The Effect of Cyclooxygenase-2 Inhibitors on Bone Mineral Density

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Contributions of Authors:

Dr. Richards was responsible for the conception of this project, the literature review, the statistical analysis and the drafting of the thesis and the paper as accepted by Osteoporosis International. Dr. Joseph contributed greatly to the statistical analysis and the drafting of both the thesis and the paper. Drs. Schwartzman and Goltzman made critical revisions to the intellectual content of the thesis and the paper. Drs. Tenenhouse and Kreiger suggested revisions to the intellectual content of the paper.

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Statement of Originality

The research presented in this thesis represents original work on behalf of the author and other contributors. This thesis contributes to the understanding of the association between cyclooxygenase-2 inhibitors and bone mineral density. To our knowledge this relationship in both men and women has not been previously reported in the literature.

Abstract

Objective: The use of cyclooxygenase-2 (COX-2) inhibitors may impair load-induced bone formation but also prevent menopause-associated bone loss. We hypothesized that COX-2 inhibitor use would be associated with an increased bone mineral density (BMD) in postmenopausal women not using estrogen therapy and conversely, a decreased BMD in men.

Methods: We used data from the Canadian Multicenter Osteoporosis Study, a longitudinal, randomly selected, population-based community cohort study. The outcome measure was percent difference in bone mineral density (g/cm^2). Using linear regression, we estimated the effect of COX-2 inhibitors on this outcome, while adjusting for important potential confounders.

Results: There were 4780 subjects available for study, of which 394 subjects reported daily COX-2 inhibitor use. In males, daily use of COX-2 inhibitors was associated with a lower BMD at all hip sites (percent difference between users and non-users at total hip: -3.1% [95% confidence interval (CI), -6.0, -0.3]. In post-menopausal women not using estrogen replacement therapy, daily COX-2 inhibitor use was associated with higher BMD at most sites (percent difference at total hip: +3.0% [95% CI, 0.3, 5.8]).

Conclusions: COX-2 inhibitor use was associated with a lower BMD in men and, on the other hand, a higher BMD in post-menopausal women not using estrogen replacement therapy. Men who have used COX-2 inhibitors may wish to seek a BMD measurement to assess their fracture risk. However, COX-2 inhibitors may have utility in post-menopausal women if bone-selective analogues can be developed.

Résume:

Objectif : L'utilisation des inhibiteurs de la cyclooxygenase-2 (COX-2) peut altérer la formation osseuse relieé à la charge gravitationalle, mais également empêcher la perte ousseuse associée à la ménopause. Nous avons soumis l'hypothèse que l'utilisation d'un inhibiteur COX-2 serait associée à une densité minérale osseuse accrue chez les femmes post-ménopausées ne prenant pas de remplacement oestrogénique, et au contraire, à une densité minérale osseuse diminuée-chez les hommes.

Méthodes : L'étude multicentrique canadienne d'ostéoporose est une cohorte longitudinale, choisie de façon aléatoire dans la population générale, qui évalua le pourcentage de différence dans la densité minérale osseuse (g/cm^2). Nous avons évalué l'effet des inhibiteurs COX-2 sur cette variable, par régression linéaire, en ajustant pour les variables confondantes principales.

Résultats : Des 4780 sujets disponibles pour l'étude, 394 sujets ont rapporté l'utilisation quotidienne d'un inhibiteur COX-2. Chez les mâles, l'utilisation quotidienne des inhibiteurs COX-2 a été associée à un densité minérale osseuse diminuée à tous les emplacements de la hanche (pourcentage de différence de -3.1% pour la hanche totale entre les utilisateurs et les non-utilisateurs [IC de 95%: -6.0, -0.3]. Chez les femmes post-ménopausées ne prenant pas de remplacement oestrogénique, l'utilisation quotidienne d'un inhibiteur COX-2 a été associée à une densité minérale osseuse plus élevée à la plupart des emplacements (pourcentage de différence de +3.0% pour la hanche totale [IC de 95% : 0.3, 5.8].

Conclusions : L'utilisation d'un l'inhibiteur COX-2 a été associée à un densité minérale osseuse inférieure chez les hommes et, d'autre part, à un densité minérale osseuse plus élevée chez les femmes post-ménopausées ne prenant pas de remplacement oestrogénique. Chez les hommes qui ont employé un inhibiteur COX-2, la mesure de la densité minérale osseuse pourrait être considérée pour évaluer leur risque de fracture. Par contre, les inhibiteurs COX-2 pourraient avoir une utilité chez les femmes post-ménopausées si des analogues spécifiques pour l'os pouvaient être développés.

Chapter 1 – Introduction

This thesis provides the first examination of the effect of cyclooxygenase-2 (COX-2) inhibitor use on bone mineral density (BMD) in men and women. While the literature review in chapter 2 will describe the role of inflammation in the development of osteoporosis in more detail, this chapter briefly introduces the aspects of osteoporosis, BMD and COX-2 inhibitors that are most relevant to this thesis. This introduction is followed by an outline of the objectives of the thesis. Our main manuscript is presented in chapter 4, examining the relationship between COX-2 inhibitor use and BMD. This chapter is preceded by a brief connecting chapter introducing the paper, and the main results and conclusions are summarized in the final chapter 5.

1.1 The importance of Osteoporosis

As humans age, the skeleton gradually loses strength and is prone to fracture. Many of these fractures are due to osteoporosis, which is defined as an increased propensity to fracture due to a loss of bone strength [1]. This increased susceptibility to fracture may be due to a decrease in bone mineral density, a loss of bone micro-architecture, changes in the material properties of bone or a combination of factors such as these [2].

Osteoporotic fractures inflict a serious economic and social burden upon developed nations [3]. The prevalence of osteoporosis is increasing due to the progressive aging of Western populations and increased rates of diagnosis likely secondary to heightened awareness of the disease and its attendant complications [4,5] . The costs of treating osteoporosis are also projected to increase substantially due to this aging phenomenon, and, in addition to this demographic phenomenon, many countries have experienced an increase in age-adjusted hip fracture incidence in both men and women older than fifty

years [6]. In the United States of America (USA), the lifetime risk of developing a hip fracture from age 50 years onward was recently estimated at 17% in white women, and 6% in white men [7]. Consequently, osteoporosis will continue to account for a sizeable proportion of total health care expenditures in most countries for the foreseeable future.

The financial burden of osteoporosis is considerable; the direct cost of osteoporotic fractures in 2001 in the USA was $17 billion per year [8]. In addition to these economic costs, osteoporotic fractures are associated with significant mortality. Indeed, the age standardized one-year mortality ratio associated with hip fracture was 2.2 in women and 3.2 in men in a study of 4,311 subjects aged 60 years and over [9]. The etiology of the observed relationship between osteoporotic fractures and mortality is still unclear and may be influenced by associated co-morbidities [3]. Despite their attendant mortality, much of the direct cost related to osteoporotic fractures is directly attributable to their resultant morbidity [3]. In 2003, the World Health Organization (WHO) reported that while hip fracture is associated with a 20% mortality rate at one year post-fracture, 50% of patients sustaining fractures had significant loss of function, with 70% of these never regaining this loss [10]. Recognition of the substantial social and economic costs of osteoporosis has led the WHO to declare this decade to be dedicated to Bone and Joint Research [11].

Overall, osteoporosis is a common disease that exacts its toll on developed societies by contributing significantly to mortality and disability. Its considerable financial and health costs have led to governments and private enterprise to invest heavily in further research.

1.2 The Utility of Bone Mineral Density Measurement

Bone mineral density (BMD) may be measured through the use of dual-energy X-ray absorptiometry (DXA). Most national and international guidelines concerned with the diagnosis and treatment of osteoporosis rest on the cornerstone of BMD testing. Bone mineral density has enjoyed popularity as a primary assessor of osteoporotic fracture risk because it may be measured with little risk to the patient [12] and confers useful information about fracture risk [13].

DXA scanning produces a measure of bone mineral content and bone area. Areal BMD is then calculated by dividing bone mineral content by bone area, measured in g/cm^2. The most clinically useful metric of BMD is the T-score, which is the number of standard deviations that the BMD deviates from the young adult mean.

The site of BMD measurement which provides the most clinical utility is currently a matter of debate. Usually, during DXA scanning, the lumbar spine (L1 to L4), total hip, femoral neck, trochanter sites and Ward's area are measured. As most guidelines recommend therapy at some BMD-based threshold it is of considerable importance to define which BMD site is most relevant. Some current guidelines, such as those of Osteoporosis Canada advocate consideration of whichever BMD site carries the lowest T-score [14] while the WHO Collaborating Centre has suggested that fracture risk be derived from femoral neck T-score [15]. Currently the International Society of Clinical Densitometry advocates use of the lowest T-score for diagnostic purposes and specifically indicates that Ward's area should not be used for diagnosis [16].

BMD as measured by DXA is correlated strongly with the mechanical strength of bone [17]. Clinically, this correlation with bone strength suggests that BMD should be an excellent predictor of fracture risk. A meta-analysis of eleven separate study populations with over 90,000 person-years of observation time revealed that a decrease in BMD by one standard deviation (SD) below the age-adjusted mean was associated with a relative

risk of fragility fracture of approximately 2.3 (95% confidence interval [CI] 1.9 to 2.8) [18]. This gradient of risk compares favourably with blood pressure as a predictor of stroke and serum cholesterol as a harbinger of cardiovascular disease [19]. Further, many clinical trials of medications designed to decrease the risk of osteoporotic fractures have demonstrated that a reduction in fracture risk is associated with an increase in BMD [20-23].

Although a low T-score provides a useful measure of fracture risk, the majority of osteoporotic fractures in women occur in those who do not have a T-score in the osteoporotic range. Indeed, of the 8,065 postmenopausal women followed in the Study of Osteoporotic Fractures, 54% of those who sustained a hip fracture did not have a T-score in the osteoporotic range [24]. The poor sensitivity of DXA scanning to predict incident fractures has led to many authors in the field of osteoporosis to call for the incorporation of clinical risk factors into the intervention criteria used to prompt therapy for osteoporosis, in order to improve the sensitivity of fracture prediction [14,15,25].

Despite the limitations of DXA scanning, such as cost, BMD remains the single best predictor of osteoporotic fractures, apart from a previous fragility fracture [15]. Due to its inherent ease of administration, useful gradient of risk and favourable safety profile, it is likely that BMD will remain pivotal to the assessment of fracture risk.

1.3 COX-2 Inhibitors: From Frenzy to Fiasco

Both rofecoxib (Vioxx) and celecoxib (Celebrex) were launched with great anticipation as selective COX-2 inhibitors in the Canadian market in 1999. The success of these medications was directly related to their perceived ability to relief inflammatory pain, while avoiding gastrointestinal ulcers—a side-effect which plagued users of non-selective non-

steroidal anti-inflammatory drugs (NSAIDs). The belief in the beneficial effect of COX-2 inhibitors stemmed from the results of two large randomized controlled trials. In the Celecoxib Long-Term Arthritis Safety Study (CLASS), users of celecoxib experienced fewer gastrointestinal ulcers at the 6-month analysis [26], however, in a subsequent publication at 12-months of use, this benefit had disappeared [27]. Further, the Vioxx Gastrointestinal Outcomes Research (VIGOR) study revealed a lower incidence of gastrointestinal ulcers after long-term use with rofecoxib when compared to naproxen [28].

A curious result of the VIGOR study troubled many researchers in the field; in the rofecoxib group there was a higher incidence of myocardial infarction when compared to naproxen users. These results were largely dismissed because, at the time of the study, there was little biologic plausibility indicating that COX-2 inhibitors could trigger myocardial infarction and there was no placebo group in the VIGOR study from which to draw controlled comparisons.

Despite these initial vascular concerns, these medications rapidly gained market acceptance and until recently accounted for $4 billion in sales per year in the United States alone [29]. During 2003 there were over 7.5 million prescriptions filled for COX-2 inhibitors in Canada [30].

Because COX-2 inhibitors had shown early promise in their ability to retard the development of colon cancer, an efficacy trial was designed to test the premise that rofecoxib could prevent the recurrence of colonic polyps. However subsequent analyses of this study revealed that rofecoxib use was associated with clear cardiovascular toxicity [31]. Rofecoxib was voluntarily withdrawn from international markets by its manufacturer, Merck, on September 30th, 2004, causing Merck to lose $28 billion USD in market capitalization that day alone [32]. The economic fallout that has stemmed from the

realization that COX-2 inhibitors likely caused myocardial infarctions has led some observers to question the financial future of Merck & Co. Inc [33]. More importantly, if even less than one percent of the tens of millions of people using rofecoxib experienced excess risk of myocardial infarction, then tens of thousands of patients may have experienced major adverse events [34].

Through modulation of the COX-2 enzyme and prostaglandin E2 production, COX-2 inhibitors may have important influences on bone strength and bone mineral density. Inflammation may play an important role in the development of postmenopausal osteoporosis and thus, some have hypothesized that the anti-inflammatory nature of COX-2 inhibitors may lessen the bone loss associated with the postmenopausal state [35]. The role of COX-2 inhibitors in bone physiology will be discussed in greater detail in the second chapter of this thesis.

Since the realization that COX-2 inhibitors may cause myocardial infarction the biologic effects of these drugs have generated an enormous body of research. It has been of paramount importance to the scientific community to understand the effects of these drugs, expected or otherwise, for two reasons: First, tens of millions of patients used these medications and thus any important side-effects of their use may have large public health sequelae. Second, although few people currently use these medications, the enzyme that they inhibit is central to human pain and suffering and thus medical researchers, and the pharmaceutical industry in particular, will undoubtedly remain preoccupied with its modulation for the foreseeable future. Finally, in order to make the best clinical decisions regarding medication use, physicians and their patients must fully understand the side-effects associated with a medication's use—whether these are harmful or beneficial.

1.4 The Canadian Multicentre Osteoporosis Study

The Canadian Multicentre Osteoporosis Study (CaMos) was initiated in January, 1996 [36]. The study was originally planned to be a five-year prospective study, however, over ten years later, follow-up is still ongoing. This study of the skeletal health of Canadians examined a randomly selected, population-based cohort of men and women ≥25 years living in, or near, one of nine regional centres distributed across Canada. The main objectives of the CaMos study were to assess the prevalence of osteoporosis and the environmental factors associated with incident fragility fractures. As well, this study was designed to examine determinants of BMD, such as gender and age.

The CaMos study is one of a handful of large randomly selected population-based studies of osteoporosis in the world. In total, 9,423 subjects were enrolled at baseline in the CaMos cohort. BMD testing was carried out on all available subjects. At five years of follow-up 7,652 (81%) subjects remained as participants. The CaMos study will be described in more detail in Chapter 4.

1.5 Objectives

The objectives of this thesis are to:

1) Review the rationale for the importance of the prostaglandin E2 pathway, and its inhibition, on BMD.

2) Estimate the association between COX-2 inhibitor use and BMD, using data from the Canadian Multi-Centre Osteoporosis Study.

In this manuscript-based thesis, chapter 2 reviews the rationale for our study hypotheses, while chapters 3 and 4 present an introduction to and our main manuscript, respectively, as accepted for publication by Osteoporosis International.

Tables and figures appear at the end of the chapters in which they are cited.

1.6 Summary of Introduction

Osteoporosis is a common, costly disease which is increasing in prevalence [4,5]. Despite certain limitations, BMD testing is the single best predictor of osteoporotic fractures, aside from previous fragility fractures [15]. As will be addressed in chapter two, there is considerable direct and indirect evidence implicating inflammation as a key modulator of bone loss. Further, the cyclooxygenase pathway may be central to the development of inflammation-induced bone loss. COX-2 inhibitors have, until recently, been used commonly in elderly populations to treat inflammation, thus any impact of COX-2 inhibitor use on BMD may have important public health sequelae. The remainder of this thesis will address the question of whether or not specific COX-2 inhibition is able to modulate bone loss.

Chapter 2 - Literature Review

The understanding of the etiology of osteoporosis has substantially increased over recent decades. Although the association between inflammatory disorders, such as rheumatoid arthritis, and osteoporosis has long been recognized, the role of inflammation itself as a primary contributor to bone loss has only been recently realized [37]. This literature review will focus on the mechanism by which estrogen withdrawal promotes the release of pro-inflammatory cytokines, which then act, at least in part, through the cyclooxygenase 2 (COX-2) pathway to cause bone loss. We will then review the role that COX-2 inhibition may play in preventing inflammatory-mediated loss of bone. On the other hand, the COX-2 pathway has also been strongly implicated in the formation of load-induced bone gain, and this process may be also inhibited by COX-2 inhibitors. Finally, after a review of these paradoxical COX-2 inhibitor effects, we will describe the potential confounders which are relevant to the epidemiologic study of the effect of COX-2 inhibitors on BMD.

2.1 Does Estrogen Withdrawal Contribute to Inflammation?

Many autoimmune diseases are modulated by physiologic changes in estrogen levels. This is exemplified clinically by observations made during pregnancy—when decreased immune surveillance diminishes the severity of most autoimmune diseases [38]. Postpartum, as estrogen levels decrease, patients with autoimmune conditions commonly worsen in severity or experience relapse [39].

Ovariectomy and menopause have consistently been shown to cause profound bone loss, which may be restored through supplementation with estrogen [40,41]. Given the observed link between estrogen withdrawal and exacerbations of autoimmune conditions,

the hypothesis has arisen that estrogen withdrawal may mediate its bone effects through the promotion of an inflammatory cascade.

T-cells are specialized lymphocytes which are able to secrete inflammatory cytokines and interact with phagocytic cells to cause cellular destruction [42]. T-cells are able to secrete tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1 (IL-1). The production of these cytokines is enhanced by the presence of IL-1 [43].

Natural and surgical menopause have been shown *in vitro* to enhance the ability of T-cells to secrete cytokines (predominantly, IL-1, IL-6 and TNF-α) [44,45]. However, the increase in cytokine levels observed during menopause is less pronounced than the mechanical-injury induced cytokine response [46]. *In vivo* studies have yielded less consistent results and this may be due to difficulties with serum assays of cytokines which are typically present at very low concentrations, often near the detection limit of the assay itself [47].

A study examining women before and after surgical menopause revealed increases in IL-1, IL-6 and TNF-α after oophorectomy [47]. Bone marrow biopsies performed on women undergoing surgery for breast cancer demonstrated that bone marrow cells from women who had recently stopped hormone replacement therapy (HRT) or had undergone menopause secreted significantly higher amounts of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 [48]. Thus, when examined both *in vivo* and *in vitro*, estrogen withdrawal has been shown to induce the secretion of inflammatory markers.

Conversely, the addition of estrogen appears to attenuate the production of immune cytokines. In a population-based study, postmenopausal women receiving HRT were found to have lower IL-6 levels than women who were receiving HRT, independent of

age, antihypertensive therapy, smoking habits, and blood pressure [49]. While *in vitro* studies of human osteoblast cells have demonstrated an estrogen dose-dependent attenuation of IL-1 mediated TNF-α production, but not IL-6 [50]. Thus it appears that TNF-α and IL-1, and perhaps IL-6, may increase after estrogen withdrawal.

The presence of estrogen also upregulates the production of an anti-inflammatory cytokine, called transforming growth factor-β (TGF-β). TGF-β is a member of a family of bone morphogenic polypeptide growth factors. This upregulation has been demonstrated consistently in osteoclasts [51,52]. TGF-β deficient mice display profound inflammation and die as a result of inflammation in the cardiac, pulmonary and gastrointestinal systems [53]. Ovariectomy induces a downregulation of TGF-β mRNA and a consequent decrease in TGF-β protein levels in rat bone, when compared to sham operated mice [54]. Estrogen may mediate some of its anti-osteoclastic effects through the induction of osteoclast apoptosis and anti-TGF-β antibodies have been demonstrated to decrease estrogen and tamoxifen mediated osteoclast apoptosis, indicating that TGF-β may mediate the anti-osteoclast actions of estrogen [52].

Despite abundant evidence for apparent pro-inflammatory effects of estrogen withdrawal, numerous studies examining the effect of estrogen supplementation have yielded conflicting results. These divergent findings may be partially due to differences in cell types, time from menopause and *in vitro* conditions employed in these studies [55,56]. Most of the discrepant studies concentrated on the effect of estrogen replacement on IL-6 production, whereas more consistent results have been demonstrated when IL-1 and TNF-α were measured as outcome variables [48,50].

Overall, it would appear that the process of estrogen withdrawal may stimulate immune cytokine production while the addition of estrogen to estrogen-deficient physiologic systems has yielded less consistent effects.

2.2 Does Inflammation Mediate Bone Loss?

Bone loss occurs through many mechanisms; however, almost all known mediators of bone loss (such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3, TNF-α, IL-1 and IL-6) exert their effect primarily on osteoblasts. Osteoblasts in turn stimulate osteoclasts to cause bone resorption by secreting paracrine hormones, the most important of which is the receptor activator of nuclear factor kappa B ligand (RANKL). Osteoblasts are able to modulate the effect on RANKL by controlling its secretion and also by secreting a decoy receptor for RANKL, termed osteoprotegerin (OPG). OPG binds to secreted RANKL and prevents activation of the RANKL receptor on the osteoclasts [57].

The role of T-cells in bone loss was first reported by Kong et al. [58]. In 1999 they described that activated T-cells themselves produce RANKL, and this agent, in turn caused osteoclastogenesis *in vitro.* Thus, in addition to being produced by osteoblasts, RANKL can be produced by T-cells. The same investigators used a T-cell dependent model for arthritis in rats and demonstrated that bone loss was decreased through the addition of OPG, the natural decoy receptor for RANKL. These experiments provided the first evidence that T-cells mediate bone loss through RANKL.

By employing the use of T-cell deficient mice, Cenci et al, demonstrated that the loss of bone caused by ovariectomy in mice could be prevented by the absence of T-cells [59]. They demonstrated that ovariectomy dramatically enhanced the production of TNF-α from T-cells. Importantly, they were able to show that in nude mice, which completely lack T-cells, there was an absence of ovariectomy-induced stimulation of RANKL-dependent osteoclastogenesis, which was demonstrated in control mice that also underwent

ovariectomy. This elegant series of experiments implicated T-cells as a central factor in the physiology of menopause induced bone loss.

In vivo experiments have demonstrated that T-cells increase TNF-α production specifically within the bone marrow by increasing T-cell mass, rather than the amount of TNF-α production per T-cell. Again, these changes were absent in T-cell deficient mice, but were restored by adoptive transfer of wild type T-cells [60].

There is considerable additional evidence implicating TNF-α as an inflammatory mediator of bone loss. In ovariectomized mice, treated for two weeks post surgery with a TNF-α inhibitor, there was a complete lack of bone loss and a reversal of osteoclast formation and bone resorption induced in ovariectomized controls [61]. Both bone marrow cells and T-cells have been shown to produce TNF-α after the withdrawal of estrogen [62,63] and TNF-α has also been demonstrated to stimulate the formation of osteoclasts and prolong their survival [64,65].

IL-1 is a pro-inflammatory cytokine produced by many cells (including T-cells) which stimulates T- and B-cells to induce inflammatory responses, such as the production of prostaglandins and TNF-α. Interestingly, both natural and surgical menopause are associated with significant increases in IL-1 secretion by monocytes and macrophages [66,67]. The inhibition of IL-1, through the use of an IL-1 receptor antagonist (IL-1ra), in ovariectomized rats prevented bone loss at eight weeks post ovariectomy almost as potently as estrogen itself [68]. This lead investigators to conclude that IL-1 is involved in menopause associated bone loss.

The balance between bone loss and bone gain involves many pathways which mediate their affects by altering the RANKL/OPG ratio. The inflammatory cascade is thought to increase this ratio and thus promotes bone loss. This relationship was demonstrated

when the addition of IL-1 and TNF-α to bone marrow stromal cells was found to increase RANKL levels by two- to three-fold in a time- and dose-dependent manner [69]. Intriguingly, OPG levels are also increased by the addition of IL-1 and TNF-α to bone marrow stromal cells [69,70]. Thus, the bone modulatory effects of IL-1 and TNF-α are likely mediated, at least in part, through the ratio of RANKL to OPG.

Thus both direct and indirect evidence strongly implicate both IL-1 and TNF-α as potentiators of menopausal associated bone loss. IL-1 is secreted from many cells including T-cells, which causes the production of TNF-α from T-cells. Both TNF-α and IL-1 act upon osteoblasts to promote osteoclastogenesis, through an increase in the RANKL/OPG ratio (Figure 1).

2.3 Does this Inflammatory Pathway Involve Cyclooxygenase?

Prostaglandin G/H synthase is an enzyme which catalyzes the transformation of arachidonic acid to potent lipid metabolites, termed prostaglandins and thromboxanes [71]. Prostaglandin E_2, (PGE_2), the prostaglandin involved in bone metabolism, can cause bone resorption when added to culture [35]. There exist two isoforms of the prostaglandin G/H synthase enzyme; cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). The actions of these downstream prostaglandins are mediated through G-protein coupled receptors [71]. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both cyclooxygenase-1 and cyclooxygenase-2, while coxibs selectively inhibit COX-2. Selective COX-2 inhibitors were developed because indiscriminate inhibition of the COX-1 pathway leads to gastrointestinal bleeding.

The COX-1 enzyme is constitutively expressed in many cells and displays the tertiary structure of a "housekeeping" enzyme, [72,73] while the COX-2 enzyme is largely expressed in response to inflammation [74]. There exists strong evidence linking COX-2

function to osteoclastogenesis. Mice lacking the COX-2 enzyme displayed a 60-70% decrease in osteoclast formation when bone marrow cultures from COX-$2^{-/-}$ mice were exposed to 1,25-dihydroxyvitamin D3 (1,25-D) or parathyroid hormone (PTH), an effect which was reversed by the addition of exogenous COX-2 [75]. Importantly, this diminution of osteoclastogenesis seemed to arise from decreased expression of RANKL in osteoblasts. Disruption of the COX-1 enzyme, through the creation of COX-$1^{-/-}$ mice resulted in no appreciable difference in PTH and vitamin D induced osteoclastogenesis compared to wild-type mice. Examination of 5-week old mice lacking the COX-2 enzyme revealed no detectable histological malformation. This series of experiments indicates that although COX-2 is not required for normal bone development, its plays a central role in vitamin D and PTH induced RANKL mediated bone loss, at least *in-vitro*.

Whether a similar role for COX-2 is also required for TNF-α action was addressed through the addition of TNF-α to a mouse osteoblastic cell line. When TNF-α was added to these cells in culture, there was a dose and time-dependent increase in both COX-2 mRNA and protein [76]. IL-1 action is also mediated, at least in part, by COX-2. Using human osteoblastic cell lines, IL-1 induced mRNA expression of COX-2 in a time- and dose-dependent manner, while this effect was not seen on COX-1 mRNA expression [77]. Using indomethacin, an indiscriminate inhibitor of both COX-1 and COX-2, the observed IL-1 mediated osteoclastogenesis in marrow *in vitro* culture, was completely inhibited, and the number of osteoclast-like cells produced was strongly correlated with the amount of PGE_2 released after induction by IL-1 [78].

Predictably, estrogen withdrawal itself increases COX-2 expression and PGE_2 production in both *ex vivo* human and rodent bone marrow cells [48,79]. Using bone marrow supernatant from ovariectomized and sham operated mice, Kawagushi et al. demonstrated that these cells produced more PGE_2 and increase COX-2 expression after surgical menopause as compared to sham operated mice [79]. These effects were

attenuated when ovariectomized mice were given estradiol. Intriguingly, the upregulation of PGE_2 and COX-2 was prevented by co-incubation with IL-1 antibodies and an IL-1 receptor antagonist, however, the addition of TNF-α antibodies did not decrease the estrogen withdrawal induced COX-2 and PGE_2 production. Despite this link between estrogen deficiency and increased COX-2 expression and PGE_2 production, no studies to date have demonstrated a direct effect of estrogen on COX-2 function. This is likely due to intermediary cytokines (namely TNF-α and IL-1) modulating the effect of estrogen, rather than a putative direct effect of estrogen alone [80].

Prior evidence has demonstrated that TNF-α, IL-1 and estrogen withdrawal can increase osteoclastogenesis and increase PGE_2 production, while PGE_2 itself can mediate bone signaling pathways in the absence of these other factors. When PGE_2 alone was added to human bone marrow stromal cells in culture, the mRNA levels of OPG were significantly decreased in a time- and dose-dependent manner, implying that PGE_2 in the absence of exogenous TNF-α and IL-1 may modulate the RANKL/OPG ratio [81]. In addition, the bone resorbing properties of PGE_2 in the absence of other cytokines can be blocked by the administration of OPG [82].

Given the central role of estrogen induced increases in IL-1 and TNF-α, which both have been shown to mediate their bone resorbing effects at least in part through COX-2, which in turn modulates the RANKL/OPG ratio in favour of bone loss, a paradigm of estrogen withdrawal-mediated bone loss may be proposed. (Figure 1.)

2.4 Does COX-2 Inhibition Prevent Bone Loss?

Using cultured human osteoblasts, Min et al. demonstrated a complete attenuation of IL-1 mediated PGE_2 production when both indomethacin and a COX-2 inhibitor, NS-398, were added to the culture media [77]. Similarly, when osteoblasts from patients with

osteoarthritis (OA) were exposed to SC-236, a selective COX-2 inhibitor, a significant decrease in IL-1 induced PGE2 production and proteoglycan destruction was observed [83].

Several investigators have examined the effect of COX inhibition on bone loss using epidemiologic methods. The first publication to address this issue analyzed a group of 7786 white women over the age of 65 [84]. Axial BMD was measured at baseline and at a subsequent follow-up visit, which was on average, four years later. As COX-2 inhibitors were not available for use, there were no patients in this cohort using COX-2 inhibitors. However, the investigators were able to examine the effect of non-selective COX inhibition on bone density using nonsteroidal anti-inflammatory drugs (NSAIDS) and aspirin (acetylsalicylic acid). In age-adjusted analysis, daily use of aspirin or NSAIDs was associated with a 2.3-5.8% increase in BMD at proximal femur and spine. However, after adjusting for weight, a variety of medications, self-reported arthritis and for radiographic findings of OA the multiply adjusted increase in BMD was decreased to 1.0-3.1% with no observed difference in fracture risk. Importantly, changes in BMD of 1% have been consistently demonstrated to be associated with decreased fracture risk in clinical trials [20,85].

There are several important limitations to this study. First, participants were accrued through media campaigns with no attempt to randomly sample the population, thus producing possible selection bias. The participants of the cohort study were almost all postmenopausal, and thus the investigators were unable to assess the effect of COX inhibition during menopause, which is when most inflammatory-mediated bone loss is thought to occur. Osteoarthritis presents a significant confounder, as its diagnosis is associated with the use of aspirin and NSAIDs as well as an increased BMD [86]. Osteoarthritis was self-reported in this sample, however, the investigators were also able to adjust for presence of OA through the use of x-rays in a small sub-sample of

participants (n=1318). Finally, the investigators were unable to assess the effect of COX-2 inhibition on bone mass, given that they had no patients using specific COX-2 inhibitors.

To more fully address the issue of whether COX-2 inhibition alters bone loss, Carbone et al. [87] divided NSAIDs based upon their relative COX-2 inhibition. As there were no participants using COX-2 inhibitors in their cohort, they divided NSAIDs into those having more effect on the COX-1 pathway and those having more effect on the COX-2 pathway. This produced a group of patients using *relative* COX-2 inhibitors, who were available only for cross-sectional analysis of BMD. Cross-sectional BMD was assessed in 2853 patients and adjustments were made for age, race, gender, weight, height, study site, calcium and vitamin D supplementation, WOMAC score (a measure of OA), history of rheumatoid arthritis, history of OA and smoking status. Using multiple linear regression, the current use of *relative* COX-2 inhibitors with aspirin was associated with a higher BMD at whole body (4.2%, 1.2-7.3 CI), total hip (4.6%, 0.5-8.8 CI) by DXA and trabecular (34.1%, 15.4-52.7 CI) and cortical spine (12.8%, 2.3-23.3 CI) by quantitative computed tomography. They concluded that relative COX-2 inhibition when combined with aspirin use is associated with a small, but clinically meaningful, increase in BMD. Interestingly, relative COX-1 inhibitors had no appreciable effect on BMD and the effect seen in users of relative COX-2 inhibitors who did not also use aspirin was insignificant. This study is severely limited by its cross-sectional design and complete lack of patients using COX-2 inhibitors.

Finally, the effect of NSAIDs on bone mineral density was addressed in the Rancho Bernardo study, a cross-sectional, population-based study of 932 Caucasian females aged 44-98 years from southern California [88]. Curiously, the authors divided NSAID use into those persons taking propionic acid-based NSAIDs and acetic acid-based NSAIDs. They provide no rationale for this division. There were 114 regular users of NSAIDs, of

which 84 used propionic- and 30 used acetic-type NSAIDs. Propionic-type NSAIDs included ibuprofen, naproxen and ketoprofen and acetic-type NSAIDs included indomethacin, diclofenac, sulindac and tolmetin. NSAID doses were converted into standard daily doses, using a previously developed method, and only regular users of NSAIDs were included [89]. Osteoarthritis was determined by self-report only. Women who used propionic acid NSAIDs, but not acetic NSAIDs had a higher BMD at all five sites, but this was statistically significant at only lumbar spine and midshaft radius. This study is limited by its cross-sectional design, artificial division of NSAIDs and use of self-reporting for osteoarthritis.

In conclusion, NSAIDs may have a small effect on BMD, particularly when used in combination with aspirin. It is also possible that COX-2 inhibitors may have a more profound effect on BMD, as Carbone et al. [87] demonstrated a more significant effect in patients using relative COX-2 inhibitors. The question of whether specific COX-2 inhibition alters bone loss has not therefore been addressed in the literature.

2.5 The Role of Prostaglandins in Load-Induced Bone Gain

Thus far, we have detailed the effect of the inflammatory pathway on bone in general, and prostaglandin production in particular, in the setting of estrogen withdrawal. However, the role of prostaglandins in bone biology is considerably more complex and involves different pathways in non-menopausal humans.

A strong inducer of bone formation is mechano-stimulation, also referred to as bone loading. When subjected to mechanical stress, such as repetitive walking, bone reacts by increasing both in strength and in BMD. Indeed, this is the reason why patients diagnosed with osteoporosis are instructed to engage in load-bearing exercise [90].

In a laboratory setting, mechanical stress is simulated by subjecting bone to pulsating fluids, to mimic the process of fluid flow in the osteocyte lacunar-canalucar network [91]. In a study of primary mouse bone cells, Klein-Nulend et al. reproduced mechanical stress using pulsating fluid and examined the induction of prostaglandin production. PGE2 production increased after 10 minutes of mechano-stimulation and continued throughout 60 minutes of stimulation. This simulation of bone loading was associated with up-regulation of the COX-2 enzyme [91]. These results implicated prostaglandins as important mediators of bone cell response to mechanical stress.

Further testing the hypothesis that prostaglandins are central to the load-induced bone gain pathway, researchers subjected healthy females to repetitive tibial trauma by having them jump off a small table onto a hard floor [92]. In this clever series of experiments the investigators inserted a microdialysis catheter into the proximal tibia metaphyseal bone. Sampling of fluid was performed every 15 minutes over a two hour period of no stimulation, which was followed by repeated measurements after subjects underwent jumping exercise and controls abstained from exercise. These investigators found that PGE2 in the microdialysate fluid increased 2.5-3.5-fold after mechanical stimulation, indicating that mechanical stimulation of the tibial plateau lead to an increase in bone PGE2 production.

Curious as to whether COX-2 inhibitors were able to inhibit load-induced bone formation, Forwood et al. gave NS-398 (a non-marketed COX-2 inhibitor) and indomethacin (a traditional NSAID) to rats prior to mechanical loading of the right tibia [93]. Sacrificing the animals at 5-8 days after loading, these investigators then examined bone gain in the animals given NS-398 and those given placebo. Bone measurement was ascertained by subtracting formation indices of the left leg (control) from those measured at the right (mechano-stimulated) tibia. The gain of bone seen in the control limb was partially

inhibited by indomethacin and completely blocked by NS-398 (the COX-2 inhibitor) at all doses.

And so, the role of prostaglandins in bone physiology is complex, and likely includes stimulation of bone formation during mechanical loading. In a non-postmenopausal environment, on the other hand, prostaglandins seemingly promote bone formation, leading to a paradoxical role of the COX-2 pathway in bone physiology: Bone formation in the setting of mechano-stimulation and bone resorption in a postmenopausal physiology.

2.6 Predictors of Bone Mineral Density—Potential Confounders

Aside from prior fragility fractures, BMD is the best predictor of future fragility fractures [15]. When analyzing the effects of any medication on BMD, it is important to consider potential variables which may confound the relationship between the medication and BMD. This section of the thesis will outline important predictors of BMD.

Peak bone mass generally decreases with age, with women displaying a more profound loss of bone mass during and after menopause [94]. Men exhibit both a higher peak bone mass in early adulthood when compared to women and maintain a higher BMD throughout life [95]. A higher body mass index has also been associated with a higher BMD, especially on load bearing sites, such as the hip [96]. The use of estrogen replacement therapy has been associated with an increased BMD in postmenopausal women [97] and estrogen replacement therapy has often been used to treat osteoporosis [98]. Increased physical activity is also associated with an increase in BMD and, as discussed above, weight-bearing exercise is often prescribed to decrease the risk of fractures in osteoporotic patients [90,99]. Although the effect of calcium supplementation on BMD has produced uneven fracture prevention results, a recent large, randomized

controlled trial of postmenopausal women confirmed that calcium and vitamin D supplementation lead to an increase in BMD in those subjects randomized to calcium and vitamin D therapy [100]. Low BMD is associated with both incident and prevalent fragility fractures [101,102].

The relationship between osteoporosis and osteoarthritis (OA) is complex and site dependent. The lumbar spine is prone to osteoarthritic changes and the development of osteophytes, which may spuriously increase BMD. Most studies examining the relationship between BMD and OA have found an increase BMD in those afflicted with OA [103-106]. Although the relationship between OA and BMD is stronger at lumbar spine, this relationship has also been demonstrated at hip sites [104,107]. Importantly, COX-2 inhibitors are efficacious in the treatment of OA and are often used by those suffering from this commonly disease [26,28].

When designing a study to assess the effects of a medication class on BMD, it is important to consider other diseases that are commonly treated by these medications, which may also have an effect on BMD. Rheumatoid arthritis is an inflammatory disease of the articular space, which may result in significant disability. COX-2 inhibitors have been often used to treat the inflammatory pain associated with rheumatoid arthritis, while this disease in turn has been associated with a decrease in BMD [108]. Systemic lupus erythematosus (SLE) is another rheumatologic condition which is associated with a low BMD and is often partially treated with COX-2 inhibitors [109].

2.7 Summary of Literature Review

Estrogen deficiency through natural or surgical menopause creates a pro-inflammatory milieu. This sub-clinical inflammatory state may potentiate post-menopausal bone loss. The inflammatory cascade activated by estrogen withdrawal is dependent, at least in part,

upon the COX-2 enzyme. Previous epidemiologic studies have demonstrated that inhibition of this enzyme, through the use of NSAIDs, is associated with an increased BMD in postmenopausal women.

On the other hand, the COX-2 pathway plays an important role in load-induced bone gain. The inhibition of this enzyme in animal models has been associated with an attenuation of mechanically-stimulated bone formation.

Given the above findings, the recent popularity of COX-2 inhibitors, and the strong association between BMD and fractures, it is of considerable public health importance to elucidate the effect of these medications on bone density. The CaMos population offers a unique opportunity to investigate this issue, as it contains a sufficiently large number of COX-2 users. To date, no studies have examined the association of COX-2 inhibitor use and BMD in men and women.

Figure 1.

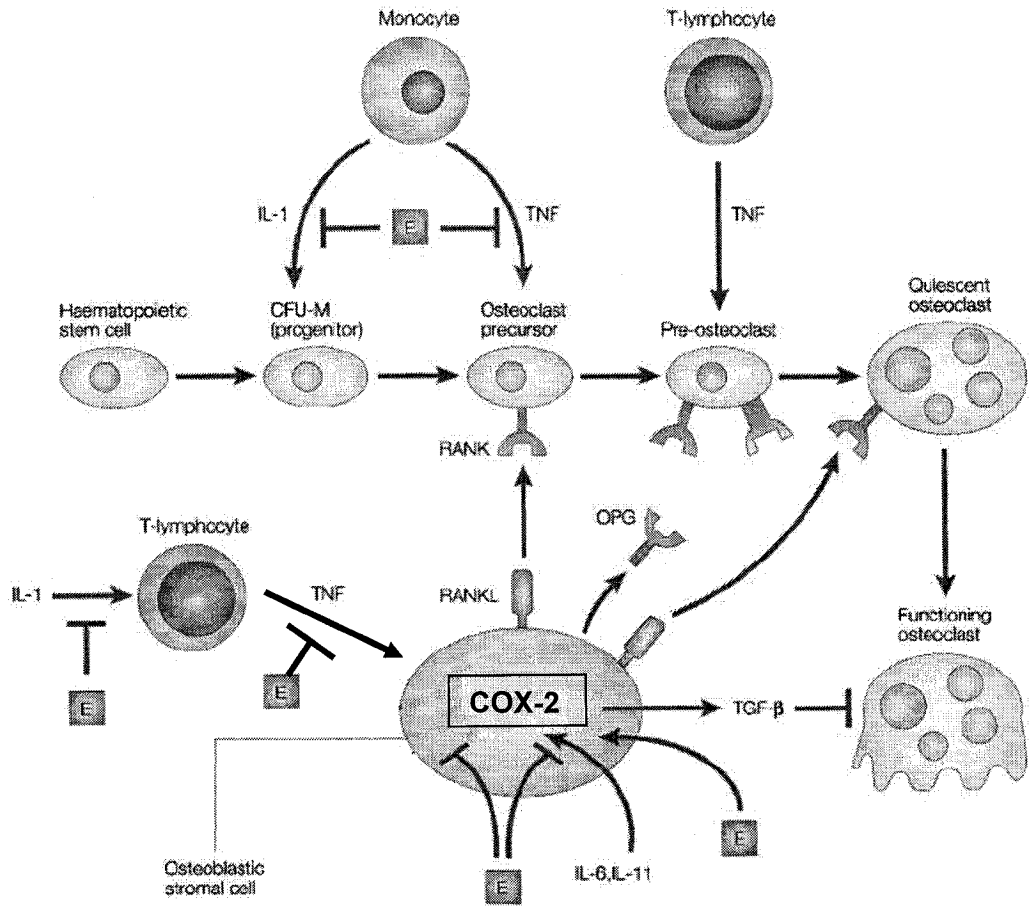

Figure 1. From Goltzman 2002, with modifications, used with permission.
Osteoclasts arise from hematopoietic stem cells. Bone resorbing agents such as IL-1, IL-6, prostaglandins of the E series (PGEs) and TNF-α act on osteoblastic stromal cells. The activity of IL-1, IL-6 and TNF-α is mediated, at least in part, through the cyclooxygenase-2 (COX-2) pathway. Most bone resorption activators seem to increase the production of RANKL, which in turn binds to its receptor RANK on osteoclasts, increasing osteoclast production and function. Osteoprotegerin (OPG) is also released from the osteoblast and can inhibit the interaction of RANKL with RANK. Estrogen (E) acts on stromal cells and decreases IL-1 and TNF-α production while increasing transforming growth factor-β (TGF-β).

Chapter 3

The next chapter presents our manuscript, entitled, "The Effect of Cyclooxygenase-2 Inhibitors on Bone Mineral Density: Results from the Canadian Multi-Centre Osteoporosis Study." This manuscript was accepted for publication by Osteoporosis International on April 6, 2006.

Although many investigators are curious about the potential relationship between COX-2 inhibitor use and bone mineral density, few epidemiologic cohorts are able to address this question. The Study of Osteoporotic Fractures, a large clinic-based cohort of postmenopausal women was able to report on the association between NSAID medications and BMD [84]. However, given the presumed distinct pharmacologic properties of COX-2 inhibitors, and their popularity, researchers from the Health ABC cohort attempted to delineate the relationship between relative COX-2 inhibiting NSAIDs and BMD. Importantly, there was only one subject in this study that used rofecoxib or celecoxib [87]. Thus, the CaMos cohort is uniquely position to address this question, given that it possesses a relatively large number of individuals who used COX-2 inhibitors and were assessed for BMD and determinants of skeletal health. Importantly, since few people currently use rofecoxib and celecoxib the CaMos cohort the results of this study provide an assessment of the association between COX-2 inhibitors and BMD which is unlikely to be granted again in the near future.

Chapter 4

The Effect of Cyclooxygenase-2 Inhibitors on Bone Mineral Density: Results from the Canadian Multi-Centre Osteoporosis Study

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Abstract

Objective: The use of cyclooxygenase-2 (COX-2) inhibitors has been demonstrated to impair load-induced bone formation but also to prevent menopause-associated bone loss. We hypothesized that COX-2 inhibitor use would be associated with an increased bone mineral density (BMD) in postmenopausal women not using estrogen therapy and conversely, a decreased BMD in men.

Methods: The Canadian Multi-center Osteoporosis Study is a longitudinal, randomly selected, population-based community cohort. We present data from men (n = 2004) and postmenopausal women aged 65 and over (n = 2776) who underwent a BMD measurement and structured interview in the fifth year of the study. The outcome measure was percent difference in bone mineral density (g/cm^2).

Results: Daily COX-2 inhibitor use was reported by 394 subjects. In males, daily use of COX-2 inhibitors was associated with a lower BMD at all hip sites (percent difference between users and non-users at total hip: -3.1% [95% confidence interval (CI), -6.0, -0.3]. In post-menopausal women not using estrogen replacement therapy, daily COX-2 inhibitor use was associated with higher BMD at most sites (percent difference at total hip: +3.0% [95% CI, 0.3, 5.8]). These effects appeared to be dose-dependent.

Conclusions: COX-2 inhibitor use was associated with a lower BMD in men and, on the other hand, a higher BMD in post-menopausal women not using estrogen replacement therapy. Men who have used COX-2 inhibitors may wish to seek a BMD measurement to assess their fracture risk. However, COX-2 inhibitors may have utility in post-menopausal women if bone-selective analogues can be developed.

Keywords: Bone Mineral Density, Cyclooxygenase -2 Inhibitors, Inflammation, Osteoporosis

Introduction

Bone is a dynamic tissue, constantly remodeling to accommodate mechanical stress and hormonal influences. Prostaglandin production is central to the processes of load-induced bone formation and menopause-associated bone loss [35,80]. Celecoxib and rofecoxib are both cyclooxygenase-2 (COX-2) inhibitors that prevent prostaglandin production, and enjoyed widespread use for arthritic disorders prior to their association with increased risk of cardiovascular events [31,110]. Rofecoxib has been withdrawn from most Western markets, while currently celecoxib remains available for prescription in many countries. Although osteoporosis is frequently observed in the same age groups in which COX-2 inhibitors have been most widely employed, there are no reports describing the effect of the daily use of these agents on human bone mineral density.

In both humans and in rodent models, repeated mechanical trauma has been demonstrated to increase prostaglandin-E_2 (PGE_2) production [91,92,111,112]. PGE_2 is produced constitutively by the COX-1 enzyme, but during mechanical stimulation, the inducible COX-2 enzyme appears to be responsible for PGE_2 production [112]. Inhibition of COX-2 has been shown to lead to decreased load-induced bone formation in rodents [93].

In contrast to the bone forming properties of PGE_2, the withdrawal of estrogen, through natural or surgical menopause, appears to lead to a PGE_2-dependent pro-inflammatory state characterized by bone loss [75,77,79,83]. In vivo and in vitro models have indicated that after menopause T-cells secrete pro-inflammatory cytokines, which enhance osteoblast production of a potent stimulus of bone resorption; the receptor activator of nuclear factor kappa B ligand (RANKL) [41,69]. This cytokine dependent production of RANKL can be decreased by the use of COX-2 inhibitors [75]. Consequently, the

inhibition of the COX-2 enzyme in postmenopausal women may prevent menopausal bone loss.

Previous epidemiologic studies involving predominantly post-menopausal women, have shown that non-selective inhibition of prostaglandin production, through the use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a small increase in bone mineral density (BMD) when compared to non-users [84,87,88]. However, a cross-sectional study found no association between markers of bone turnover and NSAID use [113].

We hypothesized that the use of COX-2 inhibitors would be associated with a lower bone mineral density in men, reflecting an inhibition of load-induced bone gain, but might conversely be associated with a higher BMD in post-menopausal women not using estrogen supplementation. Since aspirin (ASA) is a known irreversible inhibitor of the COX-1 enzyme, [114] we also assessed the combined effects of the COX-2 inhibitors and ASA to determine their impact on BMD. We estimated the magnitudes of these effects using data from a multi-center, randomly selected population-based cohort.

Materials and Methods

Study Design and Population

The Canadian Multi-centre Osteoporosis Study (CaMos) has prospectively followed a randomly selected, population-based community cohort of non-institutionalized men and women over the age of 25 living within 50 km of one of nine regional centres. Details of the purpose and methodology of the CaMos cohort have been reported elsewhere [115]. Briefly, recruitment for the cohort began in February 1996 and ended in September 1997. At the time of recruitment, BMD was measured in all available subjects and participants were interviewed by a trained interviewer to assess for osteoporosis and fracture-related risk factors. A second intensive interview was conducted five years after enrollment to reassess these risk factors and re-measure BMD. These repeat BMD measurements were conducted between July 2000 and January 2003. The female population in this current study was restricted to those 65 years and over to permit the analysis of a post-menopausal female population and to allow for direct comparison to previous studies analyzing the effect of NSAID use on bone mineral density [84,87]. The study was approved by regional institutional ethics review boards, all participants provided written informed consent and these research activities are in compliance with the Helsinki Declaration.

Assessment of Medication Use

Interviewers collected detailed drug information including type of medication, dose, route of delivery and frequency of use. When interviews were conducted in participants' homes, all contents of their medicine cabinets were assessed. For all other interviews, subjects were instructed to bring all of the contents of their medicine cabinets to the interview site. All medications reported are those at the fifth year interview. The COX-2 inhibitors

assessed in this study were celecoxib and rofecoxib, the only two COX-2 inhibitors available for use at the time of the patient interviews. Rofecoxib and celecoxib were released onto the Canadian market in 1999. Due to limitations of available data, the duration of COX-2 inhibitor use was not known. To assess dose effects, rofecoxib and celecoxib doses were standardized such that starting osteoarthritis daily doses (12.5 mg for rofecoxib and 200 mg for celecoxib) were considered equivalent [116]. For patients taking higher doses of these medications, their dose was considered as a multiple of these starting doses. For purposes of analysis we categorized each subject into non-user, low-dose daily user (25 mg equivalent dose) and high-dose daily user (50 mg equivalent dose) and assessed the relationship between dose and BMD. Persons were considered daily users of rofecoxib or celecoxib if they reported taking the medication every day. Persons were considered daily users of ASA and low-dose acetaminophen if they took at least 80 mg and less than 1g of these medications, respectively, every day. Women were considered non-users of estrogen therapy if they did not report previous estrogen therapy use. In light of our study hypotheses, women were stratified *a priori* by estrogen use. Questions as posed to study participants regarding medication use are listed in the Appendix (6.2).

Bone Mineral Density

Seven of nine centres measured bone mineral density of lumbar spine (L1-L4) and hip using dual energy X-ray absorptiometry with Hologic QDR 1000, 2000 and 4500 while two centres used Lunar DPX densitometers. All BMD results were converted to a Hologic standard, using the method described by Genant et al [117]. Each month a European spine phantom was measured systematically at each site, for standardization purposes [118]. BMD results reported are those at the year five assessment.

Other Measures

Baseline demographic information was recorded during the initial patient interview. Weight and height of each participant were measured and body mass index (BMI) was calculated by dividing the weight of the subject in kilograms, by the square of his/her height in metres. Physical inactivity was assessed by recording the average number of sedentary hours per day in the previous year. Calcium and vitamin D intake were recorded—both supplementation and dietary intake from calcium- and vitamin D-rich foods using a standardized, calcium- and vitamin D-specific diet questionnaire. All comorbidities, including osteoarthritis and rheumatoid arthritis, were based on subjects' reports of diagnoses made by their treating physicians. This was done to ensure that the presence of these diseases was confirmed by a physician and not based on the patient's self-diagnosis. Questions related to previous diagnoses of medication conditions as posed to study subjects are listed in the Appendix (6.2).

Statistical Methods

Descriptive statistics, including means and standard deviations for continuous variables, and percentages in each category for binary and categorical variables, were calculated. The effect of COX-2 inhibition on bone mineral density was examined using both univariate and multivariate linear regression (SAS institute, Inc., Cary, NC, USA). The relationships between the dependent and independent variables were assessed for nonlinear trends. Various curves were fit to examine the relationship between age and BMD, including logarithmic transformations and quadratic fits. Since we found that a simple linear relationship fit the model well, for all variables except calcium intake over the previous 12 months, only these results were reported. Calcium intake was subsequently log transformed. The residual plots for all covariates were assessed. Predictors of bone mineral density and fracture were considered as potential confounders (age, BMI, estimated number of ovulatory cycles (in females), physical activity, calcium

and vitamin D intake over the previous 12 months, previous fragility fracture, education level and centre) and added to the models. Comorbidities (osteoarthritis, rheumatoid arthritis and lupus) which are medical indications for COX-2 inhibitor use were also included as potential confounders. Interaction terms between estrogen and medications (COX-2 inhibitors, ASA and acetaminophen) were considered. The difference in BMD associated with use of COX-2 inhibitors is expressed throughout as percent difference, derived from the regression coefficients using the formula: (100% X beta/Mean BMD for non-users at year five) [119]. Thus all differences in BMD attributed to medication use are derived from a cross-sectional analysis of data at year five.

Results

In total, 9423 subjects were enrolled at baseline in the CaMos cohort. We restricted our analysis to males of all ages and females 65 years and over who had completed a year five interview and BMD measurement. After five years of follow-up our sample included 2004 men and 2776 women, of whom 394 (8.2%) subjects used COX-2 inhibitors (either rofecoxib or celecoxib) daily (Table 1). Users of COX-2 inhibitors were older, were more likely to self-report osteoarthritis or rheumatoid arthritis and had a slightly higher BMI. Daily use of ASA was reported by 1,109 subjects (23%) in the population studied, and the frequency of its use was not different between COX-2 daily users and non-users. Low-dose acetaminophen daily use was also common; 126 (2.6%) subjects reported daily use and they were more likely to also utilize COX-2 inhibitors daily. All analyses reported are adjusted for previously described confounders and predictors of BMD.

Effect of COX-2 Inhibitor use on bone mineral density

In men, COX-2 inhibitor daily use was associated with lower BMD at all hip sites, and a similar effect at the lumbar spine, although at the lumbar spine the 95% confidence interval crossed the null value (Figure 1). For example, the adjusted effect of COX-2 inhibitor daily use on total hip BMD in men was -3.1% (95% CI: -6.0, -0.3). In contrast, among post-menopausal women not using estrogen replacement therapy, COX-2 inhibitor daily use was associated with a higher BMD (Figure 2). For example, daily COX-2 inhibitor use was associated with a 3.0% (95% CI: 0.3, 5.8) higher value for BMD at the total hip, when compared to non-users. A greater difference in BMD was observed at the lumbar spine. When women *using* estrogen were analyzed, BMD in COX-2 users was no longer seen to be increased at all sites except the lumbar spine (Figure 2). Interestingly, the COX-2 associated lower BMD in males and the COX-2 associated higher in BMD in females exhibited more profound effects at higher doses of these medications, although

there was overlap in confidence intervals between the two different dose categories (Table 2). In contrast, again there was no consistent association between BMD and COX-2 inhibitor dose in females using estrogen therapy and COX-2 inhibitors (data not shown).

Daily acetaminophen is commonly prescribed to treat osteoarthritis but, at low doses, is not known to profoundly influence the COX-2 pathway [120]. To evaluate whether the described relationship between COX-2 inhibitors and BMD was due to confounding by indication we assessed the effect of daily low-dose acetaminophen use on BMD. Daily low-dose acetaminophen use was not associated with a consistent difference in BMD in men or women in this cohort (Table 3).

Effect of COX-2 Inhibitor and ASA use on bone mineral density

In total, only 31 male (1.5%) and 54 female (1.9%) subjects of the total study population reported using both COX-2 inhibitors and ASA daily. Generally, the combined adjusted effect of COX-2 inhibition and ASA use exaggerated the aforementioned relationship between COX-2 inhibition and BMD. In men, the use of both daily ASA and COX-2 inhibitors was associated with a markedly lower BMD at all hip sites, and a possible reduction in BMD at the lumbar spine, although the 95% CI crossed the null value at the lumbar site (Figure 3). On average, the effect of daily ASA and COX-2 inhibitor use was associated with a 2.4-fold (range 2.1-2.8) greater difference in BMD, when compared to the difference attributed to COX-2 inhibitor use alone. In women not using estrogen, a similar exaggeration of the effect of COX-2 inhibitors was seen when daily ASA and COX-2 users were analyzed (Figure 4). Again, on average the addition of daily ASA, further *increased* the effect of COX-2 inhibition on BMD by a factor of 2.4 (range 1.4-4.0). Daily ASA and COX-2 inhibitor use had no discernible effect on BMD at any site in women using estrogen replacement therapy (data not shown).

Effect of COX-2 Inhibitor use in patients who reported osteoarthritis

Most of the daily users of COX-2 inhibitors self-reported a physician-made diagnosis of osteoarthritis (men, 73% and women not using estrogen therapy, 78%). In order to control for confounding by indication we repeated our analyses using only those subjects who reported a diagnosis of osteoarthritis. This reduced our sample size by 64% (468 men and 1243 women). While confidence intervals were wide in this restriction analysis, the point estimates were similar to those from the analyses of COX-2 inhibitors for the total study population (Table 4).

Discussion

In this population-based study of randomly selected community dwellers, the daily use of COX-2 inhibitors was associated with a 2.4-5.3% lower BMD across hip and spine in men, after statistical adjustment for possible confounders. In post-menopausal women not using estrogen replacement therapy daily use of COX-2 inhibitors was associated with a 0.9-5.7% higher BMD at hip and spine sites after statistical adjustment for confounders. Importantly, these changes were generally consistent across multiple anatomic sites. Prostaglandins may have complex effects in bone and our findings support the hypotheses that inhibition of the COX-2 pathway and resultant reductions in PGE_2 may prevent the pro-inflammatory state associated with post-menopausal bone loss. However, PGE_2 may also exert beneficial skeletal effects by mediating mechanical load-induced bone formation, and our findings of reduced BMD in men taking COX-2 inhibitors is consistent with interference with load-induced bone gain.

In post-menopausal women using estrogen replacement therapy, we observed no consistent effect of COX-2 inhibitor daily use on BMD. Recent randomized controlled trial data indicate that estrogen replacement therapy is unable to fully return females to a biological pre-menopausal state [121-123]. Thus, post-menopausal women using estrogen replacement therapy may only have a partial reversal of the pro-inflammatory menopausal state. Hence in this population, the lack of effect of COX-2 inhibition may represent both a partial suppression of this residual inflammation and concomitant interference with load-induced bone gain, resulting in no appreciable net change in BMD.

Although COX-2 inhibitors reversibly inhibit the COX-2 enzyme, aspirin irreversibly inhibits COX-1 [124]. Thus the combined use of COX-2 inhibitors and aspirin may potentiate the effect of COX-2 inhibitors alone. We found that the combined use of daily COX-2 inhibitors and daily aspirin had an exaggerated effect on BMD, as compared with

use of COX-2 inhibitors alone in both men and postmenopausal women not using estrogen replacement therapy.

A large proportion of daily COX-2 inhibitor users reported a physician-made diagnosis of osteoarthritis (men, 73% and women not using estrogen therapy, 78%). Hence, osteoarthritis was considered a potential confounder by indication. Osteoarthritis has been reported to be associated with an increased BMD at hip and lumbar spine, however, this relationship is stronger at lumbar spine than hip sites, likely due to degenerative changes [107]. Indeed, osteoarthritis may have contributed to observed differences in lumbar spine BMD in all treatment categories—despite statistical adjustment for self-reported osteoarthritis, using multiple linear regression. Yet it is unlikely that our findings can be explained entirely by this potential confounder for several reasons. First, in our study population, and in other cohorts, osteoarthritis was associated with an *increased* BMD at total hip and spine, while in men our results indicate that COX-2 inhibitors use is associated with a *lower* BMD. Second, daily acetaminophen is also a medication commonly used to treat osteoarthritis and at low doses is not known to markedly influence the COX-2 pathway [120,125,126]. Thus, if the described relationship between BMD and COX-2 inhibitor use was due to confounding by indication, we would expect a similar relationship between low-dose acetaminophen daily use and BMD. However, low-dose acetaminophen use demonstrated no relationship with BMD. Third, when we restricted our study population to only those subjects with osteoarthritis, daily COX-2 inhibitor use was still associated with a similarly decreased BMD in men and a similarly increased BMD in post-menopausal women not using estrogen therapy. The resultant confidence intervals in this restriction analysis were wide and in most cases include zero—likely reflecting the fact that by limiting the analysis to only those subjects with osteoarthritis the sample size was reduced by 64%. Finally, osteoarthritis was controlled for in our analysis using multiple linear regression.

There are several limitations to our study. First, the duration of medication use was not known, and consequently, we cannot assess the cumulative dose effect of COX-2 inhibitors on BMD. However, rofecoxib and celecoxib were released onto the Canadian market in 1999. Hence we do know that no subjects could have used these medications for more than four years. Other reports of the effect of NSAIDs on BMD have described little effect of the duration of medication use on BMD [84]. Second, we used the subjects' report of a physician-made diagnosis of osteoarthritis and this may be prone to misclassification error. However, other large osteoporosis studies have used self-reported diagnoses of osteoarthritis and found these self-reports to be valid and reliable [84,88,127]. If misclassification error is present in our study, then it is highly likely that this is a random classification error, as the subjects and interviewers were not aware of our study hypotheses. Any such random misclassification would tend to dilute any underlying associations—not create or amplify them. Finally, as in all observational studies, it is possible that COX-2 inhibitor use may be associated with an unknown confounder which was thus not controlled for.

In a previous study of postmenopausal women, the multiply adjusted effect of daily use of aspirin or NSAIDs was associated with a 1.0-3.1% increase in BMD of hip and spine [84]. During the course of our study, COX-2 inhibitors were thought to have an improved safety profile over traditional NSAIDs and consequently many fewer subjects in our study reported daily use of traditional NSAIDs as compared to daily use of COX-2 inhibitors. Indeed, there were 40% fewer daily NSAID users compared to daily COX-2 inhibitor users. Although it would have been interesting to examine the relationship between traditional NSAIDs and BMD, we were unable to offer further insight into this relationship due the small number of subjects using these medications.

Another study stratified the relative COX-1 and COX-2 selectivity of traditional NSAIDs and compared their effect on BMD in men and women [87]. Relative COX-2 inhibitors

were found to increase BMD at whole body, total hip and cortical spine only when used in combination with aspirin. The investigators found no effect of relatively COX-2 selective NSAIDs alone. Importantly, there was only one subject in that study using a specific COX-2 inhibitor. Cauley et al, [127] conducted a cross-sectional study of predictors of BMD in a cohort of men over the age of 65. The authors found no multiply-adjusted association between COX-2 inhibitor use and femoral neck BMD and an increased lumbar spine BMD in elderly males using COX-2 inhibitors. Our study differs in several respects. Their cohort was recruited from clinical settings and not from the general community, and subjects with osteoporosis were excluded. In addition, while we analyzed daily COX-2 inhibitor use, Cauley et al included all subjects who used this medication, however infrequent. These methodological differences may at least in part explain the contrasting results between those reported in Cauley et al and those reported here.

Although there were insufficient incident clinical fractures in our study to describe the effect of COX-2 inhibitors on clinical fracture risk, many recent randomized controlled studies of fracture have indicated that small changes in BMD, of less than 5%, predict large changes in fracture rate [20,128-130]. In addition, each standard deviation decrease in BMD increased the age-adjusted risk of hip fracture 2.6 fold in a longitudinal study of fractures in postmenopausal women [131]. Thus, the differences in BMD associated with COX-2 inhibitor use described in our study indeed have relevance to the eventual development of fractures.

In conclusion, our study suggests that the daily use of COX-2 inhibitors is associated with a potentially clinically relevant higher BMD in postmenopausal females not using estrogen, and a lower BMD in men across most sites. Given these results, men currently, or previously, using COX-2 inhibitors may wish to evaluate their bone mineral density to assess their future fracture risk. On the other hand, the demonstrated effect of COX-2

inhibitors on BMD in post-menopausal women underlines the importance of inflammation in post-menopausal bone loss.

Tables

Table 1. Selected Characteristics After 5 Years of Follow-Up of Study Population.
(Mean and [SD] or count and [%])

Variable	Men		Women Not Using Estrogen Therapy		Women Using Estrogen Therapy	
	COX-2 Daily Users (*n* = 108)	**Nonusers** (*n* = 1896)	**COX-2 Daily Users** (*n* = 145)	**Nonusers** (*n* = 1392)	**COX-2 Daily Users** (*n* = 141)	**Nonusers** (*n* = 1098)
Age	70.4 (11.8)	63.3 (13.2)	76.6 (5.6)	75.2 (6.2)	73.8 (5.4)	73.4 (5.5)
BMI	28.6 (4.1)	27.4 (3.9)	28.9 (5.3)	27.3 (5.1)	27.8 (5.0)	26.7 (4.6)
Calcium Intake (mg/d previous 12 months)	913.3 (608.9)	880.8 (573.4)	885.0 (474.8)	878.9 (502.5)	925.7 (558.0)	897.6 (504.4)
Vitamin D Intake (mg/d previous 12 months)	143.8 (277.2)	160.7 (651.6)	233.3 (306)	276.7 (590)	410.0 (919.6)	340 (714.5)
OA	79 (73.2%)	389 (20.5%)	114 (78.6%)	512 (36.8%)	118 (83.7%)	499 (45.5%)
RA	10 (9.3%)	46 (2.4%)	13 (9.0%)	68 (4.9%)	7 (5.0%)	61 (5.6%)
Lupus	0 (0%)	0 (0%)	0 (0%)	5 (0.4%)	1 (0.7%)	8 (0.7%)
Number of inactive hours per day (previous 12 months)	7.0 (2.5)	7.4 (3.0)	6.6 (2.3)	6.7 (2.4)	6.7 (2.2)	6.5 (2.4)
Number of Lifetime Ovulatory Cycles	-	-	425.9 (84.3)	444.9 (78.1)	419.3 (79.0)	430.8 (85.3)
Daily Low-Dose Acetaminophen Users	9 (8.3%)	21 (1.1%)	10 (6.9%)	36 (2.6%)	10 (7.1%)	40 (3.6%)
Daily ASA Users	31 (28%)	410 (21.6%)	25 (17.2%)	326 (23.4%)	29 (20.6%)	288 (26.2%)

BMI = body mass index; OA = Osteoarthritis; RA = Rheumatoid Arthritis; ASA = Aspirin;
Daily aspirin use ≥80 mg each day; daily Acetaminophen use ≥325 mg each day.

Table 2. Multiply-Adjusted Percent Difference in BMD (g/cm^2) associated with Low Dose (25 mg) and High Dose (50 mg) standardized daily dose of COX-2 Inhibitor (95% CI). Users vs. Non-users.

	Males (n=2004)		***Females ≥65 years not using estrogen (n = 1537)***	
	Low Dose	*High Dose*	*Low Dose*	*High Dose*
Total Hip	**-4.3 (-7.6, -1.0)**	**-8.6 (-15.1, -2.0)**	**3.5 (0.2, 6.8)**	**7.0 (0.4, 13.6)**
Wards	**-7.3 (-13.1, -1.6)**	**-14.7 (-26.2, -3.2)**	3.5 (-2.5, 9.6)	7.0 (-5.0, 19.1)
Trochanter	**-5.3 (-9.2, -1.5)**	**-10.7 (-18.3, -3.0)**	3.1 (-0.7, 6.9)	6.2 (-1.3, 13.8)
Femoral Neck	**-4.3 (-7.9, -0.7)**	**-8.6 (-15.8, -1.4)**	1.5 (-2.0, 5.0)	3.0 (-4.0, 9.9)
Lumbar Spine	-3.5 (-7.6, 0.7)	-6.9 (-15.3, 1.4)	**6.0 (2.1, 10.0)**	**12.1 (4.1, 20.0)**

Differences with confidence intervals that exclude the null value are shown in **bold** type. Adjusted for: age, BMI, physical activity, number of ovulatory cycles (in females), calcium and vitamin D intake, previous fragility fracture, osteoarthritis, rheumatoid arthritis, lupus, education level and centre.

Table 3. Multiply-Adjusted Percent Difference in BMD (g/cm^2) associated with Low-dose Acetaminophen daily use (95% CI). Users vs. Non-users

Site	***Males (n = 2004)***	***Females ≥65 years not using estrogen (n = 1537)***	***Females ≥65 years using estrogen (n=1239)***
Total Hip	-4.5 (-9.4, 0.5)	2.7 (-1.8, 7.2)	1.9 (-2.4, 6.3)
Wards	-6.2 (-14.8, 2.5)	5.1 (-3.1, 13.4)	1.7 (-6.3, 9.8)
Trochanter	-5.3 (-11.1, 0.4)	4.2 (-0.9, 9.4)	1.9 (-3.2, 6.9)
Femoral Neck	-4.6 (-10.0, 0.8)	0.1 (-4.7, 4.9)	3.1 (-1.6, 7.7)
Lumbar Spine	-3.4 (-9.7, 2.8)	2.7 (-2.9, 8.3)	0.0 (-14.6, 14.6)

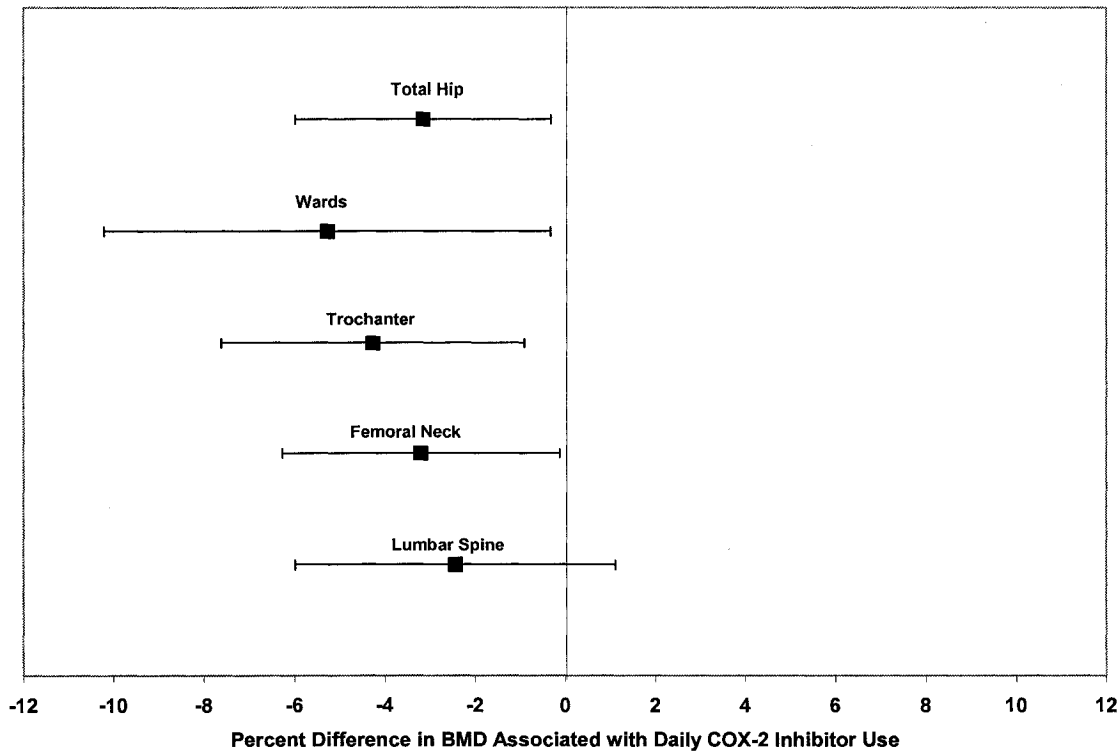
Acetaminophen daily use < 1g per day. Differences with confidence intervals that exclude the null value are shown in **bold** type. Adjusted for: age, BMI, physical activity, number of ovulatory cycles (in females), calcium and vitamin D intake, previous fragility fracture, osteoarthritis, rheumatoid arthritis, lupus, education level and centre.

Table 4. Multiply-Adjusted Percent Difference in BMD (g/cm^2) associated with COX-2 Inhibitor daily use, in only subjects with osteoarthritis (95% CI). Users. vs. Non-users.

Measurement Site	*Males (n = 468)*	*Females ≥65 years not using estrogen (n = 626)*	*Females ≥65 years using estrogen (n= 617).*
Total Hip	-2.1 (-5.6, 1.5)	2.8 (-0.5, 6.1)	-0.6 (-3.8, 2.6)
Wards	-5.4 (-11.8, 0.9)	1.5 (-4.6, 7.6)	0.7 (-5.2, 6.6)
Trochanter	-2.6 (-6.7, 1.5)	3.4 (-0.4, 7.1)	-0.4 (-4.0, 3.3)
Femoral Neck	-3.3 (-7.2, 0.6)	0.1 (-3.5, 3.4)	1.4 (-2.0, 4.8)
Lumbar Spine	-3.2 (-7.7, 1.3)	**6.1 (2.0, 10.2)**	**4.9 (0.9, 8.9)**

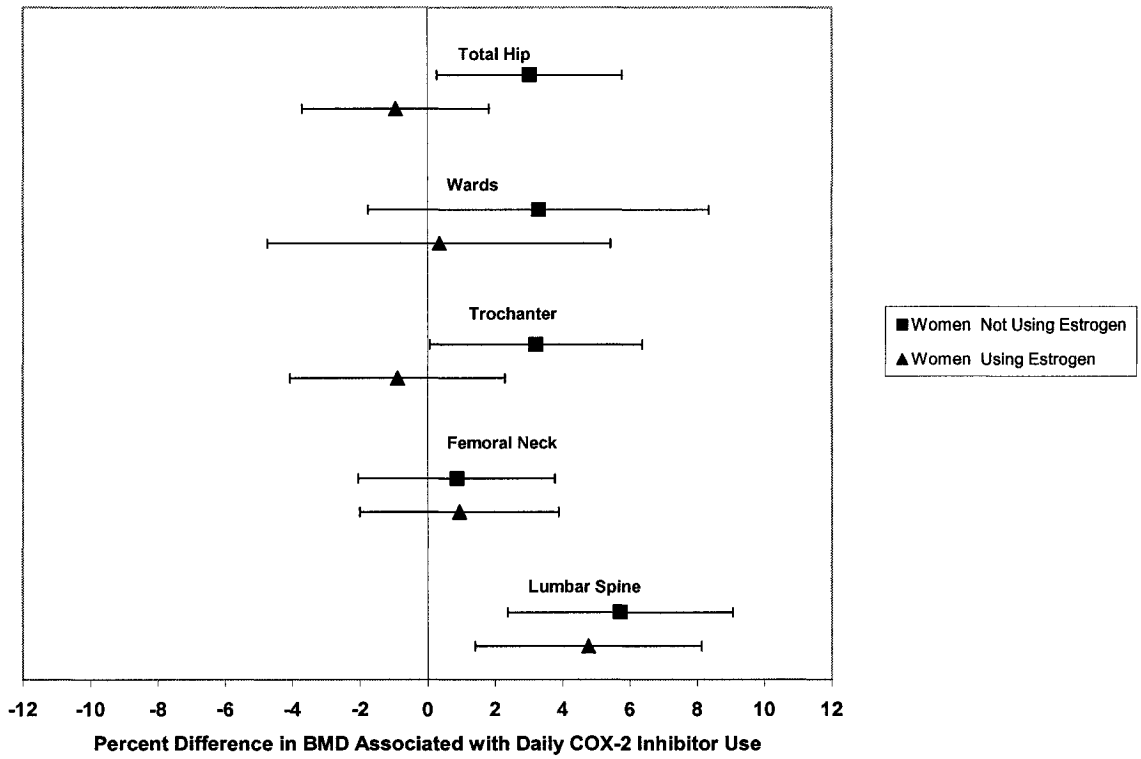
Differences with confidence intervals that exclude the null value are shown in **bold** type.
Adjusted for: age, BMI, physical activity, number of ovulatory cycles (in females), calcium and vitamin D intake, previous fragility fracture, osteoarthritis, rheumatoid arthritis, lupus, education level and centre.

Figure1. Multiply-Adjusted Percent Difference in BMD (g/cm^2) associated with COX-2 Inhibitor Use in Men (95% CI). Users vs. Non-users



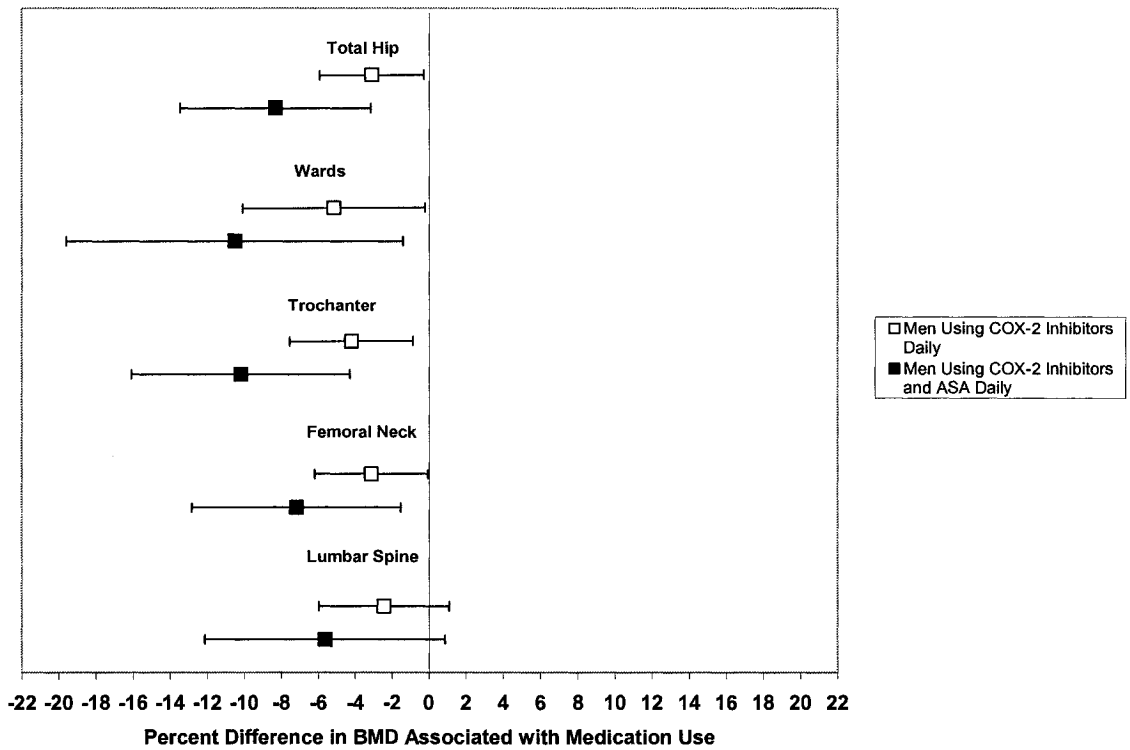
Adjusted for: age, BMI, physical activity, calcium and vitamin D intake, previous fragility fracture, osteoarthritis, rheumatoid arthritis, lupus, education level and centre.

Figure 2. Multiply-Adjusted Percent Difference in BMD (g/cm^2) associated with COX-2 Inhibitor Use in Women ≥65 Years (95% CI). Users vs. Non-users



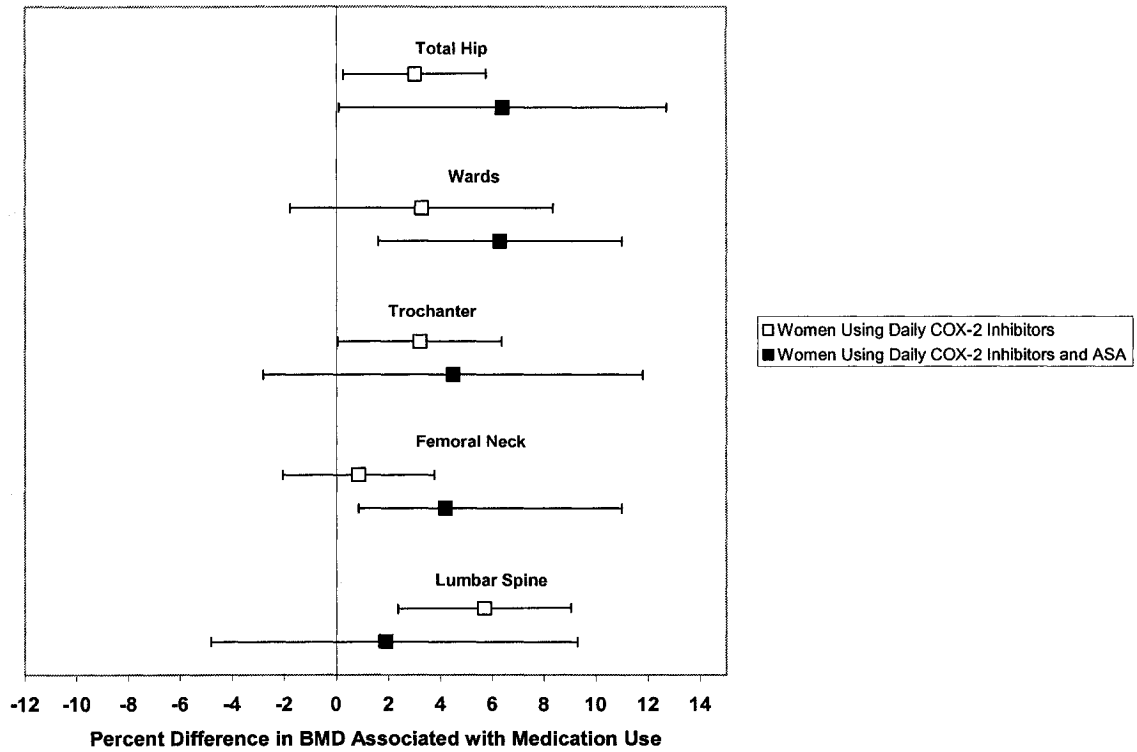
Adjusted for: age, BMI, physical activity, number of ovulatory cycles, calcium and vitamin D intake, previous fragility fracture, osteoarthritis, rheumatoid arthritis, lupus, education level and centre.

Figure 3. Multiply-Adjusted Percent Difference in BMD (g/cm^2) associated with both COX-2 Inhibitor and ASA daily use (95% CI) in Men. Users of both medications vs. Non-users of either medication.



Adjusted for: age, BMI, physical activity, calcium and vitamin D intake, previous fragility fracture, osteoarthritis, rheumatoid arthritis, lupus, education level and centre.

Figure 4. Multiply-Adjusted Percent Difference in BMD (g/cm^2) associated with both COX-2 Inhibitor and ASA daily use (95% CI) in Women ≥65 Years Not Using Estrogen Therapy. Users of both medications vs. Non-users of either medication.



Adjusted for: age, BMI, physical activity, number of ovulatory cycles, calcium and vitamin D intake, previous fragility fracture, osteoarthritis, rheumatoid arthritis, lupus, education level and centre.

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Chapter 5 – Summary

Osteoporosis is both a common and costly ailment. BMD is the single best predictor of future osteoporotic fractures in persons who have not sustained a prior osteoporotic fracture. COX-2 inhibitors were used by tens of millions of people and thus the relationship between COX-2 inhibitor use and BMD may have considerable public health importance.

In this thesis we have presented previously published evidence that estrogen withdrawal promotes a pro-inflammatory state. This postmenopausal inflammatory milieu contributes to the substantial bone loss experienced by women during and after the menopause. The inhibition of the pro-inflammatory mediators markedly reduces estrogen-withdrawal mediated bone loss. COX-2 inhibitors are potent anti-inflammatory drugs. They have been shown in rodent models to mitigate menopause-associated bone loss.

However, the COX-2 pathway may be an important mediator of load-induced bone formation. Thus, prostaglandin-2 production via the COX-2 pathway has seemingly paradoxical effects; in the postmenopausal women it may mitigate bone loss and in men it may impair load-induced bone gain. It was this basic science evidence that led us to hypothesize that COX-2 inhibition may lead to an increased BMD in postmenopausal women not using estrogen replacement therapy and in men, these same medications may be associated with a decrease in BMD.

The results of our investigations indicate that COX-2 inhibitor use was associated with an increase in BMD in postmenopausal females not using estrogen replacement therapy. In men, the converse was true; those using COX-2 inhibitors had a lower BMD. There was no discernible relationship between COX-2 inhibitor use and BMD in postmenopausal females using estrogen replacement therapy.

Due to the high prevalence of COX-2 inhibitor use across the Western world prior to their association with thrombotic events, the association demonstrated in our thesis may have important public health sequelae. As investigations continue into finding medications which can decrease the inflammation and pain experienced by humans due to activation of the COX-2 pathway, it will be important to delineate how any modulation of this pathway effects bone health.

Further investigations into this field require analysis of the longitudinal change in BMD associated with COX-2 inhibitor use and, more importantly, whether COX-2 inhibitor use is associated with osteoporotic fractures. Although data is not currently available to assess the effect of COX-2 inhibitor use on fractures, it will be incumbent upon future researchers to understand this relationship prior to marketing future COX-2 inhibitors.

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6.2 Appendix: Selected Questions from the Medication Questionnaire Administered to Subjects in the CaMos Study.

Question 2.1

Interviewers asked subjects if, "...a doctor ever told you that you have any of the following conditions?" The conditions listed were:

Osteoporosis, rheumatoid arthritis, osteoarthritis, thyroid disease (hyper and hypothyroidism), liver disease, scoliosis, eating disorder, breast cancer, uterine cancer, inflammatory bowel disease, kidney stones, hypertension, heart attack, stoke and TIA, Parkinson's disease, multiple sclerosis, diabetes (insulin dependent and non-insulin dependent), kidney disease, phlebitis, thrombophlebitis, prostate cancer, Paget's disease of bone and chronic obstructive pulmonary disease.

Subjects were also asked if they had received treatment for these disorders.

Question 3.2

Interviewers were asked to list:

"Current medications and or self administered supplements taken on a regular basis."

Questionnaire administrators were also asked to list the dose of the medication or supplement, the route of administration and its frequency of use.