Review article

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BONISTEIN™ (SYNTHETIC GENISTEIN), A FOOD COMPONENT IN DEVELOPMENT FOR A BONE HEALTH NUTRACEUTICAL

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In the discussion of the risk-benefit relation of the hormone replacement therapy (HRT) for elder women phytochemicals with estrogenic activity received a great deal of attention. Phytoestrogens are naturally occurring compounds with structural similarity to 17β-estradiol. Especially genistein, an isoflavone most abundant in soy, possess a high and selective binding-affinity to the mammalian estrogen receptors. It has been found, that genistein exert in humans both: weak estrogenic and anti-estrogenic effects, similar to the SERMs. Consequently, it was concluded, that genistein might provide an alternative to prevent postmenomausal bone-loss and ameliorate menopausal symptoms without side-effects similar to HRT. Pre-clinical experiments and results from clinical pilot studies with pure genistein confirmed its efficacy in these indications. Nevertheless, currently some open issues still exist to recommend its intake thoughtlessly.

Bonistein™, pure synthetic genistein developed by DSM Nutritional Products, was tested extensively in appropriate models for bone health. A battery of toxicological studies was conducted to determine safe intake levels. In the early clinical development pharmacokinetic studies were performed in healthy volunteers and in postmenopausal women. Now large-scale studies are in preparation to investigate Bonistein™'s efficacy in postmenopausal bone-loss and climacteric syndrome.

Key words: *bonistein, genistein, menopause, hot flushes, osteoporosis, nutraceuticals, functional foods, pharmacokinetics*

INTRODUCTION

Consumers and health authorities demand healthier food products, nutritional research evaluates certain food components for their health beneficial properties.

One common goal is the development of functional foods containing specific dietary components that exceed basic nutritional benefits to prevent certain chronic diseases. In this context we are investigating promising foodstuff and identify the most active ingredients out of the surrounding food matrix. These compounds can then be produced by either complete *de-novo* chemical synthesis or by extraction/filtration-methods. The result of both approaches is a pure substance identical to its natural origin.

One natural compound that appears to satisfy many of the requirements is the isoflavone genistein. The observation of the high abundance of isoflavones in Asian diets and the lower rates of "Western" diseases in Asia such as coronary heart disease, post-menopausal osteoporosis, climacteric symptoms as well as breast and prostate cancers has led to a proposed protective role of isoflavones (1, 2).

Estimates indicate an average intake of isoflavones of 1-20 mg/day (genistein 0.01-12 mg/day) in these regions (3-6). However, an accurate determination of the isoflavone intake is quite difficult because too many factors influence dietary isoflavone consumption like sort of soybeans, processing technique, storage conditions, regional, cultural and individual eating habits, etc. (6-9). In Western Countries soy is rarely a normal component of the diet. Although quite a lot of food products contain soy components these are mostly soy oils and soy lecithin lacking isoflavones and, consecutively, the dietary intake of isoflavones is typically negligible (<1 mg/day) (10, 11).

Within the last decades several pharmacological properties of genistein were identified and some more may follow. Especially the estrogen-like properties were recognised early and investigated systemically (12, 13). Furthermore, experiments with 13C-labelled genistein clarified the principle routes of absorption, distribution, metabolism and excretion (ADME) and other very important pharmacokinetic parameters (14-16).

Coincidentally, in the last years concerns about adverse drug reactions and the lack of cardiovascular protection of the hormone replacement therapy (HRT) became evident from clinical studies (17, 18). It seemed logical that natural estrogenic compounds such as genistein should be considered as a possible alternative. Until today, there is no evidence, that long-term intake of soy isoflavones including genistein creates side effects similar to HRT.

To investigate the pharmacological properties of isolated genistein DSM developed Bonistein™, a 99.6% pure synthetic genistein product as 100% aglycone. Due to its chemical synthesis process a constant quality could be provided in large quantities, which allows the formulation of capsules with a defined and stable genistein content to be used in biomedical research. Focus of Bonistein™ development is, first of all, to demonstrate its safety and tolerability, as well as its efficacy to prevent post-menopausal bone-loss and osteoporosis, and secondarily, relief of climacteric complaints.

Genistein's characteristics

Decades ago genistein was firstly introduced by Walz and Walter (19, 20). Genistein is most abundant in soybeans (*Glycine max*), but has also isolated from red clover (*Trifolium pratense*) and subterranean clover (*T. subterran*). It is a multipurpose biochemical of the flavonoid class and has several functions in the plant, e.g. colouring, protection against bacterial/fungal infections and hormonal cell regulation (21).

Because genistein and other isoflavones resemble the steroid hormone 17β-estradiol, can exert estrogen-like effects, and are found in plants, they have been called "phytoestrogens" (*Fig. 1*). However, many of their biological effects are unrelated to their estrogen-like properties. Moreover, the term "phytoestrogen"
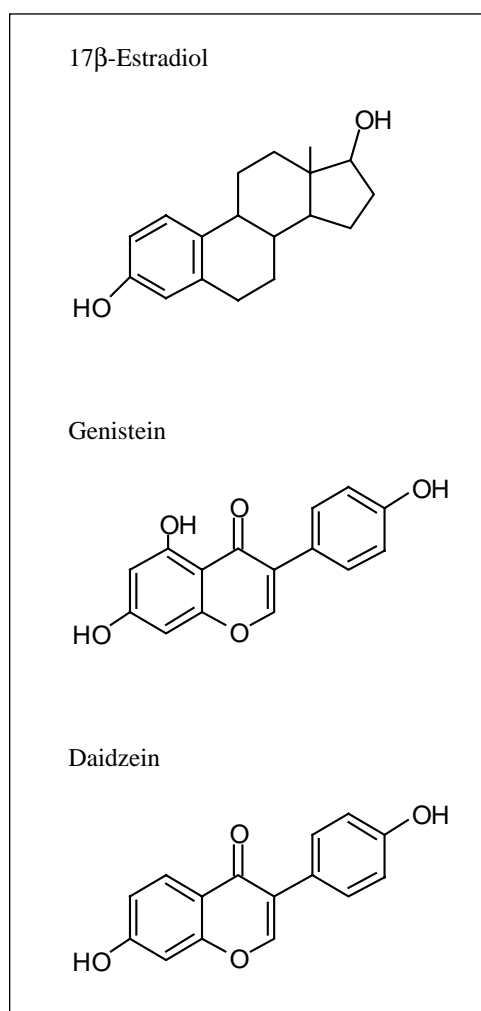

17β-Estradiol

Genistein

Daidzein

Figure 1. Chemical structure of 17β-estradiol in comparison to the two most abundant soy isoflavones genistein and daidzein (both as aglycones).

includes also substances other than isoflavones, such as lignans in flax seed and coumestans in broccoli and alfalfa sprouts. For these reasons, it is preferable to describe isoflavones in terms of their specific components rather than by one aspect of function as "phytoestrogens".

Genistein is usually present as β-glycosides (Genist*in*). Fermentation, digestion, and certain soybean processing techniques release the sugar molecule leaving the aglycone (geniste*in*). The sugar moiety is not required for biological activity but influences intestinal absorption. The aglycone is absorbed faster and achieved higher C_{max} levels, an observation consistent with additional time needed for cleavage of the glycoside moiety (15, 21, 22). Thus, the evidence indicates that intestinal β-glycosidase bacteria activity ensures that genistein in foods is readily available for absorption. Additionally, there is some suggestion that bile secretion, genistein disappearance phenotype, gut transit time, concomitant food intake and antibiotic therapy also affect the bioavailability of genistein aglycones. Once absorbed, genistein glucuronide conjugates are transported to the liver where they are removed from the portal blood. Conjugated genistein are excreted into the urine as well as into bile, which results in further metabolism and degradation in the intestine and entero-hepatic circulation (23, 24).

Most of the currently known pharmacological properties of genistein support its proposed antiatherogenic, estrogenic and anticancer effects. Briefly, plasma lipid modification, antioxidant effects, vascular reactivity changes as well as hormonal actions are to mention. On the molecular level in cancer cells genistein up-regulates apoptosis, inhibits nuclear factor (NF)-κB, downregulates transforming growth factor-β, inhibits epidermal growth factor-stimulated growth (inhibition of angiogenesis) and inhibits DNA topoisomerase II. In high concentrations genistein is a very potent inhibitor of tyrosine-kinases and has been shown to inhibit growth of cancer cells effectively. In addition, genistein has estrogen receptor (ER) binding activity. The biological response to genistein exerts both estrogenic and anti-estrogenic actions (24-26).

Genistein and bone health

The role of 17β-estradiol on bone-health emerged in 1941 when Albright *et al.* linked menopause with occurrence of osteoporosis (27). Nowadays, it is common knowledge that HRT prevents bone loss and reduces fractures (28, 29). Additional benefits of HRT were claimed in the late 1960s when it was believed to reduce risk of heart disease, colorectal cancer, as well as banish menopausal symptoms. But findings from recent studies provoke concerns: five years HRT was associated with higher risk of breast cancer, endometrial and ovarian cancers, venous thromboembolism, cardiovascular disease and stroke (17, 18). For these reasons the benefits of HRT for elderly women, especially in reducing the risk of osteoporosis, are unlikely to be realized.

In bone cells both types of ERs are present (β>> α), whereas their expression varies considerably during differentiation (30, 31). The bone-sparing effects of HRT are mainly mediated by the ERβ to which genistein has a very high and selective affinity. It is capable to displace 17β-estradiol from the ER (dose-dependently) and exerts estrogenic and anti-estrogenic effects. Therefore, genistein was assigned as a natural selective ER modulator (SERM), which anabolically modulate bone cell (ERβ-mediated) without the unwanted estrogenic activities in other tissues, eg. the blood vessels, breast, uterus, brain and heart (mainly ERα-mediated) (32, 33).

Further encouragement to investigate the bone-beneficial effects of isoflavones was given by the similarity in chemical structure between the soy isoflavones genistein and daidzein to the synthetic compound 7-isopropoxyisoflavone (INN). Initially, for Ipriflavone™ nice bone-sparing effects in postmenopausal were demonstrated, but a recent study provided negative results even suspect for severe side effects (34, 35).

First observational studies correlated the low rate of hip fractures in postmenopausal Asian women compared to Caucasians with isoflavone consumption, albeit populations differ in many ways other than diet. And indeed, a positive association between dietary isoflavone intake and reduced biomarker levels for bone resorption and, less conclusively, to higher bone mineral density (BMD) could be proposed. But, there is also great evidence, that the lower rate of hip fractures of Asian women is to be due to anatomical and biomechanical differences between races (e.g. shorter hip axis length) and a lower tendency to fall (36-39).

In parallel to observational surveys numerous pre-clinical experiments tested the response of bone cells to genistein. It has been found to stimulate protein synthesis, alkaline phosphatase release, and calcium content in rat metaphyseal tissue. In osteoclasts genistein suppressed the activation of protein phosphatases, induced apoptosis and inhibited osteoclast-like cell formation through various pathways (40-42). It was speculated that genistein decreases bone loss through modulation of the nitric oxide production in the cardiovascular system resulting in a 17β-estradiol mimicking net-effect (43-47). More recently, genistein has been found to stimulate the production of osteoprotegerin by human paracrine osteoblasts providing a further mechanism for the putative bone-sparing effects of isoflavones (48). It suppress osteoclast activity by a number of possible mechanisms, including induction of apoptosis, activation of protein tyrosine phosphatase, inhibition of cytokines, changes in intracellular calcium and membrane depolarization (49, 50).

Histomorphometic, genistein reduces both trabecular and compact bone loss. It is worth to mention, that the bone sparing effect of genistein appeared in a moderate dose range (0.5 - 1.0 mg/kg BW) and disappeared with dose escalation (> 1.7 mg/kg BW). Also a point of discussion is the reliability and relevance of the ovarectomized rodent model for human conditions. For example, some

studies with red clover isoflavones had "no effect" in this experimental set-up, whereas human studies showed surprisingly positive effects of red clover isoflavones on BMD in post-menopausal women (51, 52). Interestingly, single studies of ovariectomized monkeys and growing pigs have found no effect of soy isoflavones and genistein on BMD (53-55).

No long-term studies using phytoestrogens and looking at the fracture risk as a parameter are available until now. Only some medium-term clinical trials that lasted from 6 to 24 months have investigated the influence of phytoestrogens on BMD. Among these clinical studies, little conformity in the design and other major trial features exist. The first encouraging findings from short-term bone marker studies were not consistently confirmed in the BMD studies. Reasons for that are manyfold and were recently reviewed by Setchell and Messina (36, 37). In conclusion, available data are insufficient to recommend soy or red clover isoflavones mixtures to substitute of anti-osteoporotic medication in women at risk for accelerated postmenopausal bone-loss (56-60).

Great exception is one study investigating the effect of 54 mg genistein on BMD over 12 months compared to HRT and placebo (61, 62). In this study isolated genistein was supplied, which warranted a highly standardized bioavailability and pharmacokinetic behaviour. Additionally, the production of equol, a metabolite to daidzein with estrogenic action was ruled out. This study generated positive results: compared to placebo genistein led to a highly statistically significant increase of over 3 % in BMD of both the spine and the femoral neck. This effect was equivalent to that observed with HRT. For the first time a clinical study could show evidence that isolated genistein was effective in prevention of postmenopausal bone-loss.

Genistein and climacteric syndrome

Menopause is the natural end of menstruation and fertility for women. Menopause is confirmed when a woman has had no menstrual bleeding for 12 months, and when there are no other physical or psychological factors to account for its absence. It is characterised by a continuous decrease of free circulating 17β-estradiol and, in parallel, an increase in follicle stimulating hormone (FSH). In this phase symptoms of low estrogen levels could occur and persist, including hot flashes, insomnia, anxiety, mood swings, loss of libido and vaginal dryness. HRT is the common therapy to ameliorate peri-menopausal complaints (63, 64). Due to its estrogen receptor affinity it was suggested that genistein might be efficacious in the treatment of climacteric complaints (27, 65, 66)

Taking together the available literature of clinical trials investigating the effect of soy isoflavones on reduction of menopausal complaints no uniform picture could be drawn (see *Table 1*). Most of the studies varied relevantly in design, duration, treatment and outcome assessments and, therefore, were hardly comparable against each other (*Table 1*).

In conclusion, the nutritional intervention trials showed contradictory results. However, most of these studies, among them the largest and longest, revealed

Table 1: Human intervention trials investigating the effect of soy isoflavones on relief of peri-menopausal symptoms in peri- and postmenopausal women
(only prospective, randomised and controlled trials of at least 6 weeks of duration, published in English language were considered)

publication	n*	treatments	main efficacy parameter	results
Crisafulli 2004 (69)	90	(a) HRT[1]	hot flushes (and hot flushes scale)	(a)+(b) vs (c) decreased significantly, (b) vs (a) decreased significantly
		(b) 54 mg/d pure genistein		
		(c) placebo		
Russo 2003 (77)	50	(a) soy IF[2] extract	hot flushes, vaginal dryness, insomnia, anxiety	(a) vs (b) improved significantly
		(b) placebo	Quality of Life Questionnaire	no effect
Burke 2003 (78)	241	(a) high-IF soy extract (42 mg/d)	vasomotor symptoms incl. hot flushes	no effect
		(b) low-IF soy extract (58 mg/d)		
		(c) placebo		
Penotti 2003 (79)	62	(a) soy IF extract (72 mg/d)	hot flushes	no effect
		(b) placebo		
Van Patten 2002 (80)	157	(a) soy drink (90 mg/d)	hot flushes	no effect
		(b) placebo (rice drink)		
Albertazzi 1998 (81)	104	(a) soy protein extract (IFs 76 mg/d)	hot flushes	(a) vs (b) decreased significantly
		(b) placebo (casein)		
Germain 2001 (82)	69	(a) high-IF soy protein extract (IFs 80 mg/d)	hot flushes	no effect
		(b) low-IF soy protein extract (IFs 4.4 mg/d)		
Quella 2000 (83)	177	(a) soy IF extract (150 mg/d)	hot flushes	no effect
		(b) placebo		
Upmalis 2000 (84)	177	(a) soy IF extract (100 mg/d)	hot flushes	(a) vs (b) decreased significantly
		(b) placebo		
Kotsopoulos 2000 (85)	95	(a) soy protein extract (IFs 118 mg/d)	hot flushes	no effect
		(b) placebo		

Scambia 2000 (86)	39	(a) soy extract (50 mg/d)	hot flushes	(a) vs (c) decreased significant, (b) vs (a) & (c) decreased significantly
		(b) HRT (c) placebo	Green Climacteric Scale	(a) vs (c) improved significantly (b) vs (a) & (c) improved significantly
Washburn 1999 (87)	51	(a) soy protein extract (IFs 34 mg/d, bolus)	vasomotor symptoms incl. hot flushes	no effect
		(b) soy protein extract (IFs 34 mg/d, splitted dose)	Quality of Life Questionnaire	no effect
		(c) placebo		
Baber 1999 (88)	51	(a) soy protein extract (IFs 40 mg/d)	hot flushes	no effect
		(b) placebo	Green Climacteric Scale	no effect

* number of subjects
[1] hormone replacement therapy
[2] total isoflavones

no benefit for the affected women. Taken the few positive studies, administered isoflavones reduced occurrence of hot flashes about approximately 40% and placebo causing a 30% reduction compared with an approximate 75% reduction in hot flashes with hormone replacement therapy. Currently there is little evidence that phytoestrogens from soy foods and soy extracts do improve hot flushes or other menopausal symptoms (65, 67, 68), which reflects the general opinion. Interestingly, one trial conducted with pure genistein showed favourable effect on hot flushes, not as effective as HRT, but highly significant compared to placebo (69).

Bonistein™ pre-clinical development

Bonistein™ was tested in human bone marrow stromal cells, undergoing osteogenic differentiation (33). Profiling of the gene transcripts revealed differential stage dependent expression of the estrogen receptor isotypes and splice variants. Bonistein™ was able to regulate the lineage-determining regulator genes. The osteoblast-determining core binding factor-α1 (Cbfα1) was up-regulated, whereas classic adipogenesis regulator gene, peroxisome proliferators-activated receptor γ (PPARγ), was down-regulated by Bonistein™.

Moreover, Bonistein™ increased alkaline phosphatase mRNA levels and activity, an important late osteoblast differentiation marker. During osteogenic differentiation the OPG:RANKL ratio was highly elevated by Bonistein™ indicating an increased inhibiting effect for coupling osteoclasts. Overall, Bonistein™ enhanced the commitment and differentiation of the osteoblast precursor cells, whereas it reduced the adipogenic differentiation and maturation of bone marrow stromal cells the common precursor for both osteoblast and adipocytes in bone. Bonistein™ ADME studies were performed using 13C-labeled material in rats. The metabolism of Bonistein™ was characterised by rapid and extensive absorption of the parent compound followed by conjugation and excretion in bile and urine. The metabolites 4-OH-phenylisopropionic acid and p-ethylphenol were most probably formed by microbiological degradation of Bonistein™ in the intestine. They were subsequently absorbed and predominantly excreted in the urine. Their ratio varied widely. There was no noticeable sex difference in the routes or rates of elimination of the radioactivity. Also, there was no evidence for accumulation of radioactivity in the tissues. The potential of Bonistein™ to inhibit major human CYP450 isoforms involved in drug metabolism (1A2, 2C9, 2C19, 2D6, 3A4) was studied *in vitro* using isoform specific substrates. IC_{50} values determined were all more than 10 µM. Based on the much lower levels that are expected in human tissues relevant interactions of Bonistein™ with concomitantly administered compounds metabolized by CYP isoenzymes are not expected.

Bonistein™ was also broadly tested in toxicological studies. A comprehensive overview is given in *Table 2*. The safety of Bonistein™ was investigated by an array of studies on genotoxicity, acute and repeated-dose toxicity up to 52 weeks, developmental toxicity, skin irritation/sensitization and ADME. The no-observed-adverse-effect levels (NOAELs) were determined in two species, rats and dogs. In the mutagenicity studies and reproduction toxicity studies a weak genotoxic effect was found at extraordinary high concentrations *in vitro*, but the relevant *in vivo* genotoxicity study in mice did not show a genotoxic potential. Interestingly, Bonistein™ did not reveal any teratogenic potential. Most of the toxicological studies were done under GLP (good laboratory practice) requirement.

Bonistein™ in clinical studies

The first-entry in man was a single ascending doses study using hard-gelatine capsules filled with 10 mg or 50 mg of Bonistein™. Bolus doses of 30, 60, 150 and 300 mg were given. Secondly, a multiple ascending dose study supplying 30, 60 and 120 mg Bonistein™ per day for 14 consecutive study days was conducted. Both studies were performed in healthy subjects of either gender. Primary objectives were safety and tolerability of Bonistein™ administration. Secondarily, pharmacokinetic profiles were determined after acute dose and during steady state. Rate and extent of Bonistein™ bioavailability were compared

Table 2: Pre-clinical safety and ADME* testing battery for Bonistein™
(for the key studies scientific publication are in preparation)
* absorption, distribution, metabolism, excretion

TOXICOLOGY
    Genotoxicity
        Ames Test
        Mouse lymphoma cell mutation tests
        In vitro micronucleus test
        In vivo mouse micronucleus assay
    Acute Toxicity
        Single-dose toxicity study (Limit test)
        Single-dose toxicity study (Acute Toxic Class Method)
    Subchronic Toxicity
        28-day oral (feed admix) toxicity study in rats
        28-day oral (capsule) toxicity study in dogs
        90-day oral (feed admix) toxicity study in rats
        90-day oral (dietary) toxicity study in dogs
    Chronic Toxicity
        52-weeks oral (dietary) toxicity study in rats
        52-weeks oral (capsule) toxicity study in dogs
    Reproduction Toxicity
        In vitro reproduction toxicity study: whole embryo culture assay
        Pilot reproduction toxicity studies in rats
        Prenatal developmental toxicity study in rats

WORKERS SAFETY
        Skin irritation
        Primary skin irritation
        Skin sensitization

ADME STUDIES
        Pilot study in rats
        Main study in rats
        In vitro drug-interaction studies (CYP450)

across doses and time. To compare the characteristics of bioavailability between healthy volunteers and post-menopausal women, an open, un-controlled pharmacokinetic study was done in post-menopausal women. They received 30 mg Bonistein™ per day for one week.

It could be stated, that systemic bioavailability of oral application of Bonistein™ up to 300 mg/d was dose-proportional. However, a trend toward reduced bioavailability was detected between 150 mg and 300 mg. During repeated dosing (once daily) Bonistein™ did not accumulated in the dose range investigated (up to 120 mg). Subgroup-analysis revealed, that a small, but significant, gender-effect existed. Women seem to have a slightly better intestinal absorption of Bonistein™ resulting in a higher overall exposure. These finding

were confirmed, when the pharmacokinetic data of the healthy, young subjects were compared to the data of the post-menopausal women. The systemic bioavailability in elder women was approximately 15 % higher. Elimination half-lives showed only subtle differences.

In total 53 adverse events (AEs) were reported in the studies mentioned above. None of the AEs was serious or unexpected. Most of the AEs were of mild intensity with the exception of eight (15 %). 49 % of the AEs were judged as likely related to *Bonistein™* administration by the clinical investigators. Most common AEs were headache and slight variations of pancreas enzymes lipase and amylase, well-know effects of highly concentrated, purified isoflavones already reported in other clinical studies (16, 70). Nearly all other AEs were unspecific and might reflect the usual observation of complaints in clinical studies with healthy volunteers.

Summary and conclusion

The soy isoflavone genistein possess various interesting pharmacological properties and might help to prevent certain medical conditions. Therefore, genistein qualifies as potential candidate to be a component of functional foods or nutraceuticals. Several observational studies demonstrate positive associations between isoflavone intake and reduced biomarker for bone resorption. But these surveys did not elucidate cause and effect. Their findings should be reasonably consistent and statistically significant to justify more detailed pre-clinical and clinical research. Intervention and detailed metabolic studies are required to support and explain a causal link with health outcomes, determine an effective dose range, and characterize target groups.

During the last years numerous clinical trials tried to establish the use of isoflavones to prevent postmenopausal bone-loss and ameliorate climacteric symptoms. But, despite these efforts, today no generally accepted recommendation for the use of isoflavones could be given. The outcomes of the trials were contradictory to draw a uniform conclusion and the reasons for that are manifold.

One problem is the limited availability of pure or standardised isoflavone preparation. Pre-clinical and clinical research was done using various soy foods or beverages, soy extracts, isoflavone isolates or pure genistein in various galenic formulations. Theses preparations often relevantly differed in their isoflavone concentrations, glycone:aglycone ratios, bioavailability and other important substance characteristics-like solubility, concomitant food matrices or pharmacokinetics. Sometimes additional food/soy components, such as proteins, lignan, saponins or other were present. These might potentially influence the study by exerting independent pharmacological activities. For example, in animal studies soy protein isolates lacking isoflavones maintained BMD at levels comparable to estrogen, increased intestinal calcium absorption, Vitamin D levels

and insulin-like growth factor suggesting additional mechanistic pathways to support bone health and calcium homeostasis (71-73).

Another factor is the lack of harmonisation of study design in nutritional intervention studies. Study populations, selection criteria, sample size, dosing schemes, assessment procedures and other parameters differ substantially between the studies. Reporting and definition of adverse events only occasionally followed internationally accepted guidelines.

Finally, the ability of the people to metabolise daidzein to equol might represent a relevant confounder in isoflavone research. Roughly 35 % of humans are "equol-producers", most likely due to differences in the gut flora bacteria (74, 75). Equol, an isoflavone not found in soy itself, is by itself a very potent phytoestrogen, antioxidant and is also effective in increasing BMD in animals experiments (76). In the last years some evidence suggested that the bone-sparing effect of soy isoflavones is more significant in the "equol-producers" subpopulation than in the "non-equol-producers" group (74, 75).

By the use of Bonistein™ toxicological, pharmacokinetic and pharmacodynamic properties of pure genistein could be investigated. Prior to its general availability, an extensive set of toxicological studies was carried out to demonstrate its safety. First clinical studies proved its good tolerability and characterised its pharmacokinetic behaviour in healthy subjects and postmenopausal women. Now, large-scaled and long-term trials are in preparation to investigate the effect of Bonistein™ on post-menopausal bone loss and climacteric symptoms.

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