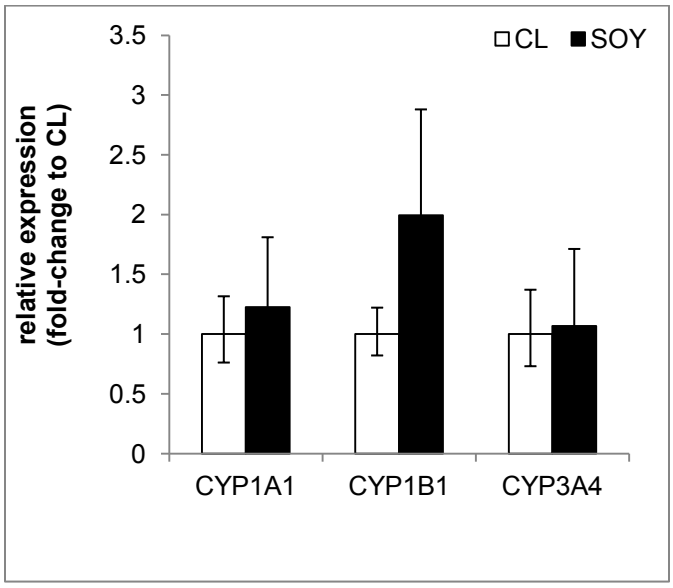
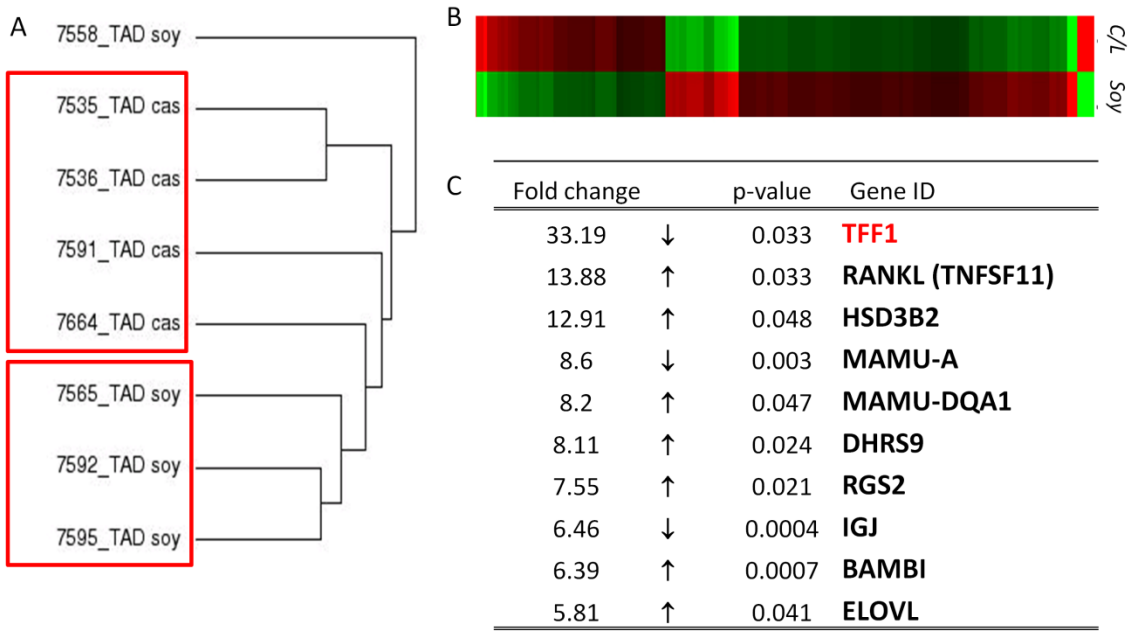
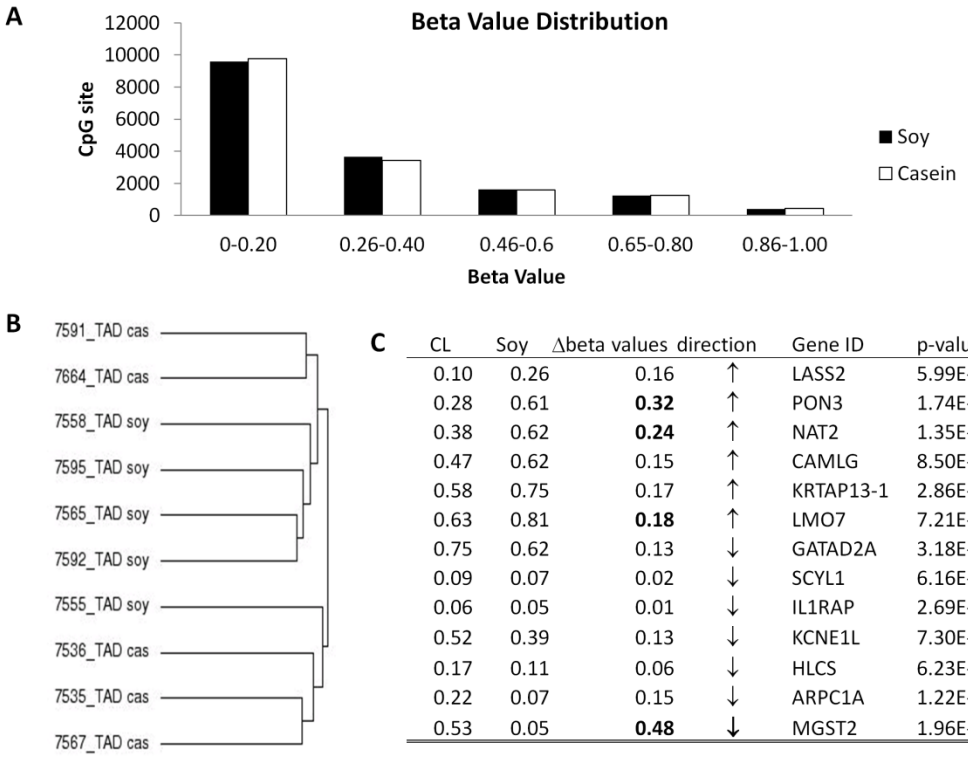



Figure 7. mRNA expression of estrogen-metabolizing enzymes, which shows no effect of dietary treatment. Values are means, bars = SEM.




| Fold change | | p-value | Gene ID |
|---|---|---|---|
| 33.19 | ↓ | 0.033 | **TFF1** |
| 13.88 | ↑ | 0.033 | **RANKL (TNFSF11)** |
| 12.91 | ↑ | 0.048 | **HSD3B2** |
| 8.6 | ↓ | 0.003 | **MAMU-A** |
| 8.2 | ↑ | 0.047 | **MAMU-DQA1** |
| 8.11 | ↑ | 0.024 | **DHRS9** |
| 7.55 | ↑ | 0.021 | **RGS2** |
| 6.46 | ↓ | 0.0004 | **IGJ** |
| 6.39 | ↑ | 0.0007 | **BAMBI** |
| 5.81 | ↑ | 0.041 | **ELOVL** |

Figure 8. Gene expression profiles in the mammary tissue of monkeys exposed to soy or CL since in-utero. (A) Unsupervised hierarchical clustering of samples ($n$=4/group) indicated that animals in the same diet group showed more similar gene expression profile. (B) Heat map for gene probes with ANOVA unadjusted $P$<0.05; fold-change>2 ($n$=122 genes), and corresponding list of the top-10 differentially expressed genes (C).




C

| CL | Soy | Δbeta values | direction | Gene ID | p-values |
|---|---|---|---|---|---|
| 0.10 | 0.26 | 0.16 | ↑ | LASS2 | 5.99E-05 |
| 0.28 | 0.61 | **0.32** | ↑ | PON3 | 1.74E-07 |
| 0.38 | 0.62 | **0.24** | ↑ | NAT2 | 1.35E-07 |
| 0.47 | 0.62 | 0.15 | ↑ | CAMLG | 8.50E-05 |
| 0.58 | 0.75 | 0.17 | ↑ | KRTAP13-1 | 2.86E-05 |
| 0.63 | 0.81 | **0.18** | ↑ | LMO7 | 7.21E-06 |
| 0.75 | 0.62 | 0.13 | ↓ | GATAD2A | 3.18E-05 |
| 0.09 | 0.07 | 0.02 | ↓ | SCYL1 | 6.16E-05 |
| 0.06 | 0.05 | 0.01 | ↓ | IL1RAP | 2.69E-05 |
| 0.52 | 0.39 | 0.13 | ↓ | KCNE1L | 7.30E-05 |
| 0.17 | 0.11 | 0.06 | ↓ | HLCS | 6.23E-05 |
| 0.22 | 0.07 | 0.15 | ↓ | ARPC1A | 1.22E-06 |
| 0.53 | 0.05 | **0.48** | ↓ | MGST2 | 1.96E-05 |

Figure 9. DNA methylation profile across monkeys exposed to soy or casein since in-utero as measured by Illumina methylation array (*n*=5/group). (A) Methylation ratio (beta-value) distribution across the study, which showed that majority of the CpG sites detected were not methylated (i.e. beta value <0.5) (B) Unsupervised hierarchical clustering of mammary samples from animals in soy or casein group based on their CpG methylation profile. (C) 13 CpG sites that showed significant difference in methylation ratio by diet groups with unadjusted $P<0.0001$.

## CURRICULUM VITAE

**Full name:** Fitriya Nur Annisa Dewi

**Born:** Jakarta, Indonesia; June 25, 1982

**Education:**

| INSTITUTION AND LOCATION | DEGREE | YEAR | FIELD OF STUDY |
|---|---|---|---|
| Bogor Agricultural University, Bogor, Indonesia | Bachelor in Veterinary Medicine (with honors) | 2000-2006 | Veterinary Medicine |
| Bogor Agricultural University, Bogor, Indonesia | Doctor of Veterinary Medicine | 2006-2007 | Veterinary Medicine |
| Wake Forest School of Medicine, Winston-Salem, NC, USA | *on going* (Ph.D. candidate, 2013) | 2009-present | Molecular Pathology/ Comparative Medicine |

**Scholastic and professional experience:**

01/2003-12/2003 Internship, Microbiology and Immunology Laboratory,
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01/2004-12/2004 Research Fellowship
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01/2005- 06/2005 Externship, Microbiology and Immunology Laboratory,
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| | |
|---|---|
| 07/2005-12/2005 | Externship, Division of Research Animal Resources (Research Animal Facility and Quarantine Facility), Bogor Agricultural University Primate Research Center (IPB-PRC).<br>Bogor, Indonesia. |
| 01/2006-10/2007 | Veterinary Technician (part-time), Bimana Indomedical.<br>Bogor, Indonesia. |
| 11/2007- 08/2009 | Research Facility Veterinarian, Bimana Indomedical.<br>Bogor, Indonesia. |
| 08/2009 – present | Post-DVM fellowship/Graduate Student<br>Department of Pathology/Section on Comparative Medicine and Wake Forest Primate Center, Wake Forest School of Medicine.<br>Winston-Salem, NC, USA |

**Awards and Honors:**

| | |
|---|---|
| 2003 | Award (1st Place) for Student with Outstanding Achievements in Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, Indonesia |
| 2003 | Award (4th Place) for Student with Outstanding Achievements in Bogor Agricultural University, Bogor, Indonesia |
| 2004 | Fellowship from Association of International Education Japan (AIEJ) to pursue one year of research training at University of Miyazaki in Miyazaki, Japan |
| 2006 | Award for Best Graduate for Bachelor in Veterinary Medicine, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, Indonesia |
| 2012 | Best Poster Award at the 7th Annual Women's Health Research Day in Wake Forest School of Medicine, Winston-Salem, North Carolina, USA |

**Professional memberships:**

| | |
|---|---|
| 2007-present | Indonesian Veterinary Medical Association, West Java II Chapter |
| 2011-present | American Association for Cancer Research |
| 2012-present | Breast Cancer Center of Excellence, Comprehensive Cancer Center of Wake Forest University |
| 2013 | International Society for Developmental Origins of Health and Disease |

**Publications:**

Peer-reviewed publications:

**Dewi FN,** Wood CE, Willson CJ, Lees CJ, Register TC, Howard TD, Huang Z, Murphy SK, Tooze JA, Miller LD, Chou JW, Cline JM. The effects of pubertal exposure to dietary soy on estrogen receptor-responsiveness in the breast of cynomolgus macaques. In preparation.

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Inagaki-Ohara K, **Dewi FN**, Hisaeda H, Smith AL, Jimi F, Miyahira M, Abdel-Aleem AS, Horii Y, Nawa Y. Intestinal intraepithelial lymphocytes sustain the epithelial barrier function against Eimeria vermiformis infection. *Infect Immun. 2006 Sep; 74(9): 5292-301.*

Scientific Poster and Oral Presentations;

**Dewi FN**, Wood CE, Register TC, Lees CJ, Howard TD, Huang Z, Murphy SK, Tooze JA, Cline JM. The effect of pubertal exposure to dietary soy on markers of estrogen activity in the breast of cynomolgus macaques. Abstract accepted for poster presentation at the 8th World Congress on Developmental Origins of Health and Disease. 17-20 November 2013. Singapore.

Willson CJ, Borgerink H, **Dewi FN**, Wagner J, Williams JK, Wood CE, Zhang L, Cline JM. Steroidogenic enzyme immunolocalization and expression in reproductive tissues of the female cynomolgus macaque. Poster presented at 2012 Annual Meeting of Society of Toxicologic Pathology. June 24-28 2012. Boston, MA.

**Dewi FN**, Wood CE, Lees CJ, Wagner JD, and Cline JM. In-utero, but not pubertal, soy exposure suppresses estrogen-regulated gene expression in non-human primate breast. Poster presented at 2012 Annual Meeting of American Association for Cancer Research (March 31-April 4 2012, Chicago, IL), and the 7th Annual Women's Health Research Day in Wake Forest School of Medicine (Winston-Salem, NC, May 1 2012).

Nishigaki T, **Dewi FN**, Hirose K, Shigematsu H. Safety and Anti-tumor Effects of *Pandanus conoideus* (buah merah) in Animals. Poster presented at 2010 International Society for Nutraceuticals and Functional Foods. October 13-15, 2010. Bali, Indonesia.

**Dewi FN,** Wood CE, Cline JM, Franke AA, Golden DL and Adams MR. Endogenous and Dietary Equol Effects in Male and Female Mice. Poster presented at the 9th International Symposium on the Role of Soy in Health Promotion and Chronic Disease Prevention and Treatment. October 16-19, 2010. Washington DC.

Villiandra, **Dewi FN**, Paramastri Y, Iskandriati D, Hayes E. Sensitivity to Ascaris suum Extract in Indonesian *Macaca fascicularis*: Skin Test Reactivity and Airway Challenge. Poster presented at the 3rd International Meeting on Asian Zoo/ Wildlife Medicine and Conservation (AZWMC) and the 10th Indonesian Veterinary Scientific Conference of Indonesian Veterinary Medical Association. August 19-22, 2008. Bogor, Indonesia.

**Dewi FN**, Yunus M, and Horii Y. Concurrent infections of *Eimeria vermiformis* and *Nippostrongylus brasiliensis* in C57BL/6 Mice. Presented at National Seminar of Association of Japan Alumni of Indonesia. August 23, 2005. Bogor, Indonesia.