Universidade Federal de Pelotas
Programa de Pós-Graduação em Epidemiologia
Doutorado em Epidemiologia

O EFEITO DA SUPLEMENTAÇÃO DE CÁLCIO DURANTE A GRAVIDEZ NOS MARCADORES BIOQUÍMICOS DE PRESSÃO ARTERIAL EM CRIANÇAS DE 13 ANOS (SEGUIMENTO DE UM ENSAIO CLÍNICO RANDOMIZADO)

Tese de Doutorado

Eduardo Bergel

ORIENTADOR: Aluisio J D Barros

PELOTAS-RS-BRASIL
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Tese apresentada ao Programa de Pós Graduação em Epidemiologia, da Faculdade de Medicina da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (D.S.)

Orientador: Prof. Aluisio J D Barros

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1 APRESENTAÇÃO

Esta tese de doutorado, conforme previsto no regimento do Programa de Pós-Graduação em Epidemiologia da Universidade Federal de Pelotas, é composta por cinco partes: projeto de pesquisa, relatório do trabalho de campo, artigos, reportagem curta para ser divulgada na imprensa e anexos.

2 PROJETO DE PESQUISA

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2.1 Resumo

A suplementação dietética de cálcio durante a gravidez tem mostrado através de ensaios clínicos reduzir a incidência de hipertensão induzida pela gravidez. Acompanhamentos longitudinais de dois destes estudos mostraram redução da incidência de elevação da pressão arterial nos filhos de mães que receberam suplemento de cálcio. Um estudo com animais confirmou estes achados. Os mecanismos relacionados a estes efeitos são desconhecidos. O presente estudo explora estes mecanismos, comparando os níveis de hormônios reguladores de cálcio nos fihos de mães que foram expostas a cálcio ou a placebo durante a gravidez, e avaliando a associação entre estes hormônios e a pressão arterial de seus filhos. Dados de um acompanhamento de crianças nascidas de mães que participaram do segundo maior estudo clínico sobre suplementação de cálcio serão utilizados para a análise. A série de dados também contém informações de variáveis perinatais, indicadores de resistência à insulina e uma avaliação antropométrica detalhada durante o seguimento. Hipertensão, obesidade e diabetes têm sido associados à chamada “síndrome x”, e esta condição está associada a prejuízos no crescimento fetal e no metabolismo de cálcio. Neste contexto, o estudo explora também os determinantes de hipertensão e resistência à insulina nestas crianças.

2.2 Artigos

Artigo 1: Suplementação de cálcio durante a gravidez e hormônios reguladores de cálcio e pressão arterial nos fihos aos 12 anos de idade.

Este estudo analisará os dados coletados durante o seguimento de um ensaio clínico randomizado sobre suplementação de cálcio durante a gravidez. Um modelo analítico com uma estratégia de análise correspondente é utilizado para explorar os mecanismos de ação da suplementação dietética de cálcio durante a gravidez na pressão arterial e no metabolismo de cálcio dos filhos. A finalidade deste estudo é a comparar os níveis de hormônios reguladores de cálcio nos fihos de mães expostas a cálcio ou placebo durante a gravidez, e o efeito modificador da suplementação de cálcio na associação entre estes hormônios e a pressão arterial dos filhos.

Artigo 2: Características perinatais e infantis preditoras da pressão arterial e resistência à insulina aos 12 anos.

O cálcio tem sido associado aos mecanismos relacionados à hipertensão, obesidade e diabetes no que se chama “síndrome x”. Alguns autores postulam que um ambiente intra-uterino inadequado pode ter um papel no desenvolvimento destas condições. Neste contexto, o estudo explora os determinantes da hipertensão e da resistência à insulina na coorte de crianças descrito no primeiro artigo. A análise inclui variáveis do período perinatal (complicações durante a gravidez, peso ao nascer, etc.), e variáveis avaliadas entre 7 e 12 anos após o parto.

Artigo 3: A ingestão dietética de cálcio durante a gravidez e pressão arterial dos filhos durante a infância e vida adulta. Uma revisão sistemática da literatura.

Diversos estudos experimentais e observacionais relatam uma associação entre a ingestão dietética de cálcio durante a gravidez e a pressão arterial da prole durante a infância e a vida adulta. Uma revisão sistemática da literatura será realizada para apresentar um resumo do conhecimento atual nesta área.

2.3 Introdução e justificativa

A hipertensão é a doença cardiovascular mais comum, e é um grande problema de saúde pública tanto em países desenvolvidos, como naqueles em desenvolvimento (Antezana F96). Doenças cardiovasculares são responsáveis por quase 20% de todas as mortes no mundo todo (Lopez90). Em países desenvolvidos, doenças cardiovasculares são as causas mais freqüentes de morte em homens e mulheres adultos. Estas doenças também emergem como um proeminente problema na saúde pública de países em desenvolvimento, sendo responsáveis por mais de 10 milhões de mortes em 2001 (Reddy and Yusuf98a,WHO03). Estratégias eficazes para a prevenção da hipertensão e das doenças cardiovasculares possivelmente causariam um impacto significativo na saúde pública.

Baixos níveis de cálcio têm sido associados a um risco crescente para o desenvolvimento de hipertensão, principalmente em mulheres grávidas. Além disto, dietas ricas em cálcio se mostraram capazes de reduzir a pressão arterial em ensaios clínicos randomizados (Appel *et al.* 97b,Craddick *et al.* 03b).

Nos últimos anos, alguns estudos forneceram evidência apoiando a hipótese de que a desnutrição que ocorre durante a vida fetal seja responsável pela programação do desenvolvimento de fatores de risco, tais como pressão arterial alta e doença cardiovascular na vida adulta (Barker97b). Sendo assim, surge a pergunta se o déficit de cálcio durante a gravidez pode ser um fator de risco para o desenvolvimento de hipertensão em um período mais tardio da vida.

Um ensaio clínico randomizado sobre a suplementação de cálcio durante a gravidez foi conduzido entre 1987 e 1990 (Belizan *et al.* 91b). Este estudo originou uma série de trabalhos, sendo o último deles uma fonte de dados a ser utilizada na presente

proposta. O estudo randomizado original recrutou 1200 mulheres grávidas com aproximadamente 20 semanas de gestação, que receberam oralmente 2 gramas diárias de cálcio ou de placebo. Os principais desfechos deste estudo foram incidência de hipertensão induzida pela gravidez, pré-eclâmpsia e prematuridade. As taxas de doenças relacionadas à hipertensão foram menores no grupo que recebeu cálcio em comparação ao grupo placebo (RR 0,67; IC95% 0,49 a 0,91). Este estudo concluiu que a suplementação de cálcio para a mulher grávida é uma estratégia promissora na prevenção de hipertensão induzida pela gravidez e de pré-eclâmpsia (Belizan *et al.* 91a)

Entre 1995 e 1996, foi realizado o seguimento de um subgrupo da coorte original. Por razões de logística, somente indivíduos de um dos 3 hospitais que participaram do estudo original foram incluídos. A randomização foi estratificada por hospital, mantendo assim a natureza experimental de comparação. O desfecho principal deste estudo foi a pressão arterial dos filhos no seguimento. O estudo mostrou que o risco de pressão arterial sistólica alta foi menor no grupo que havia feito uso de cálcio do que no grupo placebo (RR 0,59; IC95% 0,39 a 0,90), com um efeito mais significativo em crianças com sobrepeso (RR 0,43; IC95% 0,26 a 0,71) (Belizan *et al.* 97a). Estes resultados sugerem que uma estratégia simples de prevenção direcionada ao aumento da ingestão de cálcio entre mulheres grávidas pode reduzir a incidência de pressão arterial elevada nos fihos.

Depois deste primeiro seguimento um modelo animal foi desenvolvido para testar a hipótese entre a associação causal de níveis de cálcio durante a gravidez e a pressão arterial da prole (Bergel and Belizan02a). Os resultados deste estudo em ratos forneceram suporte adicional para a hipótese. Com um ano de idade a pressão arterial sistólica da prole dos ratos expostos a uma ingestão deficiente de cálcio durante a gravidez era 12 mm Hg mais alta do que a do grupo controle (Bergel and Belizan02b).

Os resultados em humanos foram recentemente confirmados. Um grupo de recém-nascidos cujas mães foram randomizadas durante a gravidez para cálcio ou placebo foram estudadas (Hatton *et al.* 03b). Suplementação de cálcio materno durante a gravidez foi associada à menor pressão arterial sistólica nos fihos de 2 anos aos idade. Estes achados são importantes para a saúde pública devido a alta incidência de hipertensão em países desenvolvidos e em desenvolvimento (Reddy and Yusuf98b,WHO03).

O próximo passo lógico para uma compreensão mais clara da relação entre o efeito do déficit do cálcio materno e a pressão arterial da prole é a exploração dos mecanismos desencadeantes de tal associação.

Existem vários modelos potenciais para explicar o efeito da suplementação de cálcio materno na pressão arterial da prole em longo prazo. Nos últimos anos houve uma expansão no conhecimento sobre o papel do cálcio, passando de apenas um componente do osso a um importante regulador de muitas funções do organismo. Em particular, o déficit de cálcio tem sido associado à hipertensão, à resistência à insulina, e mais recentemente à obesidade (Resnick99c,Zemel04b,Zemel and Miller04a). Essas três condições são altamente correlacionadas, e indivíduos que apresentam todos os três são descritos como tendo a “síndrome x" (Resnick92a,Resnick93f). Um aumento do risco para essa síndrome tem sido associado a uma nutrição deficitária durante a gravidez e à redução de crescimento fetal (Barker *et al.* 93a).

Foi postulado que o aumento do cálcio intracelular seja uma característica comum a todas estas condições (hipertensão, obesidade e resistência à insulina) (Resnick99b,Resnick94d,Zemel03d). O déficit de cálcio dispararia mecanismos compensatórios, incluindo variações em hormônios reguladores de cálcio. Alguns destes hormônios mostraram ter efeito vasoativo. Em particular, o calcitriol e o recém

descoberto fator hipertensivo da paratireóide (PHF), que tem potente efeito vasoativo (Resnick94c,Zemel01b). Este efeito vasoativo hormonal mostrou ser mediado pelo aumento do cálcio intracelular (Resnick *et al.* 91).

Foi demonstrado que o efeito da suplementação de cálcio durante a gravidez na pré-eclâmpsia é evidente apenas em populações com déficit cálcio na dieta (Hofmeyr *et al.* 03a). O efeito nos fihos também é apenas visto em associação ao déficit de cálcio na dieta materna durante a gravidez (Bergel and Belizan02c). O efeito a longo prazo do déficit de cálcio materno durante a gravidez no desenvolvimento do feto pode ser mediado por mudanças no limiar dos hormônios reguladores de cálcio. Crianças expostas a um déficit de cálcio materno durante a vida fetal podem ter maiores níveis de circulação de hormônios reguladores de cálcio vasoativos, ou maior sensibilidade a estes hormônios em suas células alvo (células endoteliais). Ainda assim, outros mecanismos que não envolvem estes hormônios poderiam explicar tal associação. Por exemplo, um déficit de cálcio materno pode induzir mudanças estruturais permanentes nos vasos sangüíneos da prole, que por sua vez poderiam aumentar a resistência periférica e hipertensão.

Em 2000, um segundo acompanhamento foi realizado com os filhos das mulheres envolvidas no ensaio clínico randomizado original de suplementação de cálcio. Aproximadamente 90% da população original elegível para um seguimento foi avaliada aos 12 anos de idade, depois da implementação de estratégias de seguimento muito intensas. Informação mais detalhada foi obtida neste último seguimento, comparado com o anterior, incluindo medidas dos hormônios reguladores de cálcio (hormônio da paratireóide (PHT), fator hipertensivo da paratireóide (PTH), calcitriol, e Peptídeo Relacionado ao Gene da Calcitonina (CGRP)), indicadores de resistência à insulina (HbA1, glicemia e glicose na urina), e indicadores hematológicos. Medidas

antropométricas detalhadas da mãe e das crianças também foram obtidas, incluindo circunferência abdominal, e medida das dobras cutâneas das regiões tricipital e sub-escapular (veja Tabela 1, na seção 7 para a lista de variáveis, e Apêndice I para mais detalhes sobre os métodos do acompanhamento). Os dados obtidos nesse estudo serão usados na análise proposta neste projeto. O objetivo desse projeto é estudar as diferenças entre as crianças de mães tratadas e não tratadas, no que se refere ao perfil bioquímico, níveis de pressão arterial e resistência à insulina.

2.4 Revisão de Literatura

2.4.1 Pressão arterial e hormônios reguladores de cálcio.

Estudos mostraram que, em casos de pré-eclãmpsia, alterações na homeostase do cálcio extra celular incluem hipocalciúria e níveis diminuídos de 1,25-dihidroxivitamina D3 sérica (August *et al.* 92a,Taufield *et al.* 87b). O aumento no hormônio da paratireóide (PTH) e a diminuição da concentração de cálcio ionizado no plasma foram observados de forma menos consistente (Seely *et al.* 92a). Foram também descritas anormalidades consistentes do metabolismo intracelular do cálcio em mulheres com pré-eclâmpsia, tais como o aumento da concentração de cálcio intracelular livre em plaquetas e em linfócitos (Hojo *et al.* 99a). O aumento de concentração de cálcio livre intracelular nas células circulantes poderia resultar da flutuação de hormônios ou de substâncias vasoativas que causasse uma alteração semelhante no músculo liso vascular. O aumento no cálcio livre intracelular aumenta a reatividade vascular, e é associado à pressão arterial alta.

Foi sugerido que todas as formas de hipertensão seriam associadas e dependentes do excesso de cálcio citosólico livre (Resnick99d), sendo as diferentes fisiopatologias de diferentes formas de doenças hipertensivas explicadas pela extensão da distorção de cátions celulares devida aos eventos iônicos extra-celulares versus intra-celulares.

Na hipertensão extracelular dependente de cálcio (identificado clinicamente como formas de hipertensão com baixa-renina), o mecanismo operante é o excesso de acúmulo do cálcio celular a partir do espaço extracelular, mediado pela ação de hormônios reguladores de cálcio como 1,25(OH)2D, e fator hipertensivo da paratireóide (PHF).

Na situação oposta, a hipertensão dependente unicamente de cálcio intracelular é mediada e clinicamente caracterizada pelo excesso de atividade de renina circulante e aumento de níveis da angiotensina II. Aqui, o excesso de cálcio citosólico livre não é resultado do acúmulo de fontes extracelulares, mas sim da liberação de angiotensina II mediada pelo cálcio do citosol a partir de locais de estocagem no retículo endoplasmático ou calciossomas. Nessa situação, níveis de PTH, 1,25 e PHF ou são suprimidos ou são indistinguíveis daqueles encontrados em indivíduos com pressão normal. Este é o perfil hormonal do cálcio observado em hipertensão com altos níveis de renina.

Resumindo, os efeitos dos sinais minerais da dieta na pressão arterial, e em particular do cálcio, são transduzidos a nível celular pelo seu efeito nos sistemas hormonais. A condição estável resultante da distribuição de cálcio mediada pelos hormônios alteraria funções hemodinâmicas cardíacas, bem como a liberação de hormônio vasoativo periférico, o tônus vasoconstrictor periférico de músculos lisos, e a pressão arterial resultante. As diferentes alterações genéticas ou adquiridas nos sistemas de transporte celular iônico resultam no “ponto limiar” metabólico do sistema renina-aldosterona e dos hormônios reguladores de cálcio entre diferentes indivíduos, o que resultará em diferentes padrões de reatividade vascular e de predisposição à pressão arterial alta. Estas são hipóteses intrigantes, sendo necessários mais estudos para compreensão do detalhado mecanismo de hormônios reguladores de cálcio envolvido na pré-eclâmpsia e no efeito programador da suplementação de cálcio na pressão arterial da prole.

2.4.2 Justificativa para a aferição de parâmetros bioquímicos

Com base na hipótese apresentada acima, foram medidos os hormônios potencialmente ligados à hipertensão que são associados à regulação de cálcio (fator

hipertensivo da paratireóide (PHF), paratormônio, calcitriol e CGRP). A resistência à insulina também foi medida, pois uma associação foi descrita entre alterações no metabolismo intracelular de cálcio, resistência à insulina e hipertensão (Resnick93e). Os mecanismos de ação para cada um desses hormônios estão descritos em mais detalhe a seguir.

2.4.3 Fator hipertensivo da paratireóide

Fator hipertensivo da paratireóide (PHF) é um fator de circulação recentemente descoberto que tem sido associado a algumas formas de hipertensão (Pang *et al.* 94d). Esta associação foi estabelecida através de vários estudos com animais e com pacientes hipertensos. PHF vem das glândulas da paratireóide (Lewanczuk *et al.* 94d). Isto explica porque indivíduos hipertensos costumam apresentar altos níveis circulantes de hormônio da paratireóide (PTH), que por si próprio é hipotensor. Isto refletiria a atividade das glândulas da paratireóide, que produziria PHF junto com PTH. Os mecanismos de ação do PHF envolvem um aumento na atividade no canal de cálcio em células de músculo liso vascular. A abertura destes canais leva ao aumento da entrada de cálcio nestas células, resultando no aumento da sensibilidade a outros vasoconstritores. Os níveis de PHF explicam porque uma dieta rica em cálcio pode ser efetiva na diminuição da pressão arterial em pacientes que respondem a bloqueadores de canais de cálcio: cálcio dietético pode inibir a produção de PHF (e PTH), enquanto bloqueadores de canais de cálcio inibiriam PHF em seu local alvo (Pang *et al.* 94c). Também foi sugerido que níveis elevados de 1,25-D e PHF coordenadamente mudam níveis de cátions intracelulares e estimulam a absorção de cálcio celular a partir do espaço extracelular (Resnick *et al.* 91).

Muitas das disfunções no metabolismo de cálcio presentes na hipertensão de baixa renina também foram descritas em diabetes mellitus não-dependente de insulina

(NIDDM). Uma pesquisa com pessoas portadoras de diabetes mellitus não-dependente de insulina mostrou que PHF estava presente em um número desproporcional destes pacientes, independentemente do nível da pressão arterial (Lewanczuk *et al.* 94c). A elevação de PHF poderia ser responsável pela resistência à insulina em uma fração de pacientes de NIDDM, e PHF poderia ser relacionado aos níveis de colesterol sérico em NIDDM (Ho *et al.* 94).

2.4.4 Calcitriol (1,25-(OH)2D3)

Alguns autores sugeriram que calcitriol tenha efeitos hipertensivos e que o efeito hipotensivo da suplementação de cálcio dietético poderia ser mediado pela redução de níveis de calcitriol (DiPette *et al.* 90a). Recentemente, maiores níveis de calcitriol que estavam relacionados à baixa ingestão de cálcio foram positivamente associados ao ganho de peso e obesidade, devido a um aumento no cálcio intracelular em adipócitos. Acredita-se que os mecanismos atuando aqui sejam similares aos descritos, dado o efeito vasoativo do hormônio (Zemel03c).

2.4.5 Hormônio da paratireóide (PTH)

O hormônio da paratireóide (PTH) tem atividade vasodilatadora que é mediada por um receptor de membrana celular específico, agrupado à adenilato-ciclase, levando então ao aumento do AMPc intracelular e à diminuição do cálcio intracelular. O peptídeo também poderia bloquear canais de cálcio sensíveis à voltagem. Entretanto, o consenso geral é que PTH não alcance níveis séricos suficientes para modular reatividade vascular (Bukoski, Ishibashi, and Bian95a).

2.4.6 Peptídeo Relacionado ao Gene da Calcitonina (CGRP)

Peptídeo relacionado ao gene da calcitonina (CGRP) é um neuropeptídeo produzido por um processo alternativo da transcrição primária do gene da calcitonina,

sendo um potente vasodilatador (Wimalawansa, Supowit, and DiPette95a). O peptídeo é liberado pelas terminações do nervo perivascular e pode normalmente ser detectado na circulação. Foi mostrado que uma deficiência no cálcio dietético acompanhada a uma diminuição do cálcio sérico ionizado diminuiu significantemente o conteúdo neuronal do CGRP no rato em crescimento. O rato espontaneamente hipertenso (SHR) é caracterizado pela diminuição dos níveis de cálcio sérico ionizado, sendo considerado o modelo mais semelhante à hipertensão essencial humana (Westlund *et al.* 91b).

Além disto, em pacientes hipertensos, a suplementação oral de cálcio aumenta os níveis do peptídeo relacionado ao gene da calcitonina (CGRP). Os efeitos anti-hipertensivos do cálcio e os níveis aumentados de CGRP na circulação retornaram ao nível original após a cessação da suplementação do cálcio, sugerindo que os efeitos do cálcio no BP e CGRP sejam específicos. É proposto que o efeito anti-hipertensivo da suplementação de cálcio dietético, pelo menos em parte, seja mediado através do CGRP (Wimalawansa, Supowit, and DiPette95b).

2.4.7 Insulina (como A1c)

Vários estudos demonstraram a existência em algum grau da resistência à insulina e/ou hiperinsulinemia em pacientes com hipertensão essencial, bem como uma relação inversa entre pressão arterial e eliminação de glicose não-mediada pela insulina (Resnick93d). É provável que exista uma relação importante entre a alteração no metabolismo do cálcio intracelular, a resistência à insulina e a hipertensão. Existem vários resultados sugerindo que níveis favoráveis de concentração de cálcio intracelular livre sejam necessários para a máxima ação celular de insulina (Resnick92b). Portanto, pode ser especulado que a redução de cálcio intracelular esperada, induzida por suplementação de cálcio oral, poderia melhorar o metabolismo de insulina celular e corrigir particularmente a resistência à insulina (Sanchez *et al.* 97b).

Não nos foi possível medir os níveis de insulina devido à necessidade de jejum antes da coleta de sangue. Assim sendo, a hemoglobina A1c foi medida como alternativa, providenciando uma informação que reflete níveis de glicose sérica pelos últimos dois meses.

2.5 Objetivos

I) Avaliar o efeito de suplementação oral de cálcio durante a gravidez nos hormônios reguladores de cálcio (CRH) da prole, e a sua relação com a pressão arterial aos 7 e 12 anos de idade. (Artigo 1).

Objetivos Específicos

I.i Avaliar o efeito de suplementação de cálcio materno durante a gravidez no CRH da prole aos 12 anos de idade.

I.ii Avaliar a relação entre CRH e pressão arterial da prole aos 7 e 12 anos de idade.

I.iii Avaliar se a relação entre CRH e pressão arterial da prole aos 7 e 12 anos de idade é diferente para filhos de mães recebendo cálcio comparadas com àquelas que receberam placebo durante a gravidez (modificação de efeito).

II) Identificar fatores preditivos da pressão arterial e da resistência à insulina em crianças aos 12 anos de idade, considerando variáveis perinatais, e características maternas e infantis no seguimento (Artigo 2).

Objetivos Específicos

II.i Avaliar o efeito na pressão arterial e HbA1c da prole considerando as seguintes variáveis:

II.i.a Características maternas durante a gravidez, incluindo idade, peso pré-gestacional, ganho de peso, indicador de nutrição materna, fumo, e complicações durante a gravidez.

II.i.b Tamanho ao nascer, incluindo peso ao nascer, restrição de crescimento intra-uterino (RCIU), índice ponderal, perímetro cefálico e razão perímetro/comprimento cefálico.

II.i.c Obesidade materna e pressão arterial dos filhos aos 12 anos de idade.

II.ii Avaliar o efeito da pressão arterial e HbA1c em obesidade nos fihos aos 12 anos de idade, e avaliar se a obesidade modifica os efeitos dos fatores preditivos do item II.i.

II.iii Avaliar se a suplementação de cálcio durante a gravidez modifica o efeito dos fatores preditivos dos itens II.i. e II.ii

III) Revisar criticamente a literatura sobre o efeito do cálcio dietético durante a gravidez no metabolismo do cálcio e na pressão arterial da prole (Artigo 3).

Modelos de análise

Figuras 1 e 2 (veja seção 8) apresentam os modelos analíticos desenvolvidos para a análise do artigo I. O efeito em longo prazo de déficit de cálcio materno durante a gravidez sobre a prole poderia ser mediado por mudanças no limiar dos hormônios reguladores de cálcio. Filhos expostos ao déficit de cálcio materno podem ter maiores níveis circulantes destes hormônios vasoativos. Os efeitos vasoativos diretos destes hormônios aumentariam a pressão arterial da prole. De acordo com esta hipótese, os níveis de hormônios reguladores de cálcio seriam significantemente diferentes entre as crianças do grupo do cálcio e as do grupo placebo. Uma alternativa é que os níveis de hormônios reguladores de cálcio sejam similares entre os grupos, mas a resposta em aumento de pressão arterial para o mesmo nível hormonal seria diferente entre crianças

do grupo do cálcio e do grupo placebo. Este efeito modificador pode ser avaliado considerando a interação entre os níveis hormonais, a pressão arterial e o grupo experimental (cálcio/placebo).

A pressão arterial foi aferida aos 7 e 12 anos de idade, mas níveis hormonais somente foram medidos aos 12 anos de idade. O mesmo modelo acima descrito para a pressão arterial de crianças de 12 anos de idade é aplicado às medidas de pressão arterial aos 7 anos de idade, assumindo que níveis hormonais aos 12 anos sejam representativos dos níveis hormonais na idade de 7 anos (figure 2).

Para o artigo II, o modelo é apresentado na figura 3 (veja seção 8). Uma análise secundária será realizada para identificação de fatores do período perinatal e da infância, que estariam associados ao desenvolvimento de pressão arterial alta e resistência à insulina aos 12 anos. Uma análise similar nos dados do primeiro seguimento foi conduzida em crianças com 7 anos de idade (Bergel *et al.* 00b). Informações muito mais detalhadas estão disponíveis no segundo seguimento. Por um lado, a resistência à insulina poderia ser incluída como uma variável dependente para identificação de indivíduos que podem expressar precocemente a síndrome X (obesidade, hipertensão e resistência à insulina). Por outro lado, o segundo seguimento inclui informação adicional sobre as variáveis antropométricas das crianças que permitiria uma melhor identificação de obesidade, o principal fator preditivo de pressão arterial. Em resumo, a base de dados fornece uma oportunidade única de identificação dos fatores preditivos de pressão arterial alta e de resistência à insulina, e sua relação à obesidade infantil. O efeito potencial da modificação dos fatores preditivos pela suplementação de cálcio durante a gravidez também é explorado.

2.6 Hipóteses

I. Suplementação de cálcio durante a gravidez levou a modificações no metabolismo de cálcio nos fihos em longo prazo. Estas mudanças modulam a pressão arterial da prole por induzirem mudanças nas concentrações de hormônios reguladores de cálcio com propriedades vasoativas, incluindo PTH, calcitriol, CGRP e Fator Hipertensivo da Paratireóide, ou mudando a reatividade do tecido alvo para os efeitos desses hormônios.

II. A pressão arterial da criança e a sua resistência à insulina aos 12 anos de idade são preditas por variáveis do período perinatal e por características maternas e infantis no seguimento.

2.7 Delineamento

O foco desta proposta de pesquisa é a análise dos dados coletados durante o segundo acompanhamento de uma coorte de crianças nascidas de mães que participaram do ensaio clínico randomizado sobre a suplementação de cálcio durante a gravidez. A Tabela 1 mostra as fases do estudo, incluindo as principais avaliações de desfecho e as variáveis que foram coletadas no estudo original, no primeiro e no segundo seguimentos. A estratégia de análise está descrita na seção 8. Veja o apêndice I para uma descrição dos métodos do ensaio clínico original.

Como método de revisão de literatura, será utilizado o modelo desenvolvido pela Colaboração Cochrane (veja http://www.cochrane.dk/cochrane/handbook/hbook.htm, último acesso Junho 2004).

Tabela 1: Variáveis coletadas no ensaio clínico randomizado original, e no primeiro e segundo estudos de seguimento, incluindo os desfechos principais e secundários.

Estudo	Participantes	Desfecho principal	Variáveis do estudo		
	Calcio / Placebo		Mãe	Prole	Pai
Estudo clínico randomizado original (1987-1990)	309 305	1) Hipertensão induzida pela gravidez 2) Pré-eclâmpsia 3) Prematuridade	***Ao entrar no estudo (20 semanas):*** idade, paridade, abortos prévios, cesáreas prévias, idade gestacional (DUM), idade gestacional (ultra-som), altura uterina, peso, altura. Exames de laboratório: glicemia, teste de tolerância à glicose oral, ácido úrico. ***Em cada visita pré-natal*** : altura uterina, idade gestacional, peso, pressão arterial, aderência ao tratamento (cálcio), tabagismo, uso de medicamentos, sintomas clínicos, condições médicas associadas (infecção urinária, anemia, ameaça de parto prematuro, diabetes, hemorragia, infecção, RCIU, outro). Exames de laboratório: Sangue: hemoglobina, hematócrito, glicose, cálcio ionizado, ***Em cada admissão hospitalar :*** dias de internação, idade gestacional, motivo para internação, medicação utilizada, pressão arterial sistólica, ***No parto:*** indução, tipo de parto	Morte fetal, morte neonatal, idade gestacional pela DUM, idade gestacional pelo exame físico, índice de apgar, peso ao nascer, sexo, altura, circunferência da cabeça, admissão em UTI neonatal, motivo para admissão.	
Primeiro seguimento (1995-1996)		1) Pressão arterial da prole	Idade, paridade, cesáreas prévias, abortos, hipertensão induzida pela gravidez, urolitíase na primeira gravidez, colelitíase na primeira gravidez, urolitíase após a primeira gravidez, colelitíase após a primeira gravidez, pressão arterial sistólica,	Idade, peso, altura, pressão arterial sistólica, pressão arterial diastólica, urolitíase, colelitíase, admissão hospitalar em UTI neonatal, admissão hospitalar durante a infância, motivos para admissão.	
Segundo seguimento (2000)		1) Pressão arterial da prole 2) Hormônios reguladores do cálcio da prole (Fator hormonal hipertensivo da paratireóide, 1,25(OH)2D, Peptídeo Relacionado ao Gene da Calcitonina	Idade, hipetensão, tratamento para hipertensão, diabetes, tratamento para diabetes, nível educacional, ocupação, peso, altura, circunferência abdominal, dobra de pele triceptal, dobra de pele sub-escapular, pressão arterial sistólica, pressão arterial diastólica.	Peso ao nascer, sexo, padrão de amamentação, nível escolar, urolitíase, colelitíase, hipertensão, tratamento para hipertensão, diabetes, tratamento para diabetes, doenças na infância (código CID-10), vive com a mãe, vive com o pai, número de irmãos, pressão arterial sistólica, pressão arterial diastólica, peso, altura, circunferência abdominal, dobra de pele triceptal, dobra de pele sub-escapular. Exames de laboratório: cálcio sérico total, cálcio sérico ionizado, colesterol, triglicérides, glicemia, hemoglobina A1c, hemoglobina, hematócrito, contagem de leucócitos, contagem de plaquetas.	Idade, peso, altura, hipertensão, tratamento para hipertensão, diabetes, tratamento para diabetes, nível educacional, ocupação.

Parâmetros avaliados nas amostras de sangue

Hormônios associados ao metabolismo de cálcio que são afetados pela ingestão de cálcio na dieta, e que também têm propriedades vasoativas

- Hormônio da paratireóide (PTH)
- Calcitriol (1,25-(OH)2D3)
- Peptídeo relacionado ao gene da calcitonina (CGRP)
- Fator hipertensivo da paratireóide (PHF)

Cálcio sérico e urinário, e creatinina urinária, para ajuste do valor do cálcio urinário

- Cálcio sérico total
- Cálcio sérico ionizado
- Cálcio urinário
- Creatinina urinária

Indicadores de possível resistência à insulina

- Glicose urinária
- Glicemia
- Hemoglobina A1

Indicadores hematológicos

- Hemoglobina
- Hematócrito
- Leucócitos
- Plaquetas

Lípides séricos

- Colesterol

- Triglicérides

2.8 Análise dos dados

2.8.1 Estratégia geral de análise

As crianças são colocadas no grupo ao qual suas mães haviam sido originalmente randomizadas, independentemente da adesão e de qualquer experiência pós-natal (intenção de tratamento). O valor médio de três aferições de pressão arterial é utilizado para análise. A distribuição das variáveis é explorada através de histogramas. Se uma variável desviar significativamente da distribuição normal, ela é transformada numa tentativa de corrigir o problema (transformação logarítmica). Para análise de regressão, variáveis contínuas independentes serão transformadas em escores z (média = 0, desvio-padrão = 1), com exceção da pressão sistólica da criança. Estas transformações permitem a comparação dos coeficientes de regressão entre as variáveis com escalas de medida diferentes. Estes coeficientes de regressão computados por escores z podem ser convertidos a coeficientes regulares de regressão pela sua multiplicação com o desvio-padrão da variável independente. A avaliação dos modelos de regressão inclui a análise residual (para verificação da distribuição residual, variação e valores notavelmente fora da média) e o teste de distância de Cook para detectar as observações mais influentes. As análises de sensibilidade serão realizadas quando variáveis de grande influência forem observadas. Os pacotes estatísticos utilizados serão o STATA (Stata Corporation, College Station, TX, USA) e o SAS (SAS Institute Inc., Cary, NC, USA).

2.8.2 Estratégia de análise da pergunta de pesquisa no artigo I

Objetivo principal: Avaliar a o efeito da suplementação de cálcio materno, por via oral, durante a gravidez, nos hormônios reguladores de cálcio (CRH)* de sua prole, e a relação destes com a pressão arterial nas idades de 7 e 12 anos. (Artigo 1).

A pergunta da pesquisa tem três componentes (veja figuras 1 e 2 para os modelos de análise).

i. Avaliação da suplementação de cálcio durante a gravidez sobre os CRH dos filhos

   Análise: regressão linear com cada um dos hormônios reguladores de cálcio como a variável dependente (um de cada vez) e uma variável independente dicotômica (Cálcio-Placebo). Esta análise é equivalente ao t-test. Se um desvio significante da distribuição normal for encontrado em qualquer um dos CRH, os grupos de cálcio e de placebo são comparados usando testes não-paramétricos. Um modelo de regressão múltipla que inclui fortes preditores da pressão arterial de crianças (como peso atual, sexo, idade, pressão arterial materna durante a gravidez e no seguimento) será relatado se a inclusão destas variáveis adicionais aumentarem a precisão das estimativas.

ii. Avaliação da relação entre os CRH e a pressão arterial dos filhos com 7 e 12 anos.

iii. Avaliar se a relação entre os CRH e a pressão arterial dos filhos com 7 e 12 anos é diferente para os filhos de mães que receberam cálcio ou placebo durante a gravidez (modificação de efeito).

   Análise: Regressão linear múltipla utilizando a pressão arterial da prole como variável dependente e os CRH como variáveis independentes. O modelo incluirá outros fortes preditores da pressão arterial (como o peso atual, sexo, idade, pressão arterial materna durante a gravidez, crescimento alcançado no seguimento) se a inclusão destes fatores aumentar a precisão das estimativas. Uma variável dicotômica do

tratamento (Cálcio-Placebo) também será incluída como variável independente.

Para investigar a questão (iii), o modelo incluirá a interação entre CRH e tratamento (Cálcio-Placebo). A interação é considerada estatisticamente significativa se o valor de p for menor que 0,10, sendo também considerado que a magnitude da interação é clinicamente significativa. Interações clinicamente significantes também serão relatadas (isto é, efeito positivo versus efeito negativo, nenhum efeito, efeito importante), mesmo que o teste para interação não seja estatisticamente significante.

2.8.3 Estratégia de análise da pergunta de pesquisa no artigo II

Objetivo principal: Identificar os fatores preditivos da pressão arterial da criança e da resistência à insulina aos 12 anos de idade, entre as variáveis e as características maternas e da criança durante o seguimento (Artigo 2). Veja figura 3 para o modelo de análise e a lista de variáveis que são incluídas na análise. O crescimento alcançado no seguimento também é incluído no modelo.

Análise: Regressões lineares múltiplas com medidas da pressão arterial e da HbA1c da prole como variáveis dependentes e potenciais fatores preditores como variáveis independentes. As interações com a atual obesidade da prole e a suplementação de cálcio na gravidez serão exploradas.

Figura 1. Modelo de análise dos mecanismos de ação da suplementação de cálcio na pressão arterial de crianças aos 12 anos de idade.

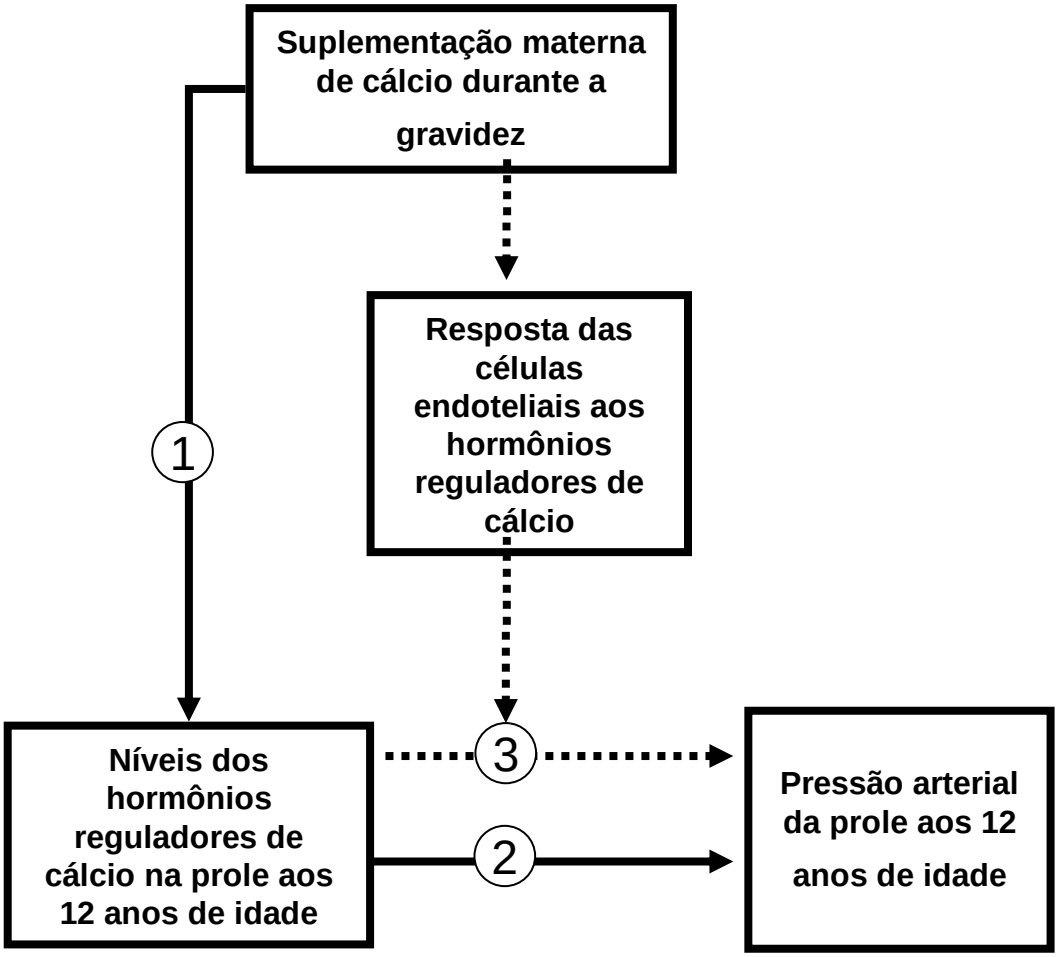


Figura 2. Modelo de análise dos mecanismos de ação da suplementação de cálcio na pressão arterial de crianças aos 7 anos de idade

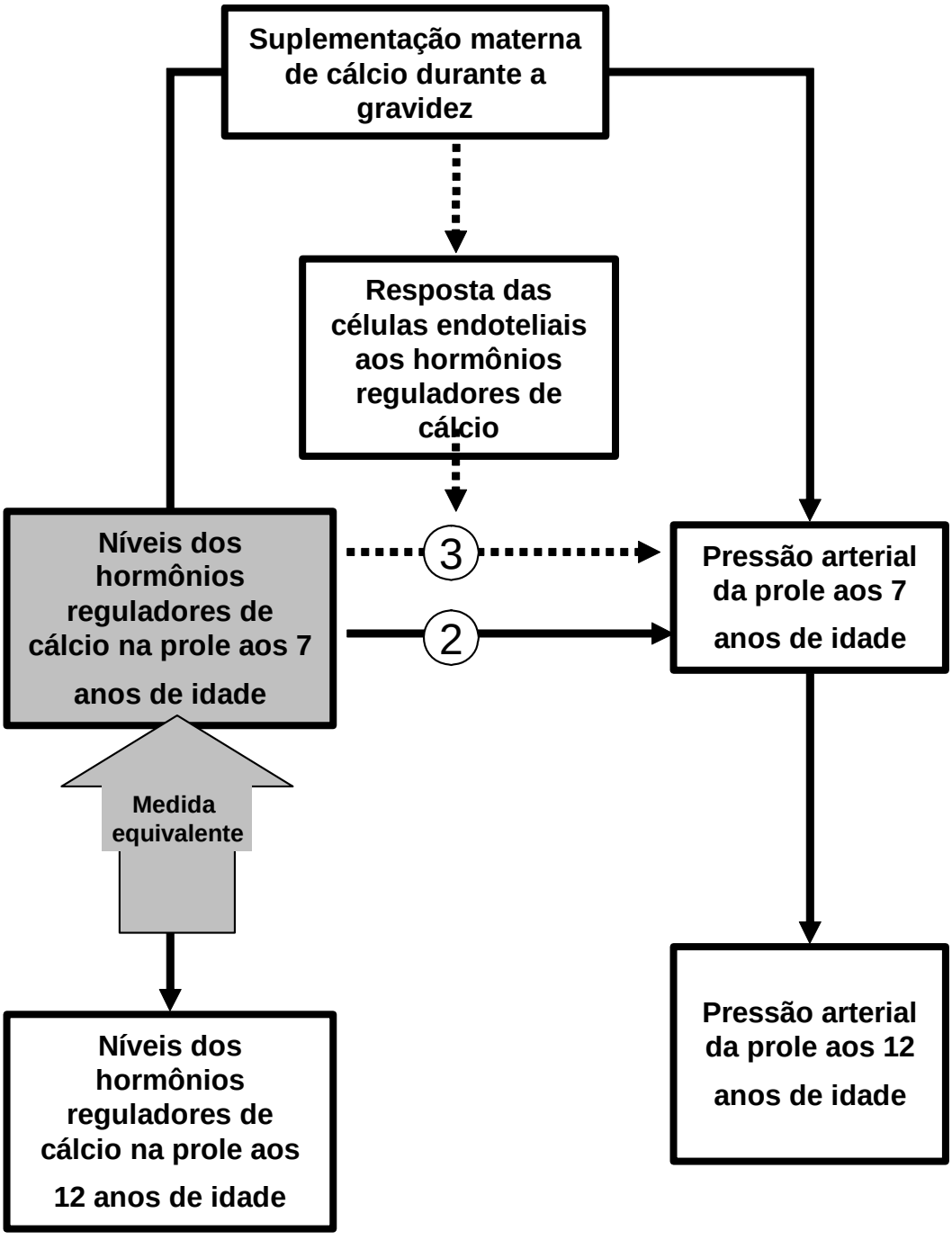
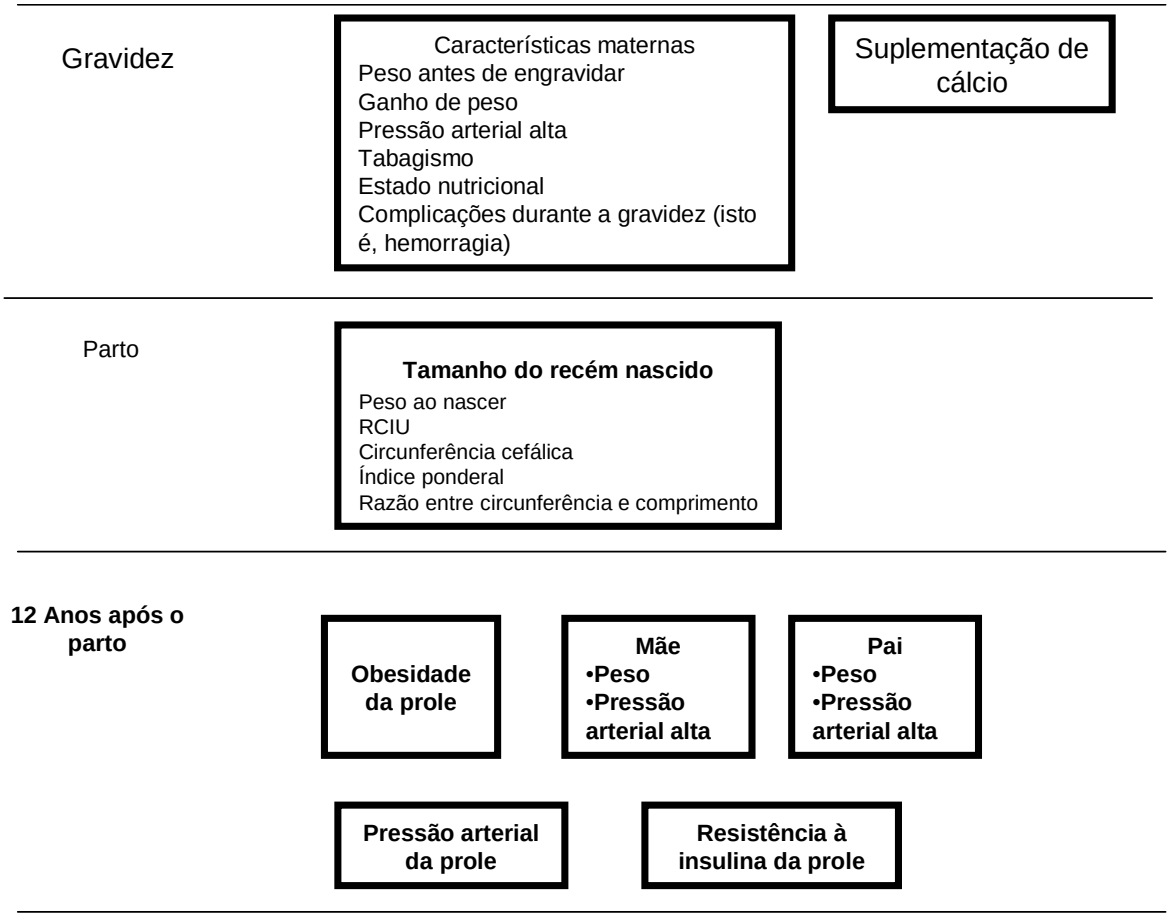


Figura 3. Modelo de análise dos preditores da pressão arterial de crianças aos 12 anos de idade.



2.8.4 Tamanho da amostra e poder estatístico

Uma grande limitação na estimativa do poder estatístico deste estudo é a dificuldade na determinação de uma diferença no valor médio do desfecho que seja clinicamente significante. Os principais desfechos são os hormônios reguladores de cálcio com distribuições e desvios-padrão diferentes. Por exemplo, PHF é apenas utilizado em estudos experimentais e os valores de referência para crianças não foram estabelecidos. Para ter uma idéia do poder do estudo, uma análise de poder estatístico é apresentada abaixo, correlacionando o poder versus escore z. Os cálculos são apresentados na Tabela e na Figura abaixo.

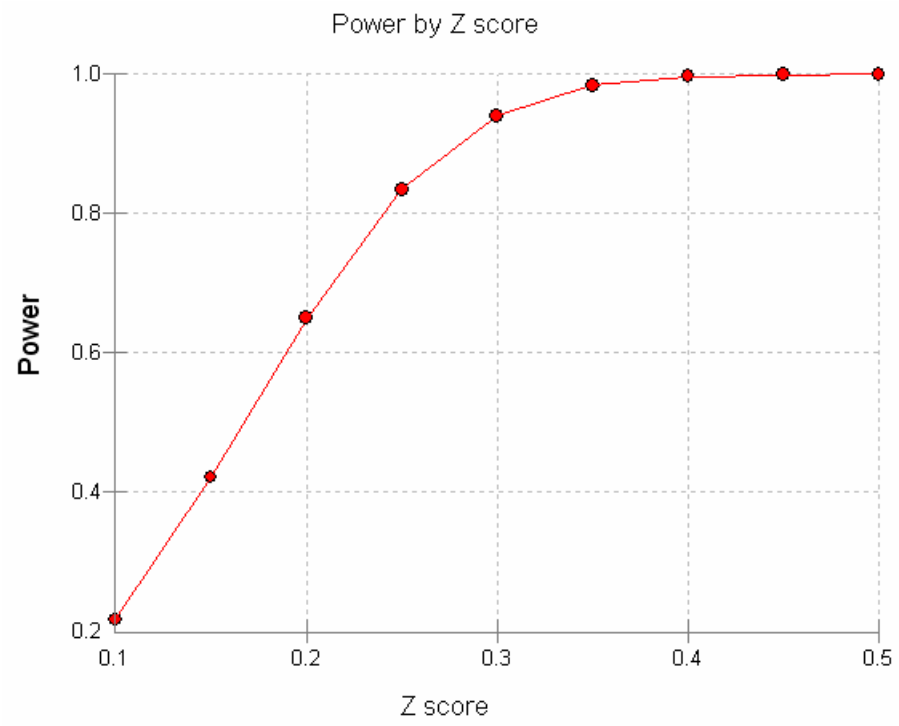


Figura 1. Análise do poder para o teste t com duas amostras

Hipótese Nula: Média 1 = Média 2. Hipótese Alternativa: Média 1 <> Média 2

Assume-se que os desvios-padrão sejam desconhecidos e iguais

Power	N1	N2	Alpha	Mean1	Mean2	S1	S2
0.21709	275	275	0.05	0	0.10	1	1
0.42092	275	275	0.05	0	0.20	1	1
0.65006	275	275	0.05	0	0.25	1	1
0.83416	275	275	0.05	0	0.30	1	1
0.94015	275	275	0.05	0	0.35	1	1
0.98387	275	275	0.05	0	0.40	1	1
0.9968	275	275	0.05	0	0.45	1	1
0.99954	275	275	0.05	0	0.50	1	1
0.99995	275	275	0.05	0	0.55	1	1

O estudo tem um poder de 80% para detectar uma variação de 0,25 do desvio-padrão entre os grupos controle e cálcio. Como um exemplo, o desvio-padrão da pressão arterial sistólica em crianças é 12 mmHg, e portanto o estudo pode detectar uma diferença de (12*0,25) 3 mmHg entre os grupos. Uma análise do poder estatístico foi incluída no protocolo.

2.9 Implicações

O desenvolvimento de um modelo teórico e do subseqüente desafio das teorias em relação aos experimentos controlados é a chave para estabelecer causalidade. Se tiver sucesso, esta pesquisa poderia fornecer evidência para uma melhor compreensão dos aspectos relacionados ao aparecimento de doença hipertensiva em seres humanos. Este conhecimento poderia ser utilizado no desenvolvimento de estratégias de prevenção ou de tratamento para hipertensão.

2.10 Limitações

Existem relatos sugerindo que o efeito da suplementação de cálcio durante a gravidez esteja presente apenas em populações com baixa ingestão de cálcio (Hofmayer et al. 2003). Este é um assunto muito importante, e um grande ensaio randomizado multicêntrico, financiado pela OMS, está sendo conduzido para avaliar o efeito da suplementação de cálcio na gravidez em populações com baixa ingestão de cálcio. Um estudo realizado nos EUA mostrou que a intervenção não reduziu pré-eclâmpsia em mães que tinham consumo adequado de cálcio. Além disto, modelos animais mostraram que o efeito do cálcio nos fihos só foi observado se as mães estivessem em uma dieta com pouco cálcio, em comparação com dieta normal (Bergel e Belizan, 2002). Existiram certas limitações para exploração destas idéias nesta base de dados. Não temos dados relativos ao consumo de cálcio no modelo. Além disto, apesar do estudo original ter incluido duas populações bastante distintas, com grande chance de apresentar diferenças em seu consumo de cálcio (hospitais públicos e privados), apenas mulheres dos hospitais privados estavam disponíveis para seguimento. Não havia dados sobre a excreção urinária de cálcio das mães, fator que está correlacionado com o consumo de cálcio na dieta. Uma análise secundária poderia ser realizada para avaliar a

existência de um efeito entre a excreção materna de cálcio (medida indireta do consumo materno de cálcio), e os desfechos nos fihos.

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3 PROJETO DE PESQUISA (English Version)

Universidade Federal de Pelotas

Programa de Pós-Graduação em Epidemiologia

Doutorado em Epidemiologia

THE EFFECT OF MATERNAL CALCIUM SUPPLEMENTATION DURING PREGNANCY ON BLOOD PRESSURE BIOCHEMICAL MARKERS OF CHILDREN AT 12 YEARS OF AGE (FOLLOW–UP OF A RANDOMIZED CONTROLLED TRIAL)

Projeto de Pesquisa

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PELOTAS-RS-BRASIL
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3.1 Abstract

Dietary calcium supplementation during pregnancy has been shown in clinical trials to reduce the incidence of pregnancy induced hypertension. A long term follow-up of two of these trials has shown a reduce incidence of high blood pressure in the offspring of calcium supplemented mothers. An animal model has confirmed theses findings. The mechanisms underlying these effects are unknown. The present study will explore such mechanisms by comparing the levels of calcium regulating hormones in the offspring of mothers exposed to calcium or placebo during pregnancy, and by assessing the relationship between these hormones and offspring blood pressure. Data from a follow-up of children born from mothers that participated in the second largest trial of calcium supplementation will be used for the analyses. The dataset also contain information on perinatal variables, indicators of insulin resistance and a detailed anthropometry evaluation at follow-up. Hypertension, obesity and diabetes, had been linked in what is called “syndrome x”, and the risk for this condition has been associated to impaired fetal growth and calcium metabolism. In this context, the study will also explore the determinants of hypertension and insulin resistance in these children.

3.2 Papers

Paper 1: Calcium supplementation during pregnancy and calcium regulating hormones and blood pressure in the offspring at 12 years of age.

This study will analyze data collected during the long-term follow-up of a randomized trial of calcium supplementation during pregnancy. An analytical model with a corresponding analysis strategy will be used to explore the mechanisms of action of dietary calcium supplementation during pregnancy on offspring blood pressure and calcium metabolism. To this aim the study will compare the levels of calcium regulating hormones in the offspring of mothers exposed to calcium or placebo during pregnancy, and the effect modification of calcium supplementation on the relationship between these hormones and offspring blood pressure.

Paper 2: Perinatal and childhood factors as predictors of blood pressure and insulin resistance at 12 years.

Calcium has been implicated in the mechanisms underlying hypertension, obesity and diabetes in what is called “syndrome x”. Some postulate that an inadequate intra-uterine environment might play a role in the development of these conditions. In this context, the study will explore the determinants of hypertension and insulin resistance in the cohort of children describe in paper one. The analysis will include perinatal variables (i.e. complications during pregnancy, birth weigh, etc.), and variables measured at 7 and 12 years post-partum.

Paper 3: Maternal calcium dietary intake during pregnancy and offspring blood pressure during childhood and adult life. A systematic review of the literature.

A number of experimental and observational studies have reported an association between maternal calcium dietary intake during pregnancy and offspring blood pressure

during childhood and adult life. A systematic review of the literature will be conducted to summarize the current knowledge in this area.

3.3 Introduction and rationale

Hypertension is the most common cardiovascular disease and is a major public health problem in both developed and developing countries (Antezana F96). Cardiovascular diseases are responsible for nearly 20% of all deaths world-wide (Lopez90). In developed countries, cardiovascular diseases are the leading causes of death for adult men and women. They are also emerging as a prominent public health problem in developing countries, responsible for over 10 million deaths in 2001 (Reddy and Yusuf98c,WHO03). Effective strategies for the prevention of hypertension and cardiovascular disease are likely to have a significant public health impact.

Calcium deficit has been associated with an increased risk for the development of hypertension, in particular among pregnant women. Furthermore, diets with an increased calcium content have been shown in randomized trials to reduce blood pressure (Appel *et al.* 97a,Craddick *et al.* 03a).

In recent years, a number of studies have provided evidence supporting the hypothesis that undernutrition during fetal life programmes the development of risk factors, such as high blood pressure, for cardiovascular disease in adult life (Barker97a). In line with these hypotheses, the question that arises is whether a calcium deficit during pregnancy might be a risk factor for the development of hypertension later in life.

A randomized controlled trial of calcium supplementation during pregnancy was conducted between 1987 and 1990 (Belizan *et al.* 91c). This trial originated a series of studies, the last of them being the source of the data to be used in this proposal. The original randomized trial enrolled 1200 pregnant women around 20 weeks gestation to either 2 daily grams of oral calcium or placebo. The main outcomes for this study were the incidence of pregnancy induced hypertension, pre-eclampsia and prematurity. The rates of hypertensive disorders of pregnancy were lower in the calcium group than in the

placebo group (RR 0.67; 95% CI 0.49 to 0.91). This study concluded that calcium supplementation provided to pregnant women is a promising preventive strategy for pregnancy induced hypertension and pre- eclampsia (Belizan *et al.* 91d)

Between 1995 and 1996 the follow-up of a subgroup of the original cohort was carried out. For logistic reasons, only subjects from one of the 3 hospitals that participated in the original study were included. The randomization was stratified by center, so the experimental nature of the comparison was preserved. The main outcome for this study was offspring blood pressure at follow-up. The study showed that the risk of high systolic blood pressure was lower in the calcium group than in the placebo group (RR 0.59; 95% CI 0.39 to 0.90), with a stronger effect among overweight children (RR 0.43; 95% CI 0.26 to 0.71) (Belizan *et al.* 97b). These results suggest that a simple prevention strategy directed to increase calcium intake among pregnant women might reduce the incidence of high blood pressure in the offspring.

After this first follow-up an animal model was developed to test the hypothesis of a causal association between calcium levels during pregnancy and offspring blood pressure(Bergel and Belizan02d) . The results of this study in rats provided additional support for the hypothesis. At one year of age systolic blood pressure in the offspring of rats exposed to a deficient calcium intake during pregnancy was 12 mm Hg higher than the control group (Bergel and Belizan02e).

The results in humans have been recently confirmed. A group of newborns from mothers randomized during pregnancy to calcium or placebo were studied (Hatton *et al.* 03a). Maternal calcium supplementation during pregnancy was associated with lower systolic blood pressure in the offspring at 2 years of age. These finding have important public health implication given the high incidence of hypertension in both developed and developing countries (Reddy and Yusuf98d,WHO03).

The next logical step to obtain a clearer understanding of the effect of maternal calcium deficit on offspring blood pressure is to explore the underlying mechanisms.

There are a number of possible models that can potentially explain the long term effect of maternal calcium supplementation on offspring blood pressure. In recent years the role of calcium has been expanded from the traditional role as a bone component to a key regulator in many body functions. In particular calcium deficit has been associated with hypertension, insulin resistance, and more recently with obesity (Resnick99e,Zemel04a,Zemel and Miller04b). These three conditions are highly correlated, and individuals presenting all three are described as having the “syndrome x" (Resnick92c,Resnick93c). An increase risk for this syndrome has been associated to impaired nutrition during pregnancy and reduced fetal growth (Barker *et al.* 93b).

An increased intracellular calcium has been postulated as a common characteristic of these conditions (hypertension, obesity and insulin resistance) (Resnick99a,Resnick94b,Zemel03b). Calcium deficit triggers compensatory mechanisms, including changes in calcium regulating hormones. Some of these hormones have been shown to have vasoactive effects. In particular calcitriol and the newly discovered parathyroid hypertensive factor (PHF) have strong vasoactive effects (Resnick94a,Zemel01a). This vasoactive effect of the hormones has been shown to be mediated by increasing intracellular calcium(Resnick *et al.* 91).

It has been shown that the effect of calcium supplementation during pregnancy on pre-eclapmsia is evident only in populations with a dietary calcium deficit (Hofmeyr *et al.* 03b). The effect on the offspring is also seen only in association with dietary maternal calcium deficit during pregnancy (Bergel and Belizan02f). The long term effect of maternal calcium deficit during pregnancy on the developing fetus might be mediated by changes in the "Set Point" of calcium regulating hormones. Children

exposed during fetal life to a maternal calcium deficit might have higher circulating levels of vasoactve calcium regulating hormones, or higher sensitivity to these hormones in target cell (i.e. endothelial cells). Still, other mechanisms not involving these hormones might explain the association. For example, maternal calcium deficit might induce permanent structural changes in the offspring blood vessels that might in turn increase peripheral resistance and hypertension.

During year 2000 a second follow-up was carried out with the offspring of the women enrolled in the original randomized trial of calcium supplementation. Approximately 90% of the eligible original population for follow-up was assessed at 12 years of age, after the implementation of very aggressive follow-up strategy. More detailed information was obtained in this last follow-up, as compared with the former one, including measurements of calcium regulating hormones (parathyroid hormone (PHT), parathyroid hypertensive factor (PTH), calcitriol, and Calcitonin Gene Related Peptide (CGRP)), indicators of insulin resistance (HbA1, glycemia and glucose in urine), and hematological indicators. Detailed Maternal and children anthropometric measures were also obtained, including abdominal circumference, and triceps and subscapular skinfolds (see table 1, in section 7 for a list of all variables, and Appendix I for further details on the methods of the follow-up). The data obtained in this study will be used in the analyses proposed in this project. The objective of this project is to study the differences between children of treated and non-treated mothers in terms of their biochemical profile, blood pressure levels and insulin resistance.

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3.4 Background

3.4.1 Blood pressure and calcium regulating hormones.

Studies shown that alterations in extra cellular calcium homeostasis in pre-eclampsia include hypocalciuria and decreased serum levels of 1,25- dihidroxyvitamin d3 (August *et al.* 92b,Taufield *et al.* 87a). Increase parathyroid hormone (PTH) and decrease plasma ionized calcium concentration have been less consistently observed (Seely *et al.* 92b). Also, consistent abnormalities of intracellular calcium metabolism have been described in pre-eclamptic women, such as increase intracellular free calcium concentration in platelets and lymphocytes (Hojo *et al.* 99b). Increases in intracellular free calcium concentration in circulating cells are hypothesized to result from fluctuation in hormones or a vasoactive substance that causes similar alteration in vascular smooth muscle. Increase intracellular free calcium increases vascular reactivity and is associated with high blood pressure.

It has been suggested that all forms of hypertension are associated with and dependent on cytosolic free calcium excess (Resnick99f), and that what underlies the different pathophysiologies present in different forms of hypertensive disease is the extent to which this distortion of cellular cations is due to extra cellular vs. intracellular ionic event.

In extracellular calcium-dependent hypertension (identified clinically with low-rennin forms of hypertension) the operative mechanism is excess net cellular calcium accumulation from the extracellular space, mediated by the action of calcium regulating hormones such as 1,25(OH)2D, and parathyroid hypertensive factor (PHF).

The opposite, pure intracellular calcium dependent hypertension is mediated and clinically characterized by excess circulating renin activity and increased angiotensin II

levels. Here, the excess cytosolic free calcium is not a result of accumulation from extracellular sources but is from angiotensin II mediated release of calcium into the cytosol from storage sites in the endoplasmic reticulum or calciosomes. In this situation, PTH, 1,25 and PHF levels are either suppressed of indistinguishable from those in normotensive subjects. This is the calcium hormonal profile observed in high renin hypertension.

In summary, the blood pressure effects of dietary mineral signals, and in particular calcium, are transduced at the cellular level by their effect on hormone systems. The resultant hormone mediated steady state alteration in the distribution of calcium would alter cardiac hemodynamic function and peripheral vasoactive hormone release, peripheral smooth muscle vasoconstriction tone, and the resultant blood pressure. Different genetic or acquired alterations in cellular ion transport systems result in the metabolic “set-point” of the renin-aldosterone system and of calcium regulating hormones among different individuals that will result in different patterns of vascular reactivity and predisposition to high blood pressure. These are intriguing hypotheses and the detailed mechanism of calcium- regulating hormone involvement in pre-eclampsia and in the programming effect of calcium supplementation of offspring blood pressure await further studies.

3.4.2 Rationale for measuring the biochemical parameters

Based on the hypotheses presented above, hormones potentially linked to hypertension that are associated with calcium regulation (parathyroid hypertensive factor (PHF), parathormone, calcitriol and CGRP) were measured. Insulin resistance was also measured because an association has been described between alterations in intracellular calcium metabolism, insulin resistance and hypertension (Resnick93b). The mechanisms of action for each of these hormones is described in more detail below.

3.4.3 Parathyroid hypertensive factor

Parathyroid hypertensive factor (PHF) is a newly discovered circulating factor that has been implicated in some forms of hypertension (Pang *et al.* 94b). Such an involvement has been established in several animal models and in hypertensive patients. PHF comes from the parathyroid glands (Lewanczuk *et al.* 94b). This explains why hypertensive individuals often have high levels of circulating parathyroid hormone (PTH), which is hypotensive by itself. This would reflect the activity of the parathyroid glands, which would produce PHF concomitantly with PTH. The mechanism of action of PHF involves an increase in calcium-channel activity in vascular smooth muscle cells. The opening of these channels would lead to increase calcium entrance into these cells, resulting in increased sensitivity to other vasoconstrictors. PHF level explains why a high calcium diet may be effective in lowering blood pressure in patients who respond to calcium-channels blockers: dietary calcium might inhibit the production of PHF (and PTH), whereas calcium-channels blockers would inhibit PHF at its target site (Pang *et al.* 94a). It has also been suggested that elevated levels of 1,25-D and PHF coordinately shift intracellular cation levels and stimulate cellular calcium uptake from the extra-cellular space (Resnick *et al.* 91).

Many of the abnormalities in calcium metabolism present in low-renin hypertension have also been described in non-insulin dependent diabetes mellitus (NIDDM). A survey of human non-insulin dependent diabetes mellitus has revealed that PHF was present in a disproportionate number of these patients independently of the blood pressure level (Lewanczuk *et al.* 94a) . . Elevated PHF may be responsible for insulin resistance in a fraction of NIDDM patients and PHF may be related to serum cholesterol levels in NIDDM (Ho *et al.* 94).

3.4.4 Calcitriol (1,25-(OH)2D3)

Some authors have suggested that calcitriol has hypertensive effects and that the hypotensive effect of dietary calcium supplementation may be mediated by reductions in calcitriol levels (DiPette *et al.* 90b).Recently higher calcitiol levels associated with low calcium intake have been positively associated with weight gain and obesity, due to an increase in intracellular calcium in the adiposites. The mechanisms acting here are believed to be similar to the ones described for the vasoactive effect of the hormone (Zemel03a).

3.4.5 Parathyroid hormone (PTH)

Parathyroid hormone (PTH) has vasodilator activity that is mediated by a specific cell membrane receptor coupled to adenylate cyclase thus increasing intracellular cAMP and lowering intracellular calcium. The peptide may also block voltage-sensitive calcium channels. However, the general consensus is that PTH does not achieve sufficient levels in the serum to modulate vascular reactivity (Bukoski, Ishibashi, and Bian95b).

3.4.6 Calcitonin Gene-Related Peptide (CGRP)

Calcitonin gene-related peptide (CGRP) is a neuropeptide, produced by alternative processing of the primary transcript of the calcitonin gene, and is a potent vasodilator (Wimalawansa, Supowit, and DiPette95c). The peptide is released from perivascular nerve endings and can normally be detected in the circulation. It has been shown that dietary calcium deficiency accompanied by decreased serum ionized calcium significantly decreases the neuronal content of CGRP in the growing rat. The spontaneously hypertensive rat (SHR) is characterized by decreased serum ionized

calcium levels and is thought to most closely resemble human essential hypertension (Westlund *et al.* 91a).

Furthermore, in hypertensive patients, oral calcium supplementation increases the levels of calcitonin gene-related peptide (CGRP). The antihypertensive effects of calcium and the increased levels of CGRP in the circulation returned to baseline levels following cessation of calcium supplementation, suggesting that the effects of calcium on BP and CGRP are specific. It is proposed that the antihypertensive effect of dietary calcium supplementation, at least in part, is mediated through CGRP(Wimalawansa, Supowit, and DiPette95d).

3.4.7 Insulin (as A1c)

Several studies have demonstrated the existence of some degree of insulin resistance and/or hyperinsulinemia in patients with essential hypertension and an inverse relation between blood pressure and insulin-mediated glucose disposal (Resnick93a). An important relationship is likely to exist between alteration in intracellular calcium metabolism, insulin resistance and hypertension. There are several results suggesting that optimal levels of intracellular free calcium concentration are necessary for maximal cellular action of insulin (Resnick92d). Therefore, it could be speculated that the expected reduction in intracellular calcium induced by oral calcium supplementation could improve cellular insulin metabolism, and particularly correct insulin resistance (Sanchez *et al.* 97a).

It was not possible for us to measure insulin levels due to need of fasting before blood collection. Thus, hemoglobin A1c was measured instead, providing information that reflects serum glucose levels for the past two months.

3.5 Objectives

IV) To asses the effect of maternal oral calcium supplementation during pregnancy on offspring calcium regulating hormones (CRH), and its relation to blood pressure at 7 and 12 years of age. (Paper 1).

Specific Objectives

IV.i To asses the effect of maternal calcium supplementation during pregnancy on offspring CRH at 12 years of age

IV.ii To assess the relation between CRH and offspring blood pressure at 7 and 12 years of age

IV.iii To assess whether the relation between CRH and offspring blood pressure at 7 and 12 years of ages is different for the offspring of mother receiving calcium compared to those receiving placebo during pregnancy (effect modification).

V) To identify predictors of children's blood pressure and insulin resistance at 12 years of age, among perinatal variables and maternal and child's characteristics at follow- up (Paper 2).

Specific Objectives

V.i To assess the effect on offspring blood pressure and HbA1c of the following sets of variables:

V.i.a Maternal characteristics during pregnancy, including age, pre-pregnancy weight, weigh gain, indicator of maternal nutrition, smoking, and complications during pregnancy.

V.i.b Size at birth, including birthweight, IUGR, ponderal index, head circumference and head circumference/length ratio.

V.i.c Maternal obesity and blood pressure at offspring 12 years of age.

V.ii To assess the effect on offspring blood pressure and HbA1c of offspring obesity at 12 years of age, and to assess if obesity modifies the effects of predictors in item II.i.

V.iii To asses if calcium supplementation during pregnancy modifies the effect of predictors in items II.i. and II.ii

VI) To critically review the literature on the effect of dietary calcium during pregnancy on offspring calcium metabolism and blood pressure (Paper 3).

Analytical models

Figure 1 and 2 (see section 8) present the analytical models developed for the analysis of paper I. The long term effect of maternal calcium deficit during pregnancy on the offspring might be mediated by changes in the "Set Point" of calcium regulating hormones. Offspring exposed to maternal calcium deficit might have higher circulating levels of these vasoactive hormones. The direct vasoactive effect of the hormones will in turn increase blood pressure in the offspring. Under this hypothesis, levels of calcium regulating hormones will be significantly different between children in the calcium and placebo group. An alternative is that levels of calcium regulating hormones are similar between groups, but the pressor response for the same hormone level is different between children in the calcium and placebo group. This effect modification can be

assessed by looking at the interaction between hormone levels, blood pressure and experimental group (calcium/placebo).

Blood pressure was measured at 7 and 12 years of age, but hormone levels only at 12 years of age. The same model described above for children blood pressure at 12 years of age will be applied to blood pressure measurements at 7 years of age, assuming that hormones levels at 12 years are a proxy of hormone levels at age 7 (figure 2).

For paper II the model is presented in figure 3 (see section 8). A secondary analysis will be conducted on the database to identify perinatal and childhood factors associated with the development of high blood pressure and insulin resistance at 12 years. A similar analyses on the data of the first follow-up was conducted on children at 7 years of age (Bergel *et al.* 00a). Much more detailed information is available in the second follow-up. On one hand insulin resistance can be included as a dependent variable to identify individuals that might be early expressers of the syndrome X (obesity, hypertension and insulin resistance). On the other hand the second follow-up includes additional information on children anthropometric variables that would allow for a better identification of obesity, the main predictor of blood pressure. In summary the database provides a unique opportunity to identify predictor of high blood pressure and insulin resistance, and it relation to obesity during childhood. The potential effect modification of calcium supplementation during pregnancy on predictor effect will also be explored.

3.6 Hypotheses

III. Calcium supplementation during pregnancy induced long term changes in calcium metabolism in the offspring. These changes modulate offspring blood pressure by inducing changes in the concentration of calcium regulating

hormones with vasoactive properties, including PTH, calcitriol, CGRP and Parathyroid Hypertensive Factor, or by changing the reactivity of the target tissue to the effects of these hormones.

IV. Children blood pressure and insulin resistance at 12 years of age are predicted by perinatal variables and maternal and child's characteristics at follow- up.

3.7 Study design

The focus of this research proposal is the analyses of the data collected during the second follow-up of a cohort of children born from mothers that participated in a randomized controlled trial of calcium supplementation during pregnancy. Table 1 summarizes the study stages, including the main outcome measures and the variables that were collected in the original trial, in the first follow-up and in the second follow-up. The analysis strategy is described in section 8. See appendix I for a description of the methods of the original trial.

For the literature review the methods developed by the Cochrane collaboration will be used (see http://www.cochrane.dk/cochrane/handbook/hbook.htm, last accessed June 2004).

Table 1: Variables collected in the original randomized trial, and in the first and second follow-up, including main and secondary outcomes.

study	Subjects		Main Outcomes	Study Variables		
	Calcium	Placebo		Mother	Offspring	Father
Original randomized clinical Trial (1987-1990)	309	305	1) Pregnacy Induced Hypertension 2) Preeclampsia 3) Prematurity	***At trial entry (20 weeks):*** age, parity, previous abortions, previous cesarean, gestational age (LMP), gestational age (ultrasound), uterine high, weight, height. Laboratory exams: glicemia, Oral Glucose Tolerance Test, uric acid. Urine: proteinuria, haematuria. ***At each antenal visit:*** uterine high, gestational age, weight, blood pressure, treatment compliance (calcium), smoking, medications used, clinical symptoms, medical conditions (urinary infection, anemia, threatened preterm labor, diabetes, hemorrhage, infection, IUGR, other). Laboratory exams: Blood: hemoglobin, hematocrit, platelets, glucose, ionized calcium, total calcium, inorganic phosphate, Magnesium, Total protein, Albumin, Uric Acid. Urine: Density, proteinuria, pyuria, haematuria, calciuria, creatininuria ***At each hospital admission:*** days in hospital, gestational age, reason for admission, medication used.systolic blood pressure, diastolic blood pressure. ***At delivery:*** induction, way of delivery (vaginal-cesarean)	Fetal death, neonatal death, gestational age by LMP, gestational age by physical exam, apgar score, birthweight, sex, height, head circumference, admission to NICU, reason for admission.	
First Follow-up (1995-1996)	257	261	1) Offspring blood pressure	Age, weight, parity, previous cesareans, abortions, pregnancy induced hypertension, urolithiasis in first pregnancy, Cholelithiasis in first pregnancy, urolithiasis after first pregnancy, Cholelithiasis after first pregnancy, blood pressure systolic, blood pressure diastolic.	Age, weight, height, systolic blood pressure, diastolic blood pressure, urolithiasis, Cholelithiasis, Admission to NICU, hospital admission during childhood, reason for admission.	
Second Follow-up (2000)	275	282	1) Offspring Blood pressure. 2) Offspring Calcium Regulating Hormones. (Parathormone, paratiroid hypertensive factor, 1,25-(OH)2D, Calcitonin Gene Related Peptide).	Age, hypertension, treatment for hypertension, diabetes, treatment for diabetes, education, work, weight, height, abdominal circumference, triceps skinfold, subsapular skinfold, systolic blood pressure, diastolic blood pressure.	Birthweight, sex, breastfeeding pattern, school grade, urolithiasis, Cholelithiasis, hypertension, treatment for hypertension, Diabetes, treatment for diabetes, disease during childhood (ICD-10 code), Lives with mother, lives with father, Number of brothers, systolic blood pressure, diastolic blood pressure, weight, height, abdominal, circumference, triceps skinfold, subsapular skinfold. Laboratory exams: Serum total calcium, Serum ionic calcium, colesterol, trigliceridos, glicemia, Hemoglobin A1c, hemoglobin, hematocrit, White cells count, platelets	Age, weight, Height, hypertension, treatment for hypertension, diabetes, treatment for diabetes, education, work.

Parameters measured in the blood samples

Hormones associated with calcium metabolism that are affected by dietary calcium intake, and also have vasoactive properties

- Parathyroid hormone (PTH)
- Calcitriol (1,25-(OH)2D3)
- Calcitonin Gene-Related Peptide (CGRP)
- Parathyroid hypertensive factor (PHF)

Serum and urinary calcium, and urinary creatinine, to adjust urinary calcium

- Serum Total Calcium
- Serum Ionized calcium
- Urinary calcium
- Urinary Creatinine

Indicators of possible insulin resistance

- Glucose in urine
- Glycemia
- Hemoglobin A1

Hematological indicators

- Hemoglobin
- Hematocrit
- Leucocytes
- Platelets

Serum lipids

- Cholesterol
- Triglycerides

3.8 Data analysis

3.8.1 General analytical strategy

Children will be assigned to the group their mothers were originally randomized to, regardless of compliance and any postnatal experience (intention to treat analysis). The mean of the three blood pressure measurements will be used as the blood pressure for the analyses. The distribution of the variables will be explored using histograms. If a variable is found to deviate significantly from the normal distribution it will be transformed to attempt to correct the problem (i.e. log transformation). For regression analyses, continuous independent variables will be transformed to z-scores (mean = 0, standard deviation = 1) with the exception of child's systolic pressure. These transformations allow the comparison of regression coefficients between variables with different scale of measurement. These regression coefficients computed using z-scores can be converted to regular regression coefficients by multiplying them by the standard deviation of the independent variable. Assessment of regression models will include residual analyses (to check residual distribution, variance and outliers) and Cook's distance to detect influential observations. Sensitivity analyses will be performed if highly influential variables are found. STATA (Stata Corporation, College Station, TX, USA) and SAS (SAS Institute Inc., Cary, NC, USA). statistical packages will be used for the analyses.

3.8.2 Analytical strategy for the research question in paper I

Main objective: To assess the effect of maternal oral calcium supplementation during pregnancy on offspring calcium regulating hormones (CRH)*, and its relation to blood pressure at 7 and 12 years of age. (Paper 1).

The research question has three components. (see figures 1 and 2 for the analytical models).

iv. To assess the effect of maternal calcium supplementation during pregnancy on offspring CRH

Analyses: linear regression with each of the calcium regulating hormones as the dependent variable (one at a time) and a dichotomous indicator variable (Calcium- Placebo) as the independent variable. This analysis is equivalent to a t-test. If a significant deviation from the normal distribution is found in any of the CRH, the calcium and placebo group will be compared using non-parametric tests. A multiple regression model including strong predictors of children blood pressure (i.e. current weight, sex, age, maternal blood pressure during pregnancy and at follow-up) will be reported if the inclusion of these additional variables increases the precision of the estimates.

v. To assess the relation between CRH and offspring blood pressure at 7 and 12 years of age.

vi. To asses whether the relation between CRH and offspring blood pressure at 7 and 12 years of ages is different for the offspring of mother receiving calcium or placebo during pregnancy (Effect modification).

Analyses: Multiple linear regression with offspring blood pressure as the dependent variable and the CRH as independent variables. The model will include other strong predictors of blood pressure (i.e. current weight, sex, age, maternal blood pressure during pregnancy, catch-up growth and at follow-up) if their inclusion increase the precision of the

estimates. A dichotomous treatment indicator variable (Calcium-Placebo) will also be included as an independent variable.

To explore question (iii) the model will include the interaction between CRH and treatment (Calcium- Placebo). The interaction will be considered statistically significant if the p-value is less than 0.10, and is also considered that the magnitude of the interaction is clinically significant. Clinically significant qualitative interaction will also be reported (i.e. positive vs. negative effect, no effect vs strong effect), even if the test for interaction is not statistically significant.

3.8.3 Analytical strategy for the research question in paper II

Main objective: to identify predictors of children's blood pressure and insulin resistance at 12 years of age, among perinatal variables and maternal and child characteristics at follow- up (Paper 2). See figures 3 for the analytical model, and the list of variable to be included in the analyses. Catch-up growth will also be included in the model.

Analyses: Multiple linear regressions with offspring blood pressure and HbA1c as the dependent variable and potential predictors as independent variables. Interactions with current offspring obesity and calcium supplementation during pregnancy will be explored.

Figure 1. Analytical model for the mechanisms of action of calcium supplementation on children blood pressure at 12 year of age.

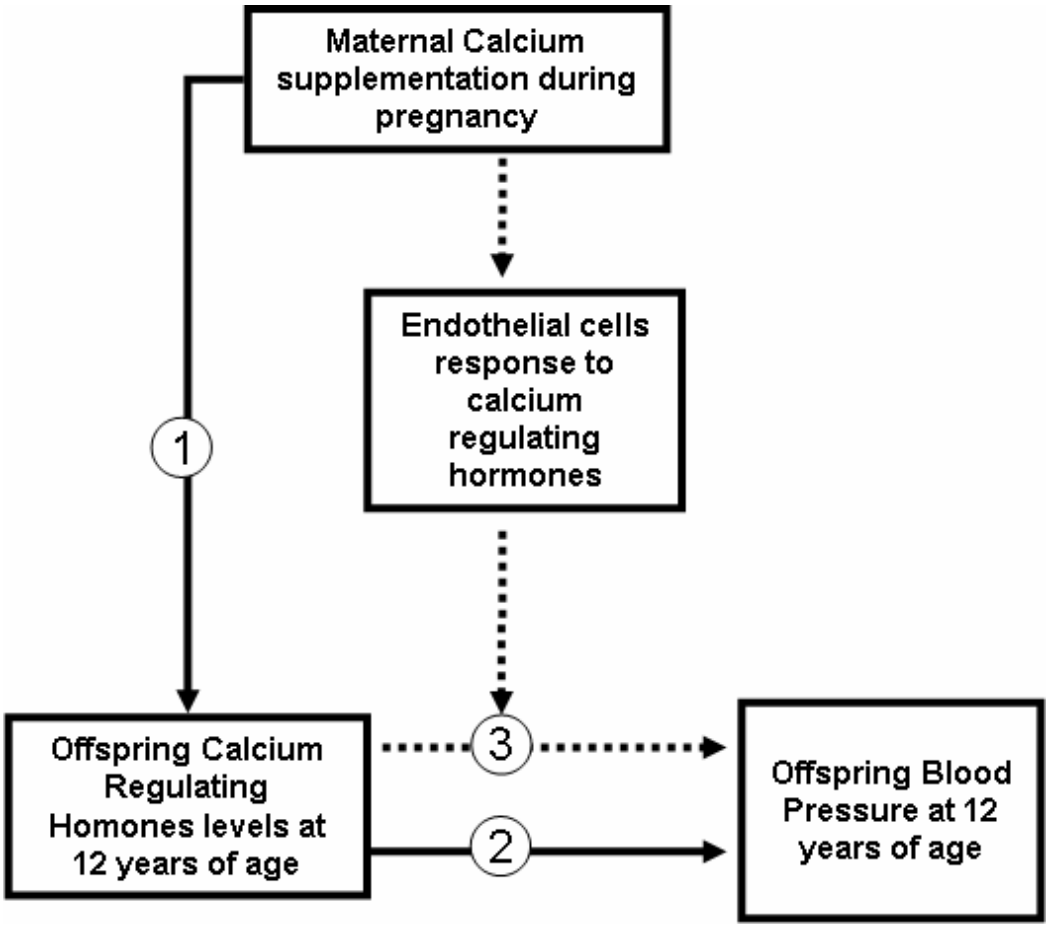


Figure 2. Analytical model for the mechanisms of action of calcium supplementation on children blood pressure at 7 year of age.

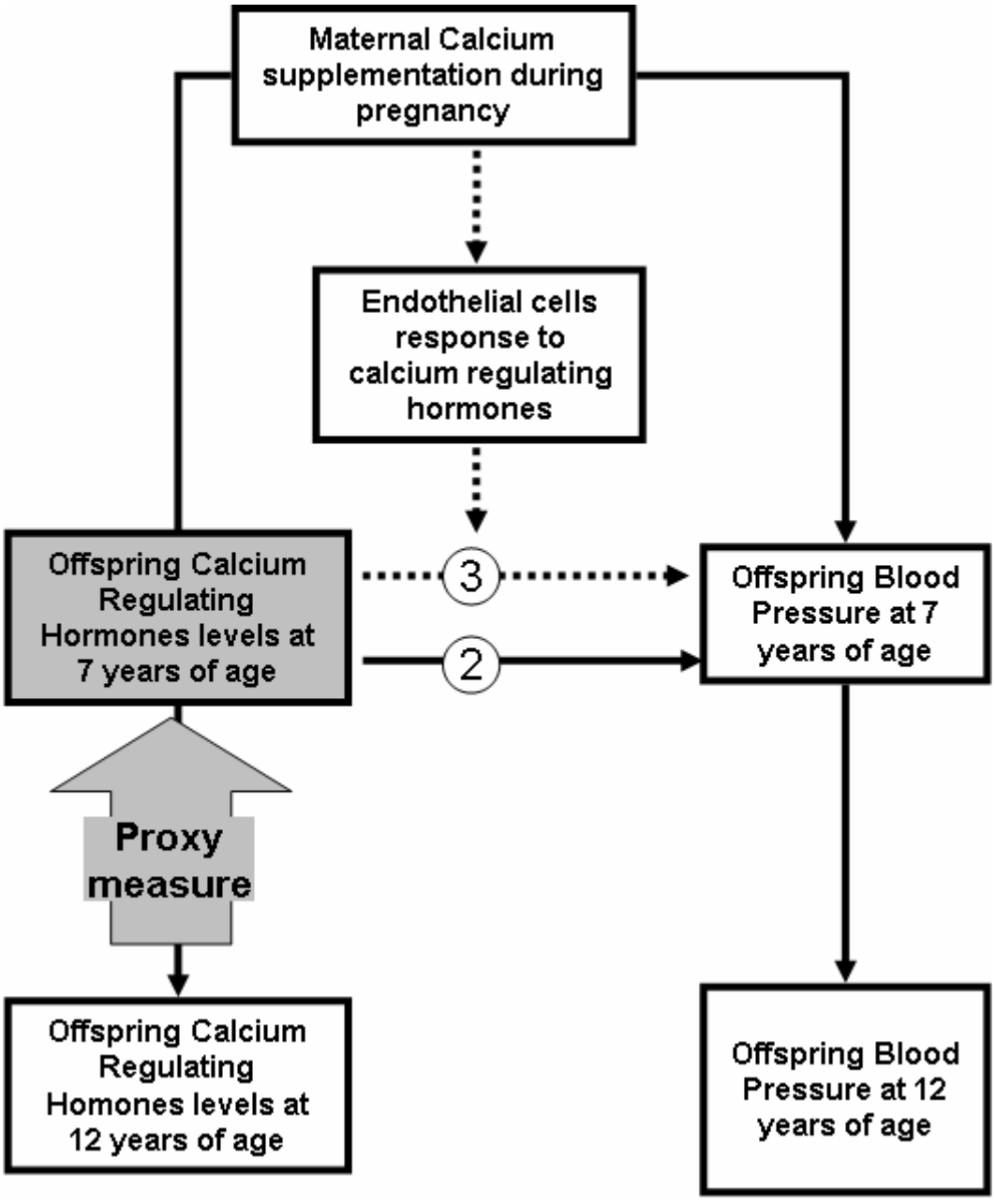
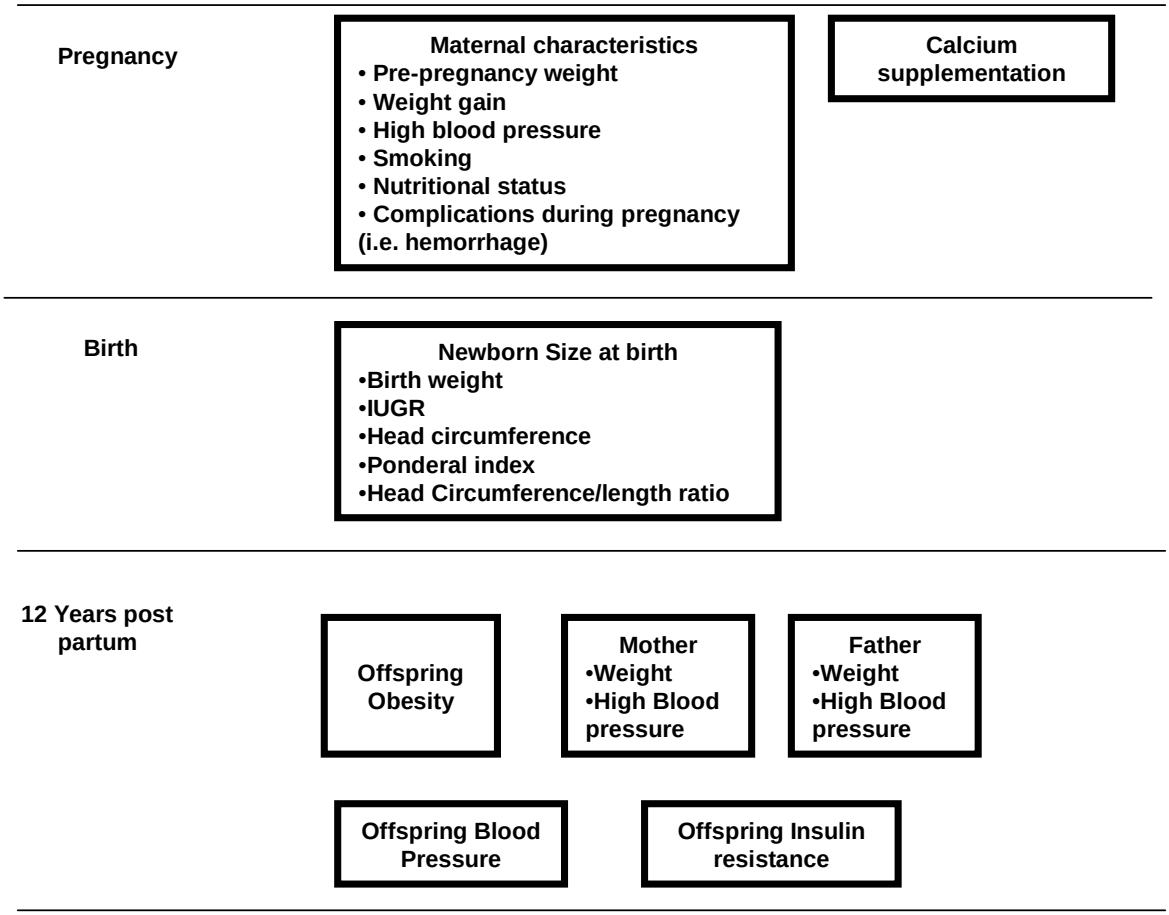
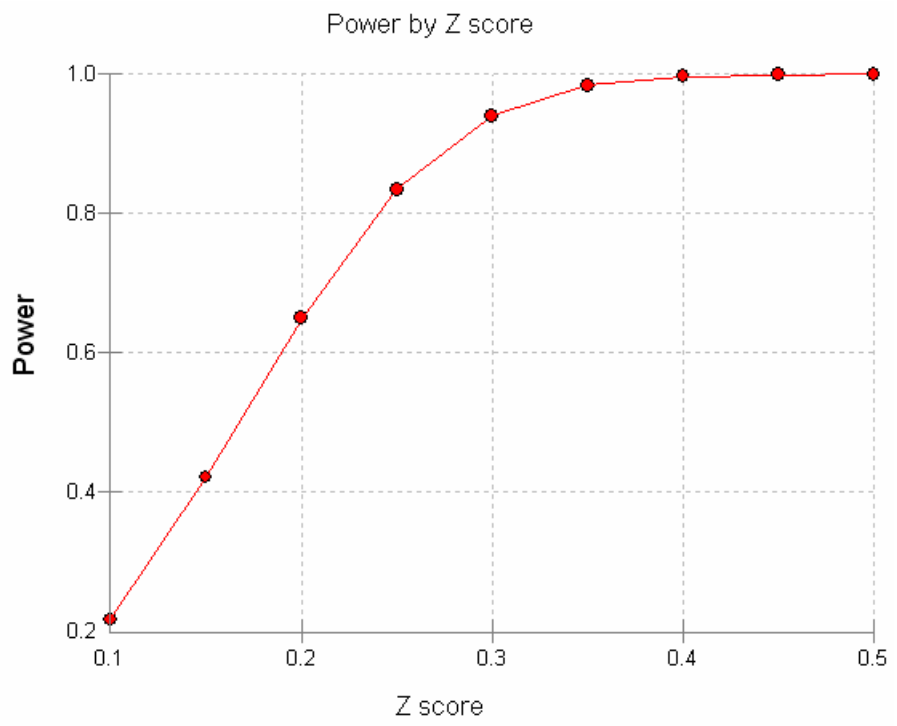


Figure 3. Analytical model for predictors of children blood pressure at 12 year of age.



3.8.4 Sample size and power

A main limitation to estimate the power of this study is the difficulty to determine a difference in outcome mean that is clinically significant. Main outcomes are calcium regulating hormones with different distributions and standard deviations. For example PHF is only used in experimental settings and the reference values in children have not been established. To have an idea of the power of the study a power analyses is presented by plotting power vs zscore. Calculations are presented in the Table and Figure below.



Power	N1	N2	Alpha	Mean1	Mean2	S1	S2
0.21709	275	275	0.05	0	0.10	1	1
0.42092	275	275	0.05	0	0.20	1	1
0.65006	275	275	0.05	0	0.25	1	1
0.83416	275	275	0.05	0	0.30	1	1
0.94015	275	275	0.05	0	0.35	1	1
0.98387	275	275	0.05	0	0.40	1	1
0.9968	275	275	0.05	0	0.45	1	1
0.99954	275	275	0.05	0	0.50	1	1
0.99995	275	275	0.05	0	0.55	1	1

The study can detect with 80 % power a shift of 0.25 standard deviations between the control and calcium group. As an example, the standard deviation for children systolic blood pressure is 12 mmHg, so the study can detect a difference of (12*0.25) 3 mmHg between groups. A power analysis was included in the protocol.

3.9 Implications

The development of a theoretical framework and the subsequent challenge of the theories against controlled experiments are key to establish causality. If successful, this research might provide evidence to better understand aspects of the underlying mechanisms that lead to the development of the hypertensive disease in humans. This knowledge might be used to develop preventive or treatment strategies for hypertension.

3.10 limitations

There are reports suggesting that the effect of calcium supplementation during pregnancy is present only in population with a low calcium intake (Hofmayer et al. 2003). This issue is very important, and a large multicenter randomized trial sponsored by WHO is being conducted to assess the effect of calcium supplementation during pregnancy in populations with low calcium intake. A study in the US has shown that the intervention does not reduce pre-eclampsia in mother with adequate calcium consumption. Furthermore it has been shown in an animal model that the effect on the offspring is observed only if mothers are in a low calcium diet, compared to a normal diet (Bergel and Belizan, 2002). We have some limitations to explore these ideas in this dataset. We do not have in the dataset information on calcium consumption. Furthermore, although the original study included two rather distinct populations, likely to have differences in calcium consumption (public and private hospitals), only women from the private hospitals were available for follow-up. We do have data on urinary calcium excretion in the mothers, which is correlated with dietary calcium intake. A secondary analyses can be undertaken to see if there is an effect associated between maternal calcium excretion (proxy for maternal calcium consumption), and offspring outcomes.

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4 RELATÓRIO DO TRABALHO DE CAMPO

Universidade Federal de Pelotas
Programa de Pós-Graduação em Epidemiologia
Doutorado em Epidemiologia

O EFEITO DA SUPLEMENTAÇÃO DE CÁLCIO DURANTE A GRAVIDEZ NOS MARCADORES BIOQUÍMICOS DE PRESSÃO ARTERIAL EM CRIANÇAS DE 13 ANOS (SEGUIMENTO DE UM ENSAIO CLÍNICO RANDOMIZADO)

.

Relatório do trabalho de campo

Eduardo Bergel

ORIENTADOR: Aluisio J D Barros

PELOTAS-RS-BRASIL
2006

4.1 Introduction

The study was conducted at the Centro Rosarino de Estudios Perinatales in Rosario, Argentina (CREP) from March 2000 to December 2000. The study was funded by The Nestle Foundation.

The main challenge for this study was to locate women and their offspring 13 years after birth of the index child. An aggressive follow-up strategy was implemented and more than 90% of the eligible original cohort was found and contacted. The study included a detailed biochemical evaluation, and children blood samples were required. A multidisciplinary advisory team was invited to our centre in to discuss children and parents' reaction for blood sample withdrawal and to suggest strategies to improve compliance. A blood sample was obtained in more than 70 % of the sample.

4.2 Search strategy for locating and contacting women and children.

A database with all contact information from patients of the original calcium supplementation trial and the first calcium follow-up trial was an important tool for locating and contacting women. The existing database at CREP created for the first follow-up contained detailed subjects contact information. That information included mother's name and surname, address, telephone number, mother's identity card number, and name, surname and children's birth date. A computer program was developed to manage the follow-up procedures. The program contained all available information on the study subjects, and contained a module to gather information during phone calls and schedule phone visits (see appendix). The program was able to generate a detailed report of the status of the follow-up for all subjects in the cohort (i.e. follow-up

completed, visit scheduled, contact information missing, etc). To update the contacts database telephone calls were conducted to all women, to relatives and neighbors registered in our files. Letter with an invitation to participate in the study were sent to all families using a private courier. This courier returned letter that could not be delivered and this information was used as indication of an invalid address. For women that could not be located with the previous strategy, a computerized address index was used (DATEL 99, Datel S.A. 1999, Argentina). This program is a database listing the address and telephone numbers for all subjects with a telephone line in the country. Hospital files where mothers delivered their babies were also consulted to search for phone numbers and addresses. With the information recovered by all these sources, a new updated database was created. If a successful contact was made, an appointment for a home visit was made. For women without a valid phone number a visit to the neighborhood of the last known address was conducted. I the women could no be found, neighbors were contacted for information related to the current woman's address. This strategy was very effective for locating missing subjects as we located more than 90% of the subjects in original cohort.

4.3 Strategies to improve patient participation.

Pediatricians, a pediatric psychiatrist, mothers of teenage children, social communicators and teachers were invited to our centre in order to discuss children and parents' reaction for a blood sample withdrawal and to suggest strategies to improve compliance. During several meetings some strategies were proposed in order to improve the recruitment rate.

Based on these recommendations information brochures were developed for parents, children and their pediatrician (see appendix). A mug with the logo of the trial was given as a gift to all participating children in acknowledgement for their contribution to the trial, and was very well received.

4.4 Activities at home visits.

Three research nurses and a medical student were responsible for completing home visits, which included filling the study forms, measuring blood pressure and obtaining anthropometric indicators for mothers and children and blood samples. It was decided that specialized personnel were responsible for obtaining blood samples and these personnel was selected because they had experience in obtaining blood samples in children. Examiners were blinded to the assignment group (calcium or placebo).

Blood pressure measurements were taken on the right arm, after a 15-minute rest, with the child seated in a quiet room at the home visits. No measurements were taken if the child was sick or under drug treatment, leaving this procedure to be done at a follow-up visit. An OMRON HEM 705-CP sphygmomanometer was chosen as the equipment to be used in this trial for blood pressure measurements, as it is the only model supported by the British Hypertension Society. Since there was no local provider for this device they were purchased in USA.

4.5 Blood samples and laboratory determinations.

A concern during the follow-up was to obtain blood samples from as many subjects as possible. In order to achieve this objective we offered mothers a wide time-band to schedule home visits. Because we wanted to process sample within 3 hours from withdrawn, we hired two biochemists, with cell phones, that were responsible for samples processing from 6 AM to 11 PM, 7 days a week. This was particularly important because for many families with very busy schedules, the only time available for the interview and to obtain blood samples was late in the evening or during holidays.

Blood was withdrawn by vein punction and collected in a vacuottainer. Samples were sent following standardized procedures for sample transportation within four hours of sample extraction to the laboratory. Samples were centrifuged at 3,500 revolutions per minute during 10 minutes. Serum and plasma (with heparin) samples were obtained. Two hours after the withdrawal, glycaemia, cholesterol and triglycerides were determined. For urine analysis, a midstream urine sample of the child's first morning micturition was collected in a sterile collector. Urine samples were stored at a conventional refrigerator (4 centigrades) for no more than 12 hours before processing.

Samples for the remaining biological markers were stored at -190 centigrade in a liquid nitrogen dewar. In Rosario City power outages are a common problem. A nitrogen dewar does require electricity supply and is immune to power outage.

Biochemical determinations were performed at a research clinical laboratory at the Pharmacology and Biochemistry Faculty, Rosario National University, Rosario, Argentina, with the exception of PHF, 1,25-dihydroxyvitamin D3 and CGRP. There is no commercial laboratory kit for measuring PHF, and the analysis were carry out by the Division of Endocrinology and Metabolism, University of Alberta, Edmonton, Canada. 1,25-dihydroxyvitamin D3 and CGRP were measured at a specialized laboratory at the

Institute of Nutrition in Mexico. Samples were sent to Mexico and Canada using a courier specialized in shipping biological samples.

4.6 Data management

A computer program for data entry and data management was developed at the centre. All study forms were double entered in this database. Once the forms arrived at CREP a visual check was performed by the data-manager to missing data or inconsistencies, both in the individual form and between different forms from the same subject. If errors were found the forms were given back to the field supervisor for correction. A data manager was also in charge of the codification of all the qualitative variables using standardized procedures. She used the International Statistical Classification of Diseases and Related Health Problems (ICD-10), 10th Revision for disease classification and for treatments and interventions, the International Classification of Procedures in Medicine, Vol. 1 and 2, World Health Organisation, Geneva, 1978.

After coding, batches of five forms each were made for data entry. Two independent data clerks double entered all data in the database. A program for data validation prepared at the Centre checked double data entrance consistency. A list of errors was produced after running this program, and the resulting queries were solved going back to the original form. When necessary the field supervisor contacted the woman again for missing or inconsistent data. A similar procedure was used for the laboratory results.

5 ARTIGO 1

Effect of maternal calcium intake during pregnancy on children's blood pressure: A systematic review of the literature

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Abstract

Background

Calcium supplementation during pregnancy has been shown to reduce the incidence of hypertension in the mother, but the effects on the offspring are uncertain. Assessing the impact on the offspring is very important given the now large body of evidence indicating that blood pressure levels in childhood and young adulthood can be influenced by factors operating during fetal life. We conducted a systematic review of the literature to summarize the evidence supporting an association between maternal dietary calcium intake during pregnancy and blood pressure in the offspring.

Methods

A systematic review was performed to identify randomized, quasi-randomized and cohort studies reporting the relationship between offspring blood pressure or incidence of hypertension and levels of maternal dietary calcium intake during pregnancy, either from supplements (i.e. pills) or food. MEDLINE, EMBASE and the Cochrane Library Registry were searched for relevant trials.

Results

Two randomized trial and three observational studies were identified and included in this review. In 4 of the 5 studies, loss to follow-up was a serious concern. There was heterogeneity between the studies, particularly those conducted on children below 12 month of age. Results were more consistent among the studies including older children (1 to 9 years) where a higher maternal calcium intake was associated with a reduction of -1.92 mm Hg (95% CI -3.14 to -0.71) in offspring systolic blood pressure. One large randomized trial found a clinically and statistically significant reduction in the incidence of hypertension in 7-year-old children (RR = 0.59, 95% CI 0.39 to 0.90).

Conclusions

There is evidence in the literature to support an association between maternal calcium intake during pregnancy and offspring blood pressure. However, more research is needed to confirm these finding given the small sample sizes and the methodological problems in many of the studies conducted so far. More studies on populations with calcium deficit are also needed. If confirmed, these findings could have important public health implications. Calcium supplementation during

pregnancy is simple and inexpensive and may be a way to reduce the risk of hypertension and its sequels in the next generation.

Background

Increased dietary calcium intake has been associated with lower blood pressure among children, adults and pregnant women [1, 2]. The effect seems to be more evident among individuals with low calcium intake [3-6]. Some recent experimental and observational studies in humans and animals have reported an association between maternal calcium intake during pregnancy and blood pressure in the offspring [5, 6], but others have not [1, 7]. These findings follow a large body of evidence indicating that blood pressure levels in childhood and young adulthood are influenced by factors operating early in life [7-10] and are associated with later cardiovascular disease and mortality [11]. We conducted a systematic review and meta-analysis of studies reporting blood pressure levels in offspring of mothers who were either enrolled in a trial of calcium supplementation during pregnancy or included in a study on maternal calcium intake during the index pregnancy.

Methods

Types of studies

This review includes randomized and quasi-randomized controlled trials. We pre-specified that if the evidence from randomized trials was insufficient (i.e. small trials, trials of bad quality, etc.) to assess the effect of the intervention, then data from observational studies (e.g. cohort studies) would be considered for inclusion. Studies with historical controls and ecological studies were excluded, as the data provided by these are unreliable for determining causation and/or association.

Studies should provide an estimate of the incidence of hypertension, or the mean difference in offspring blood pressure between levels of maternal calcium dietary intake during pregnancy, or should enable this information to be computed from data extracted from the article.

Types of participants

Offspring of mothers included in studies assessing the association between calcium intake during pregnancy and offspring blood pressure.

Types of intervention or exposure

Maternal dietary calcium intake during pregnancy, from supplements (i.e. pills) or food.

Types of outcome measures

Offspring diastolic and systolic blood pressure in mm Hg; incidence of hypertension.

Search strategy for identification of studies

1. MEDLINE and EMBASE (1966 to 2005) were searched in December 2005 using the following search strategy

#1	Search calcium[Title]	84110
#2	Search pregnan*	568541
#3	Search "blood pressure"	247471
#4	Search hyperten*	241702
#5	Search #1 AND #2 AND (#3 OR #4)	253

2. All databases included in The Cochrane Library (issue 4, 2005) were searched with a search strategy equivalent to the Medline strategy.

3. Reference lists of all the studies that went into the pool of retrieved studies, including those of other reviews, were examined to identify any further studies. We did not implement a standardized strategy to find unpublished studies.

Selection of studies and data extraction

The titles, abstracts and descriptor terms of all material downloaded from the electronic searches were read and irrelevant reports were discarded. All citations identified were then inspected to establish the relevance of the article according to the inclusion criteria. Where there was uncertainty about relevance, the full article was obtained. Studies were reviewed for relevance on the basis of study design, types of participants, exposures and outcome measures. Standardized data extraction forms were used, one for clinical trials and one for cohort/cross-sectional studies. The following characteristics were extracted from each study included:

a) Administrative details: identification; author(s); published or unpublished; year of publication; year in which study was conducted; details of other relevant papers cited.

b) Details of study: study design; method(s) of recruitment; inclusion and exclusion criteria; number of participants assessed for eligibility, number excluded, number enrolled, number analyzed; type, duration, frequency and completeness of follow-up in the case of cohort studies; country and location of the study.

c) Characteristics of participants: age; location; details of intervention.

d) Crude and adjusted measures of effect, confidence intervals and p-values were extracted. When an adjusted analysis was performed, type of analysis and the list of covariates adjusted for were recorded.

e) The amount of maternal calcium intake was extracted and reported as the amount of elemental calcium in mg.

Details of analysis

One study [12] did not report regression coefficients, but reported the correlation coefficient between maternal calcium intake and offspring blood pressure. Standard errors for the correlation coefficients were computed using the Fisher r-to-z transformation [13]. The correlation coefficients were transformed to standardized mean differences and multiplied by blood pressure standard deviation to estimate the effect [14].

The meta-analysis was conducted using the Revman computer software package [15]. The amount of heterogeneity between studies was assessed by a formal statistical test (chi-square test), and by I2 [16]. The chi-squared test assesses whether observed differences between results are compatible with chance alone [17]. A low p-value provides evidence of heterogeneity of treatment effects [17]. The results of the chi-squared test might be misleading because it has low power for trials with small sample size, and I2 has been proposed as an additional tool for assessing heterogeneity [16]. I2 describes the percentage variability in effect estimates that is due to heterogeneity rather than sampling error (chance) and is not affected by the number of trials in the meta-analysis [17]. A value greater than 50% may be considered substantial heterogeneity. Mathematically, it is defined as $I2= [(Q - df)/Q] \times 100\%$, where Q is the chi-squared statistic and df is the number of degrees of freedom [18, 19].

Quality assessment

Quality of randomized controlled trials was assessed following the method described in the Cochrane Collaboration Handbook [17]. For observational studies, quality was assessed following the recommendations of the Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines [20].

Results

Two randomized trials [21, 22] and three observational studies [12, 23, 24] were included in the review. Three studies included infants less than one year of age [12, 22, 23] and four included children between 1 year and 9 years of age [12, 21, 22, 24]. Table 1 describes the characteristics of the two randomized trials. Both studies were long-term follow-ups of randomized trials of calcium supplementation during pregnancy to prevent pre-eclampsia. In both studies the randomization procedure was adequate, subject and health professionals were blinded regarding calcium or placebo status, and baseline characteristics were similar between study arms. Belizan et al. [25] randomized 1194 women during early pregnancy to either 2000 grams of oral calcium supplementation or placebo. This study included only healthy primiparous women. Compliance with calcium supplementation was acceptable (more than 80%), and increased calcium intake in the intervention group was confirmed by measurements of urinary calcium excretion. A follow-up of this experimental cohort was conducted 7 years after the original trial [21]. The original study was conducted in three hospitals, two public and one private, but the follow-up only included the 614 participants from the private hospital (approximately 50% of the original sample). Randomization was stratified by center, and the baseline characteristics of those included in the follow-up were comparable between the trial arms. However, women excluded from the follow-up were younger and had lower socioeconomic status than those included. Loss to follow-up (16%) was acceptable for a long-term follow-up. This study used cut-off points for systolic and diastolic pressure, specific for sex, age and height, that corresponded to the 95th centiles given in tables developed by the US National Institutes of Health.

Hatton et al. [22] randomized 4,589 women during early pregnancy to either 2000 grams of oral calcium supplementation or placebo. A detailed clinical and laboratory evaluation was conducted before trial entry, and women with complications during pregnancy or signs or history of calcium metabolism disorders were excluded. Furthermore, a baseline compliance test was used to exclude women with low compliance before trial entry. Two follow-up studies were conducted at 3 and 24 months post-partum. Patients from only one out of five medical centers were included in the follow-up (559 out 4589 subjects). Randomization was stratified by center, but no data are available on the baseline characteristics of the follow-up sample. Loss to follow-up was 53% at 3 months and 90% at 2 years. The authors acknowledge this to be a problem, adding that that a large proportion of the cohort had not reached two years of age by the end of the study. This is the main methodological limitation of this

study. Sample size is also an issue, given the small number remaining for analysis. Calcium intake from other sources (i.e. prenatal supplements, diet) in the population from Belizan et al. [25] was estimated at 600 mg per day, well below the recommended level during pregnancy. In contrast, the reported calcium intake for the population in Hatton et al. [22] was over 1200 mg per day, within recommended levels. In summary, the two randomized trials included [21,22] were similar in respect of the characteristics of the intervention, study design and inclusion criteria, but differed in an important population characteristic (baseline calcium intake); also, loss to follow-up was a problem in one study [22].

Table 2 describes the characteristics of the three observational studies included in the review [12, 23, 24]. McGarvey et al. [12] designed their study to explore the association between infant blood pressure and maternal dietary intake of calcium, potassium and magnesium. Data on maternal prenatal diet were obtained by conducting a post-partum 116-item semi-quantitative food frequency questionnaire (FFQ). The authors measured the offspring blood pressure in hospital when the babies were 2-4 days old, and at home at 1, 6 and 12 months.

Gillman et al. [23] used the data from a cohort study of pregnant women conducted in the US. This study was designed to assess the effects of mother's diet on mother's and offspring's health. It assessed maternal calcium intake during the first and second trimesters using a validated semi-quantitative FFQ, and measured offspring blood pressure at birth and at 6 months. The authors reported figures for calcium from food sources and from prenatal supplements, and then performed two independent analyses accordingly. This seems to have been a non-pre-specified analysis. The strength of the evidence for this analysis is not clear because it was not stated whether this analysis strategy was pre-specified [26].

Morley et al. [24] used the data from a population-based survey in Tasmania designed to investigate sudden infant death syndrome. Mothers of all live-born twins during the study period were approached after birth for data collection, including nutritional supplement consumption during pregnancy. Data on calcium consumption from other sources (i.e. foods) were not available. Children were assessed at a mean age of 9 years and blood pressure was measured.

All three studies were conducted in developed countries, and average calcium intake was higher than the recommended calcium intake during pregnancy for the two studies with quantitative estimates [12, 23].

It is not clear whether outcome assessment in any of the three observational studies was blinded to levels of exposure. Loss to follow-up was large (see table 2 for details). One of the studies [12] conducted follow-ups at 4 time points, but the

sample size decreased for older infants. Less than 40% of the sample was available in the last follow-up at 1 year of age. The authors reported that one of the primary reasons for the decrease in sample size with age was that not all infants had reached 1 year by the end of the study.

All three observational studies attempted to adjust for confounding variables, but they differed markedly in the set of variables included . Two of them adjusted for blood pressure measurement conditions [12, 23]. All three adjusted for socioeconomic factors (e.g. maternal education) and child age at assessment. One [12] reported estimates from more than one model, including one adjusted for dietary potassium and magnesium. Crude and adjusted estimates were similar in all three studies, suggesting that confounding was not a problem in exploring this association.

A funnel plot was produced to assess publication bias [27]. Because of the small number of studies, the figure was not very informative and was not included in this report. Publication bias cannot be excluded.

In summary, all five studies seem to have been well conducted. The main limitation in all the observational studies was loss to follow-up. For two of these studies, small sample size was also a problem. In one study, the focus of the analysis was a sub-group analysis.

Higher maternal calcium intake during pregnancy was associated with lower offspring systolic blood pressure in all studies, but the effect was statistically significant in only 3 of them [12, 22, 23] (see figure 1). There was heterogeneity between studies ($I^2 > 50\%$); this was large for studies conducted on children below 12 months of age ($I^2 = 53\%$), and small for studies on older children ($I^2 < 10\%$). Because of this heterogeneity, the analysis was stratified according to age, and a summary measure (meta-analysis) was obtained only for studies that reported on children aged 1 year or more. The results of this analysis are compatible with a reduction in SBP in the calcium group of -1.92 mm Hg (95% CI -3.14 to -0.71) (see figure 1).

In the largest randomized trial [21] there was a modest, statistically insignificant effect on systolic blood pressure, but a clinically and statistically significant effect on the incidence of high systolic blood pressure at 7 years of age. This study also reported that the effect is stronger among overweight children; this was not observed by others, though the sample sizes were too small to exclude such a difference.

For children under 12 months, the data are less consistent (see table 4). In the same cohort, one study [12] found no effect at birth, a strong effect at 1 month and a moderate effect at 6 months. Another [23] reported that no effect was associated with calcium from foods, but a strong effect was found in association with (prenatal)

calcium supplements. This analysis strategy did not seem to be pre-specified, and the authors failed to provide a convincing hypothesis to account for the finding. Finally, the follow-up at 3 months conducted by Hatton et al. [22] was too small to draw any conclusion.

Discussion

This systematic review of the literature identified two randomized trials and three observational studies. The evidence provided by this body of research suggests an association between dietary calcium intake during pregnancy and offspring blood pressure. A good quality randomized trial found a large reduction in the incidence of hypertension in children at 7 years of age [21]. However, the same trial found a smaller effect on blood pressure as a continuous variable. A possible explanation for these findings is that the intervention produced a change in the shape of the blood pressure distribution, as opposed to a shift in mean blood pressure.

A meta-analysis that combined four studies on children over one year of age found a reduction in mean systolic blood pressure. This finding should be viewed with caution, since evidence obtained by combining small studies has previously been shown to be unreliable because of publication bias [28]. For infants under one year of age the evidence is contradictory and difficult to summarize.

Among the 5 studies reported, only two were randomized trials. The validity of the evidence from observational studies for assessing the effect of interventions is controversial [17]. The two randomized trials included in the review were multi-center trials and the randomization was stratified by center. The authors chose to follow up subjects from only one center. It can be assumed that because the randomization was stratified by center, the effect estimate will not be biased. However, an impact on external validity can be expected. For example, in one study, participants from the selected hospital were of higher socioeconomic status than those from the centers not included in the follow-up [21]. Apart for the methodological problems of the original articles, other limitations of this analysis should be pointed out. Four out of the five studies included in the review were conducted in developed countries, and on populations in which maternal calcium intake was adequate or even higher than the recommended levels during pregnancy. This is clearly not the ideal target for a nutritional intervention. Given the evidence that the effect of calcium might be apparent only when there is a deficit, the external validity of these results might be compromised [5].

The heterogeneity between studies also creates difficulties for interpreting the results. The sources and dose of dietary calcium vary widely among the

observational studies, and so do the methods used to assess the amount consumed. There are also large differences between studies in infants' ages at assessment. It is well known that the determinant of blood pressure varies with age, and it has been shown that the impact of factors affecting the fetal environment are seen particularly after adolescence [29]. This problem is magnified because of the difficulties in measuring blood pressure accurately at early ages [30].

Conclusions

In summary, there is evidence in the literature to support an association between maternal calcium intake during pregnancy and offspring blood pressure. However, more research is needed to confirm these findings, given the small sample sizes and the methodological problems of many of the studies conducted so far. New evidence should be derived from the long-term follow-up of large and well-conducted randomized trials of calcium supplementation during pregnancy. More studies on populations with calcium intake deficit are also needed. Assessing the effect of the intervention on other cardiovascular risk factors would also be an asset for future research. Calcium supplementation during pregnancy is simple and inexpensive, and if these findings are confirmed it could be a way to prevent hypertension and its sequels in the next generation.

Competing interests

One of the authors (EB) authored one of the reports included in the systematic review [21].

Authors' contributions

EB was the principal author responsible for the conception and design of the systematic review, screening of citations, data auditing, analysis and interpretation, and drafting and editing of the manuscript. AB drafted and edited the manuscript and made major contributions to the interpretation of the evidence and manuscript revisions. Both authors read and approved the final manuscript.

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Figure legends

Figure 1. Maternal calcium supplementation during pregnancy and offspring systolic blood pressure, by offspring age at follow-up.

Key: Calcium source: F from food, S from supplements. RCT, Randomized Controlled Trial. Lines are 95% confidence intervals. Boxes are proportional to the sample size of the trial.

Tables

Table 1. Characteristics of randomized controlled trials included in the review.

	Belizan, 1997	Hatton, 2003
Methods		
Randomization	Numbered, sealed opaque envelopes, containing randomization codes.	Numbered treatment packs in computer-generated simple randomization sequence.
Lost to follow-up - women	Of 593 (calcium) vs. 601 (placebo) enrolled, 14 vs. 13 were lost before starting treatment and excluded from analysis; 577 vs. 588 had at least partial follow up. Follow up was incomplete for 52 vs. 46, but delivery data were available in 17 vs. 12 of these, giving delivery data for 544 vs. 554.	calcium 132/2,295 vs. placebo 121/2,294.
Lost to follow-up - offspring	Of 614 randomized in one center (calcium 309/placebo 305), 301/299 completed the first study, 2/6 infant deaths and 1/0 maternal deaths had occurred, leaving 298/293 eligible for follow up. 289/285 were contacted, 10/5 refused to participate, 22/19 lived outside the country, and 257/261 were assessed.	559 randomized in study site. Of 497 invited to participate in the follow-up study, BP was measured in 260 infants at 3 months of age and 57 toddlers at 2 years of age.
Participants		
Mothers	Nulliparous women, < 20 weeks pregnant; blood pressure < 140/90 mmHg (mean of 5 measurements); no present or past disease; not taking medication; normal oral glucose tolerance tests.	Pregnant nulliparas (45% black, 35% non-Hispanic white, 17% Hispanic white). Passed compliance test (took 75% of placebo over 6-14 days); BP 134/84 mmHg or less; urine protein dipstick negative or trace; 13-21 weeks pregnant. Exclusion criteria: taking medications; obstetric or pre-existing diseases or personal characteristics which could influence study end-points, absorption or metabolism of calcium; any risk associated with calcium supplementation, or compliance; elevated serum creatinine (1.0 mg per decilitre or more) or calcium (10.6 mg per decilitre or more); renal disease; haematuria; history or family history of urolithiasis; frequent use of calcium supplements or antacids. Of 11,959 women screened, 5,703 excluded initially and a further 1,667 after the compliance test. The remaining 4,589 women were enrolled.
Offspring	Two public and one private hospital participated in the original trial, but only babies born in the private hospital were included in the follow-up study at 7 years of age.	Five participating medical centers participated in the original trial, but only babies born in one center (Oregon) were included in the follow-up study. Additional criteria for inclusion: mothers who completed the original trial, delivered an infant without serious problems, and who read english. Follow-up was completed at 12 week postpartum and at 2 years of age.
Interventions	2 g calcium as 500 mg calcium carbonate tablets, vs. identical looking placebo tablets. Compliance was 84% (calcium) and 86% (placebo).	2 g/day elemental calcium as calcium carbonate, or placebo. Taken until delivery, development of pre-eclampsia or suspicion of urolithiasis. All women took 50 mg calcium per day as normal supplementation and were asked to drink 6 glasses of water per day. Compliance was 64% in the calcium group and 67% in the placebo group. 20% of women took > 90% of the allocated treatment.
Outcomes	Systolic blood pressure, diastolic blood pressure, kidney stones, gall stones, hospital admissions	Systolic blood pressure, left ventricular wall mass. Diastolic blood pressure was measured at 2 years but group means were not reported.
Allocation Concealment	A	A

Table 2. Characteristics of observational studies included in the review.

	McGarvey, 1990	Gillman, 2004	Morley, 2004
Country, region	Rhode Island, USA	Massachusetts, USA	Tasmania, Australia
Age at Follow-up	At birth - 1 month - 6 month - 12 month	6 month	9 years
Sample size	212 - 184 - 114 - 70	936	294
Outcome measures	Systolic and diastolic blood pressure	Systolic blood pressure	Systolic and diastolic blood pressure, insulin, fasting glucose, triacylglicerol, cholesterol (total, HDL and LDL)
Average maternal calcium intake during pregnancy (mg/day)	1712	Total:1494 first trimester (FT), 1330 second trimester (ST). From prenatal supplements: 264 (FT), 203 (ST). From food: 1230 (FT), 1128 (ST).	NA
Exposure measurement	Maternal prenatal diet assessed postpartum in hospital by a 116 item semi quantitative food frequency questionnaire.	Maternal diet assessment with a semi quantitative food frequency questionnaire validated in pregnancy during the first trimester and second trimester. Analysis stratified by calcium from food and calcium from prenatal supplements.	Mothers of all live born twins in Tasmania were approached soon after birth. They were asked whether they had taken any nutritional supplements during pregnancy.
Exposure categories, maternal calcium intake in mg	Quartiles (1380, 1722, 2048) and correlation coefficients	Quartiles (Q4-Q1= 424 mg) and linear regression	Maternal calcium supplements during pregnancy (yes/no). Calcium content of supplements not stated.
Blinding of exposure status and ascertainment of outcome.	Unknown	Unknown	Unknown
Lost to follow-up or invalid exclusions	Total eligible population not stated. 212 mothers with prenatal dietary data included. Lost to follow-up by age at follow-up (%): 0 13 46 67	2128 total population. 462 missing maternal diet assessment, 232 did not consent, 381 did not attend follow-up visits, 77 outcome missing, 39 missing covariates.	Total eligible population not stated. 463 twin children recruited. 11had co-twin not available for recruitment. 23 pairs left Tasmania. 14 pairs could not be traced and 42 declined to participate.
Adjustment	Cuff size, observer, sleep/activity status. Age in days (At birth), body weight (at 6 and 12 month). Maternal race, parents education and occupation. Dietary potassium and magnesium	Energy intake, BP measurement conditions (cuff size, infant position, appendage used, machine model, infant state, clinic site) Maternal race, education, number of previous pregnancies, marital status, pregnancy body mass index and third trimester systolic blood pressure. Infant age and sex.	Maternal age and education, twin pair birth order (in family), child age at assessment and sex. Study in twins.
Comments		Study reported two sets of results, for calcium from food and from prenatal supplements.	Study in twins.

Table 3. Maternal calcium intake during pregnancy and blood pressure in the offspring. Summary of results from the long term follow-up of randomized controlled trials.

Outcome measure		Age at follow-up	calcium		placebo		Mean Difference (95%CI)	p value
			N	mean	N	mean		
Systolic Blood Pressure (mm Hg)								
	Belizan 1997	7 years	257	103.9 (10.6)	261	105.3 (11.0)	-1.4 (-3.2 to 0.5)	0.14
	Hatton 2003	2 years	35	95.4 (7.6)	18	100.2 (7.9)	-4.8 (-9.2 to -0.3)	0.036
	Hatton 2003	3 month	130	111.4 (14.3)	130	113.6 (12.6)	-2.2 (-5.5 to 1.1)	0.20
Diastolic Blood Pressure (mm Hg)								
	Belizan 1997	7 years	257	65.4 (9.3)	261	65.8 (9.3)	-0.4 (-2.0 to 1.2)	0.63
			n/N	**(%)**	**n/N**	**(%)**	**Relative Risk (95%CI)**	
High Blood Pressure - systolic								
	Belizan 1997	7 years	29/257	(11.4)	50/261	(19.3)	0.59 (0.39 to 0.90)	0.01
High Blood Pressure - diastolic								
	Belizan 1997	7 years	26/257	(10.2)	33/261	(12.7)	0.80 (0.49 to 1.30)	0.41

Table 4. Maternal calcium intake during pregnancy and blood pressure in the offspring. Summary of results from observational studies.

Outcome measure	Age at follow-up	Calcium Source†	Maternal calcium intake (mg)	N	Offspring blood pressure in mm Hg, by quartiles of maternal calcium intake				Crude effect size (95%CI)‡	p value	Adjusted effect size (95% CI)*	p value
					low	med-low	med-high	high				
Systolic blood pressure (mm Hg)												
McGarvey 1990	At Birth	F+S	1712	212	71.5	70.8	70.2	69.9	-0.84 (-3.69 to 2.01)	0.56	NA	
McGarvey 1990	1 month	F+S	1712	184	82.4	81.8	77.9	75.5	-6.13 (-9.32 to -2.94)	< 0.01	-4.28 (-7.12 to -1.44)	< 0.01
McGarvey 1990	6 month	F+S	1712	114	87.0	85.7	83.5	84.1	-3.84 (-7.81 to 0.13)	0.06	-3.08 (-6.66 to 0.50)	0.08
Gillman 2004	6 month	F	1494	936	91.5	90.2	90.4	88.4	-0.30 (-1.29 to 0.69)	0.55	-0.04 (-1.10 to 1.00)	0.55
	6 month	S			91.1	90.3	91	90.3	-3.10 (-4.78 to -1.42)	< 0.01	-3.00 (-4.90 to -1.10)	< 0.01
McGarvey 1990	12 month	F+S	1712	70	89.6	85.8	85.3	86.2	-4.28 (-8.94 to 0.38)	0.72	-3.40 (-8.40 to 1.69)	0.14
Morley 2004	9 years	S	NA	294	96.4		95.6		-0.80 (-4.17 to 2.57)	0.64	-0.70 (-4.20 to 2.70)	0.70
Diastolic blood pressure (mm Hg)												
McGarvey 1990	At Birth	F+S	1712	212	42.1	42.6	41.8	41.7	0.21 (-2.64 to 3.06)	0.88	NA	
McGarvey 1990	1 month	F+S	1712	184	41.0	43.1	41.2	40.9	-0.95 (-3.72 to 1.82)	0.50	-1.91 (-4.96 to 0.87)	0.18
McGarvey 1990	6 month	F+S	1712	114	56.9	51.9	48.6	50.3	-5.89 (-9.95 to -1.83)	< 0.01	-4.28 (-7.90 to -0.66)	0.01
McGarvey 1990	12 month	F+S	1712	70	53.1	49.1	47.6	49.7	-4.80 (-9.39 to -0.01)	0.05	-5.19 (-10.37 to 0.01)	0.07
Morley 2004	9 years	S	NA	294	52.6		51.8		-0.80 (-2.80 to 1.20)	0.40	-0.90 (-2.90 to 1.10)	0.40

‡ Regression coefficient between maternal calcium intake and blood pressure.
* See table 2 for the list of variables of variables included in the models for each study.
† F calcium from food sources, S calcium from supplements.

6 ARTIGO 2

The effect of maternal calcium supplementation during pregnancy on offspring calcium regulating hormones at 13 years of age. Follow–up of a randomized controlled trial.

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RUNNING HEADS

Blood pressure, calcium, pregnancy.

Abstract

Background: An altered calcium metabolism was associated with hypertension, an altered lipid profile, obesity and insulin resistance. These risk factors have also been shown to be affected by an adverse fetal environment. The aim of this study is to assess whether the exposure to high calcium levels during fetal life has a long-term effect on indicators of calcium metabolism during early adolescence.

Methods and Results: This is the follow-up of 440 children at a mean age of 13 years, whose mothers during pregnancy were randomly assigned to receive 2g/day calcium supplementation (n=214) or placebo (n=226). Main outcome measures were offspring parathyroid hypertensive factor, calcitonin gene regulating peptide, parathormone, 1,25-dihydroxyvitamin D3, total serum calcium, ionic serum calcium and urinary calcium-creatinine ratio. Urinary calcium-creatinine ratio levels were higher among children in the placebo group (Mean Difference (MD) between groups: 0.315; 95%CI: 0.087 to 0.543; p=0.007). All other biochemical markers were similar between groups. 1,25-dihydroxyvitamin D3 was a strongest predictor of offspring SBP, and a significant interaction was found between calcium supplementation status, 1,25-dihydroxyvitamin D3 and SBP (p=0.031). 1,25-dihydroxyvitamin D3 was associated with children SBP in the placebo group (regression coefficient: 2.29 mm Hg (95%CI: 0.73 to 3.86), p = 0.004), while no association was observed in the calcium group (regression coefficient: 0.68 mm Hg (95%CI: -1.03 to 2.39), p = 0.44).

Conclusions: This study suggests that maternal calcium supplementation during pregnancy induces permanent changes in offspring calcium metabolism. Changes seem to be mediated by modification of the sensitivity to calcium regulating hormones at the target cells, rather than absolute changes in hormone levels.

Key Words: pregnancy, calcium, blood pressure, epidemiology.

Background

Evidence from randomized controlled trials have shown that dietary calcium supplementation during pregnancy can reduce the incidence of pregnancy induced hypertension, in particular in populations with low calcium intake[1]. Such intervention during pregnancy might also have an impact on the developing fetus. A study using the population of a large multicentre randomized placebo-controlled trial suggested that calcium supplementation during pregnancy could be associated at age 7 with lower systolic blood pressure in the offspring[2]. A second follow-up of this study was conducted at age 13 to explore if the observed association between maternal calcium intake during pregnancy and children blood pressure is also observed in early adolescence. The results of this study could not corroborate the finding of the first follow-up (manuscript submitted). This study was also designed to explore the effects of calcium supplementation on the biochemical profile of the offspring. An altered calcium metabolism was associated not only with hypertension, but also with other risk factor for cardiovascular disease, including an altered lipid profile, obesity and insulin resistance[3-5]. All these risk factors have also been shown to be affected by fetal programming[6-8].

The present study reports a detailed biochemical profile of offspring of mothers that participated in a randomized trial of calcium supplementation during pregnancy. Calcium regulating hormones levels, indicators of insulin resistance, serum lipids and a detailed children anthropometric evaluation are compared between subjects exposed intra-uterus to maternal calcium supplementation and a placebo control group.

Methods

This is the second follow-up of a population of children and their mothers, who were originally enrolled in a randomized double blind placebo controlled-trial of calcium supplementation to prevent hypertensive disorders of pregnancy[2, 9].

A detailed description of the methodology of the original trial has been published elsewhere[9]. In short, the trial examined the effectiveness of 2g of elemental calcium supplementation a day, for the prevention of hypertensive disorders of pregnancy. Women were eligible for the study if they were nulliparous, had singleton pregnancies, and had blood pressure values below 140/90 mm Hg at the time of random allocation. Supplementation was started at 20 weeks' gestation and continued until delivery. In all, 1194 nulliparous pregnant women were enrolled in two public hospitals (580 women) and one private hospital (614 women) affiliated to the Centro Rosarino de Estudios Perinatales (CREP), Rosario, Argentina, between August 1987 and November 1990. The women were randomly assigned to receive 2 g of calcium or similar placebo orally. The randomization was stratified by hospital. Women in the two public hospitals are of low socioeconomic status, with a significant proportion living in "shanty towns" were houses do not have a formal address. The mobility of these populations is very high and it was assume that lost to follow-up among them would be too large and were not included in the study. All women from the private hospital had a known address at the time of enrollment in the trial. For these reasons the first follow-up study of this trial was restricted to the 614 women and children born at the private hospital[2]. The same population was used for the present follow-up, when the children were 13 years-old. There were 309 women in the calcium group and 305 in the placebo group randomized at the study hospital. The mothers and children were contacted using information from: (1) the original database created

during the first follow-up including name and surname, address, telephone number, identity card number of the mothers as well as the name, surname and birthday of their children; (2) telephone searching calls to relatives or neighbors that are registered in our files; (3) census and polls lists; (4) computer files from the hospital where mothers delivered; (5) telephone phone-books for a new telephone number and the corresponding new address; and (6) site visits to neighbors of the original address for information of where the mother is presently living.

From March to December 2000 mothers and their children were contacted and invited to participate in the study. If mothers and children gave written consent they were included in the study. Examiners specifically trained for this follow-up study, who were blinded to the assignment group completed the health questionnaire, conducted the physical examination in mother and children and extracted the blood sample in the children. The procedures were conducted at the patient's home. Three consecutive measurements of the children's systolic and diastolic blood pressure (Korotkoff V sound) were taken at one minute intervals with an automated blood pressure measuring device (Omron HEM 705 CP). The examiners also took three consecutive blood pressure measurements in the mothers, following the same methodology as described for the children using the same device. The examiners measured height and weight with the children without shoes, and additional anthropometric measures including abdominal circumference, skinfold triceps and skinfold subscapular.

For laboratory determinations, 10 ml of blood were withdrawn by vein punction and collected in a vacuottainer. Correctly identified samples were sent following standardized procedures for sample transportation within four hours of sample extraction to the laboratory. Samples were centrifuged at 3,500 revolutions per

minute during 10 minutes. Serum and plasma (with heparin) samples were obtained. Two hours after the withdrawal, glycaemia, cholesterol and triglycerides were determined. Samples for the remaining markers were stored at -190 °C in a liquid nitrogen dewar. For urine analysis, a midstream urine sample of the child's first morning micturition was collected in a sterile collector. Urine samples were stored at a conventional refrigerator (4 °C) for no more than 12 hours before processing. Determinations were performed at a research clinical laboratory at the Pharmacology and Biochemistry Faculty, Rosario National University, Rosario, Argentina, with the exception of PHF, 1,25-dihydroxyvitamin D3 and CGRP. There is no commercial laboratory kit for measuring PHF, and the analysis were carried out by the Division of Endocrinology and Metabolism, University of Alberta, Edmonton, Canada. 1,25-dihydroxyvitamin D3 and CGRP were measured at a specialized laboratory at the Institute of Nutrition in Mexico.

Statistical analysis

For the present analysis, children remained in the group to which their mothers were originally assigned at random, regardless of the women's compliance with the treatment and any postnatal experience (intention to treat analysis). The mean of the three blood pressure recordings was used as the blood pressure measure for the analyses.

The distribution of the variables was explored using histograms. If a variable was found to deviate significantly from the normal distribution it was transformed in an attempt to correct the problem (i.e. log transformation). For regression analyses, continuous independent variables were transformed to z-scores (mean = 0, standard deviation = 1) with the exception of child's systolic pressure. These transformations allowed the comparison of regression coefficients between variables with different

measurement scales. These regression coefficients computed using z-scores can be converted to regular regression coefficients by multiplying them by the standard deviation of the independent variable. Assessment of regression models included residual analyses (to check residual distribution, variance and outliers) and Cook's distance to detect influential observations. If the inspection of the residuals revealed a deviation from linearity, a quadratic term was introduced in the model. It was stated in the study protocol that interaction between biochemical markers, systolic blood pressure and calcium supplementation status was going to be explored using regression models with an interaction term. STATA (Stata Corporation, College Station, TX, USA) statistical package was used for the analyses.

The Ethics Committee of the Centro Rosarino de Estudios Perinatales approved the study protocol.

Results

The number of subjects randomized, the number contacted at the first and second follow-up, and the number of children for whom blood samples were obtained is presented in table 1. More than 90% of the eligible original population selected for follow-up was assessed in the calcium and the placebo group at a mean age of 13 years and for more than 70% blood samples were obtained.

Tale 2 shows maternal and offspring characteristics of children with valid blood samples, according to randomization group during pregnancy. Maternal characteristics during the index pregnancy, children characteristics at birth, and maternal and children characteristics 13 years after delivery were similar between groups.

Mean biochemical values for children in the calcium and placebo group are shown in table 3. Calcium-creatinine ratio levels were higher among children in the placebo group. All other biochemical markers were similar between groups .

The association between biochemical markers and children systolic blood pressure at 13 years of age is presented in table 4. Among all variables 1,25-dihydroxyvitamin D3 was the strongest predictor of SBP. One standard deviation increase in the logarithm of 1,25-dihydroxyvitamin D3 was associated with 1.6 mm Hg increase in children systolic blood pressure (see table 4). The two indicators of glucose metabolism, glycaemia and HbA1, were also predictor of systolic blood pressure. An interaction term was introduced in the regression analysis to assess if the association between the biochemical markers and children systolic blood pressure was different for children in the calcium and placebo group. Only 1,25-dihydroxyvitamin D3 showed a significant interaction term. 1,25-dihydroxyvitamin D3 was associated

with children systolic blood pressure for children in the placebo group (regression coefficient: 2.29 mm Hg (95%CI: 0.73 to 3.86), p = 0.004), while no association was observed in the calcium group (regression coefficient: 0.68 mm Hg (95%CI: -1.03 to 2.39), p = 0.44). A quadratic term was introduced in the model because of signs of curvature in the relationship between the 1,25-dihydroxyvitamin D3 and systolic blood pressure. Figure 1 shows the relationship between these two variables, for the calcium and placebo group. The same analysis was conducted for offspring systolic blood pressure at 7 years of age (see figure 1), with similar results.

Discussion

We have presented a detailed biochemical profile of children at 13 years of age exposed to high levels of maternal calcium intake or placebo during pregnancy. Among the biochemical markers we reported values of four hormones associated with calcium metabolism, serum calcium levels, indicators of insulin resistance and serum lipids, and none of them showed an association with fetal exposure to high calcium. However, urinary calcium excretion was reduced in children in the calcium group and 1,25-dihydroxyvitamin D3 was a strong predictor of systolic blood pressure, but only among children in placebo group. These finding are related as 1,25-dihydroxyvitamin D3 plays a central role in the regulation of urinary calcium excretion[10].

Calcium can be seen as astrong candidate for fetal programming. There is now a substantial body of evidence supporting a key role of calcium in the regulation of blood pressure, blood glucose and lipids metabolism[11]. In particular 1,25-dihydroxyvitamin D3 has been shown to be highly responsive to variations in dietary calcium consumption and can stimulate rapid increases in intracellular calcium[5]. It has been postulated that the hypotensive effect of dietary calcium supplementation is mediated by reductions in 1,25-dihydroxyvitamin D3 levels [12]. Hypertension has been

associated with and dependent on cytosolic free calcium excess, as increase intracellular free calcium increases vascular reactivity and blood pressure[13, 14]. Vitamin D metabolites appear to enhance the expression of Ca-ATPase, increase the entry of calcium into the cell, raise cytosolic free calcium and induce the expression of contractile proteins, all of which may directly or indirectly affect arterial tone and increase blood pressure[11]. A similar mechanism has shown to operate in the regulation of lipids metabolism and obesity [5].

More recently, an enzymatically active 25-Hydroxyvitamin-D3-1alfa-hydroxylase, the final enzyme in the biosynthetic pathway of 1,25-dihydroxyvitamin D3, have been shown to be expressed in human vascular smooth muscle cells. This finding suggests that human vascular smooth muscle cells might regulate the production of 1,25-dihydroxyvitamin D3 directly, as opposed to the circulating vitamin, which is regulated entirely by systemic calcium homeostatic factors[15]. In another line of research studies in rats have shown that 1,25-dihydroxyvitamin D3 is an inhibitor of renin expression in the juxtaglomerular apparatus as well as an inhibitor of vascular smooth muscle cell proliferation[16, 17]. These results suggests that 1,25-dihydroxyvitamin D3 is a negative regulator of the renin-angiotensin system and play a critical role in blood pressure homeostasis[18]. Our data confirmed an association between 1,25-dihydroxyvitamin D3 and blood pressure, but such association does not seem to be present when subjects are exposed to a high maternal dietary calcium intake during pregnancy. We also observed that offspring hormone levels in the calcium and placebo group were similar, and not affected by the fetal exposure. This might be an indication that changes in urinary calcium excretion and blood pressure are mediated by changes in hormone sensitivity at the target cells, rather than absolute changes in hormones levels. A similar mechanism was proposed for programming of

blood pressure induced by protein restriction during pregnancy. Offspring of rats which receive low amounts of protein during pregnancy remain hypersensitive into adult life, and have increased glucocorticoids receptor numbers at several sites, including the vasculature, and an increased sensitivity to angiotensin II[19-21].

Morley et al reported that children whose mothers took calcium supplements during pregnancy had a better lipid profile at 9 years of age and suggested that calcium availability could permanently programmed lipid metabolism during fetal life levels[3]. Our data does not support such findings as levels of cholesterol and triacylglycerol in the calcium and placebo groups were similar.

At the time of writing the protocol for this study there was strong interest in the newly described paratiroid hypertensive factor[22-24]. It was a strong candidate to explain the hypotensive effect of calcium supplementation. Our results could not support these findings as PHF had no vasoactive effect in this population. In contrast with previous reports CGRP does not seem to play a role as a determinant of systolic blood pressure (10).

We have found an association between and indicator of insulin resistance, HbA1, and systolic blood pressure. However our study did not find an association between this parameter and calcium supplementation during pregnancy.

The results observed in this report are unlikely to be the result of confounding or other sources of bias. As shown in table 2 the mothers and children in the calcium and placebo groups were very similar in characteristics measured during pregnancy, after birth, and at 13 years of age. Furthermore, lost to follow-up was small and mother, children and the individuals conducting the assessments were blinded to treatment status. We have conducted a large number of hypothesis tests, and although

they were all pre-specified, we can not exclude the fact that one or more of our observation can be just a random variation of the biological outcomes.

Conclusions

This study suggests that maternal calcium supplementation during pregnancy induces permanent changes in offspring calcium metabolism. Changes seem to be mediated by modification of the sensitivity to calcium regulating hormones at the target cells, rather than absolute changes in hormone levels. Because of the exploratory nature of this analysis, these results should be taken with caution and need confirmation by future studies.

Competing interests

None declared.

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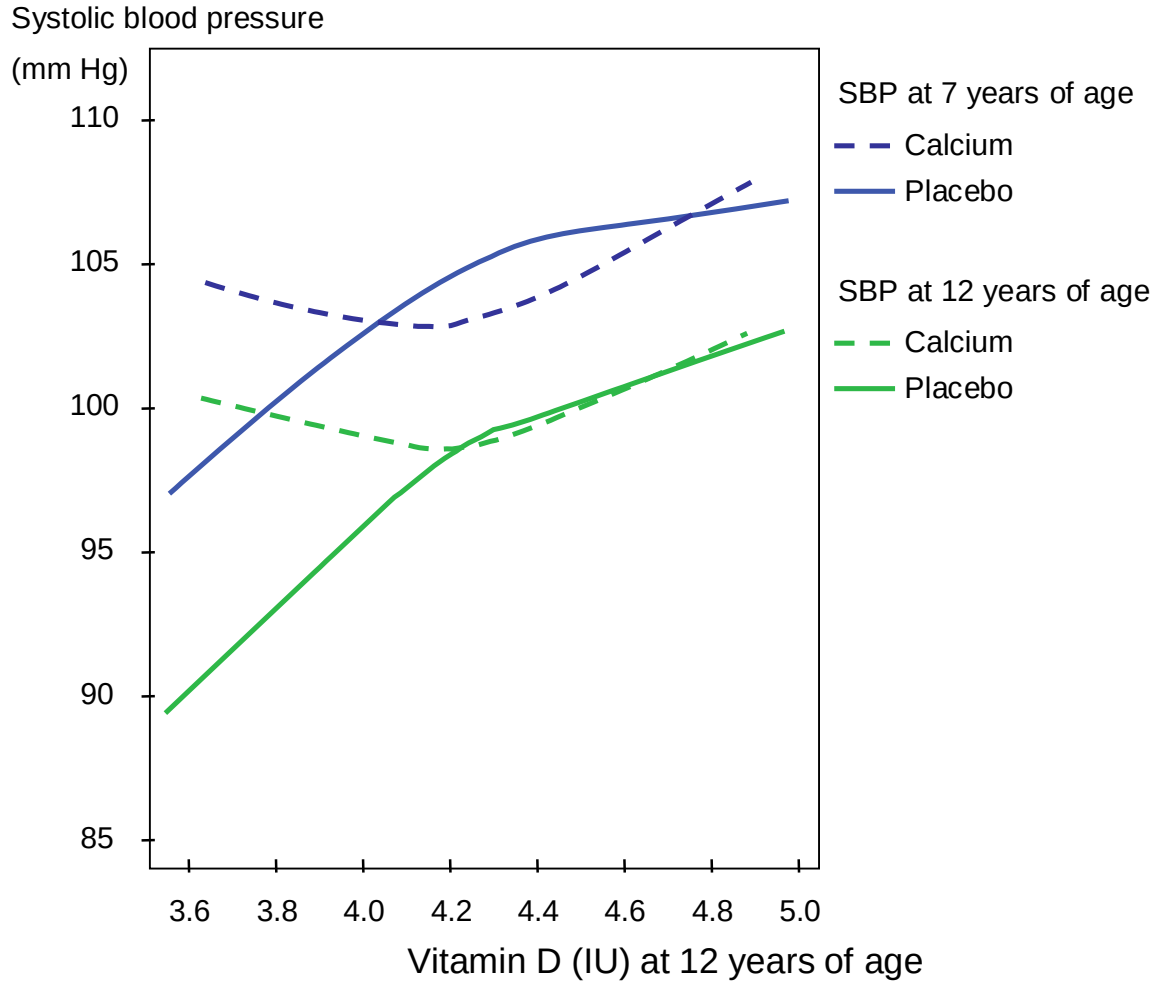
Figure legends

Figure 1. Relation between levels of 1,25-dihydroxyvitamin D3 in children at 13 year of age and systolic blood pressure at 7 and 12 years of age, by calcium supplementation group.

Figures


Systolic blood pressure
(mm Hg)
110
105
100
95
90
85
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
Vitamin D (IU) at 12 years of age
SBP at 7 years of age
Calcium
Placebo
SBP at 12 years of age
Calcium
Placebo

Tables

Table 1. Study population at the first and second follow-up.

Population	Calcium group	Placebo group	all
Women randomised in original study	593	601	1194
Women randomised at selected hospital	309	305	614
Death of infant at selected hospital	5	6	11
Death of mother	1	0	1
Children eligible for follow-up	303	299	602
First follow-up (7 years after delivery)			
Children contacted	289	285	574
Refuse to participate	10	5	15
Children with blood pressure meassurments	254	260	514
Second follow-up (12 years after delivery)			
Children contacted second follow-up	296	295	591
Refuse to participate	21	13	34
Children with blood pressure meassurments	274	280	554
Children with blood samples (% of elegible)	214 (70.6%)	226 (75.6%)	440

Table 2. Maternal and offspring characteristics of children with blood samples 13 years after delivery, according to randomisation group during pregnancy.

Characteristic	Calcium (n = 214) mean	SD	Placebo (n=226) mean	SD	Mean difference (95% CI)	p value
Maternal characteristics during index pregnancy						
Third trimester systolic blood pressure (mmHg)	116.4	11.2	116.5	9.7	-0.06 (-2.11 to 1.99)	0.953
Third trimester diastolic blood pressure (mmHg)	76.4	8.9	76.7	9.1	-0.28 (-2.03 to 1.48)	0.758
Pre-pregnancy weight (kg)	57.5	8.1	57.5	8.1	0.10 (-1.48 to 1.67)	0.905
Height (cm)	162.9	6.2	162.6	5.8	-0.30 (-1.47 to 0.88)	0.623
Smoking during pregnancy (% yes)	8.6		12.4			0.860
Maternal characteristics 13 years after delivery						
Age (years)	39.7	4.0	39.5	4.6	-0.15 (-0.96 to 0.66)	0.711
Weight (kg)	62.4	10.9	63.5	12.9	1.15 (-1.11 to 3.40)	0.318
Systolic blood pressure (mmHg)	111.4	14.2	111.9	15.4	0.53 (-2.26 to 3.33)	0.707
Diastolic blood pressure (mmHg)	72.4	9.4	72.8	10.5	0.38 (-1.51 to 2.27)	0.691
Abdominal circumference (cm)	76.4	9.3	77.7	10.8	1.30 (-0.62 to 3.23)	0.183
Children characteristics at birth						
Birth weight (grams)	3221	486	3240	453	19 (-74 to 112)	0.692
Gestational age at birth (weeks)	39.4	1.5	39.5	1.6	0.16 (-0.15 to 0.47)	0.313
Lenght at birth (cm)	49.9	2.1	49.8	2.4	-0.08 (-0.54 to 0.39)	0.750
Head circunference (cm)	34.7	1.3	34.7	1.4	0.02 (-0.27 to 0.30)	0.905
Sex (% males)	56.9		52.3			0.754
Children characteristics 12 years after delivery						
Age (years)	12.8	0.6	12.7	0.6	-0.07 (-0.18 to 0.05)	0.257
Weight (kg)	47.4	10.6	45.7	10.4	-1.74 (-3.72 to 0.24)	0.084
Height (cm)	153.2	8.0	152.0	8.1	-1.18 (-2.70 to 0.33)	0.125
Abdominal circumference (cm)	68.1	9.6	67.0	8.6	-1.03 (-2.73 to 0.68)	0.237

Table 3: Characteristics of mothers and children at assessment 13 years after delivery, according to randomisation group during pregnancy. Values are means (SD) unless indicated otherwise.

Characteristics	Placebo		Calcium		Mean difference (95% CI)	p value
	N	Mean (SD)	N	Mean (SD)		
PHF†	212	-1.64 (0.90)	201	-1.74 (0.95)	0.105 (-0.074 to 0.284)	0.251
CGRP†	180	3.14 (1.10)	178	3.11 (1.18)	0.028 (-0.209 to 0.266)	0.814
Parathormone†	174	3.51 (0.45)	158	3.45 (0.45)	0.060 (-0.037 to 0.157)	0.224
Vitamin D†	195	4.17 (0.28)	189	4.21 (0.26)	-0.037 (-0.091 to 0.016)	0.170
Total serum calcium	226	9.49 (0.53)	215	9.51 (0.55)	-0.022 (-0.124 to 0.079)	0.665
Ionic serum calcium	222	1.07 (0.12)	211	1.07 (0.13)	0.000 (-0.024 to 0.025)	0.988
Urinary calcium*†	260	-2.57 (1.35)	249	-2.89 (1.26)	0.315 (0.087 to 0.543)	0.007
HbA	219	5.90 (0.69)	212	5.87 (0.64)	0.022 (-0.104 to 0.148)	0.733
Glicemia	226	89.0 (10.8)	214	88.5 (10.8)	0.512 (-1.515 to 2.540)	0.620
Colesterol	226	159.8 (26.3)	214	159.2 (28.9)	0.590 (-4.585 to 5.765)	0.823
Trigliceridos†	226	4.46 (0.51)	214	4.45 (0.46)	0.005 (-0.087 to 0.096)	0.917

* Expressed as urinary calcium/creatinine ratio

† The natural logartihm of the variable was used in the analyisis.

Table 4: Association between laboratory parameters and children systolic blood pressure. Figures are regression coefficients and 95% confidence intervals.

Parameter	Regression coefficient** (95% CI)	p value	Interaction with calcium p value
PHF†	-0.10 (-1.21 to 1.01)	0.861	0.658
CGRP†	0.47 (-0.76 to 1.70)	0.457	0.138
Parathormone†	0.31 (-0.95 to 1.56)	0.633	0.732
Vitamin D†	1.60 (0.45 to 2.75)	0.006	0.031‡
Total serum calcium	0.71 (-0.37 to 1.79)	0.197	0.809
Ionic serum calcium	0.17 (-0.93 to 1.27)	0.757	0.455
Urinary calcium*†	-0.70 (-1.73 to 0.34)	0.186	0.367
HbA	1.37 (0.28 to 2.45)	0.014	0.647
Glicemia	1.51 (0.44 to 2.59)	0.006	0.100
Colesterol	-0.37 (-1.45 to 0.72)	0.509	0.291
Trigliceridos†	0.61 (-0.48 to 1.69)	0.273	0.280

* Expressed as urinary calcium/creatinine ratio.
† The natural logartihm of the variable was used in the analyisis.
** Laboratory parameters were transformed to z scores for regression analysis. The regression coefficients represent the change in children's systolic pressure in mm Hg for 1 standard deviation increase in the independent variable.
‡ The regression model included a quadratic term.

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7 ARTIGO 3

Associations of pregnancy, birth, and early life characteristics with systolic blood pressure at 13 Years of Age.

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Abstract

Background

We explore whether pregnancy, birth, and early life characteristics are associated with offspring systolic blood pressure at age 13.

Methods

This study is a secondary analysis of data from a prospective cohort of children born to healthy nulliparous women enrolled in a randomized controlled trial. Information from 554 children at a mean age of 13 years was included, comprising 91.7% of subjects eligible for the follow-up. The study was conducted at a private hospital affiliated to a maternal and child research unit in Rosario, Argentina.

Results

In crude analysis the only perinatal factors associated with children's systolic blood pressure were maternal weight at 20 weeks gestation (beta: 2.1 mmHg, 95%CI = 1.1 to 3.1, p=<0.001, maternal diastolic blood pressure in the third trimester (beta: 1.0 mmHg, 95%CI = 0.01 to 2.1, p=0.043) and newborn head circumference (beta: 1.2 mmHg, 95%CI = 0.1 to 2.4, p=0.035). Such association's disappeared after adjustment for offspring age, sex, current weight and maternal blood pressure at follow-up. Other factors related to maternal nutrition and well being during pregnancy, including weight gain, glycemia, serum albumin, smoking and blood pressure in the third trimester showed weak and inconsistent relations with children's blood pressure. Furthermore, there was no consistent association between offspring blood pressure and small-for-gestational age, ponderal index or breastfeeding. A U-shaped rather than a linear relation between birthweight and systolic blood pressure was found. The regression coefficient between systolic blood pressure and birthweight was negative for birthweights below the median (beta: -1.95 mmHg, 95%CI = -7.22 to 3.31, p=0.47), and

positive for birthweights above the median (beta: 5.35 mmHg, 95%CI = 0.04 to 10.7, p=0.043) with a statistically significant interaction (p=0.043).

Conclusions

In this healthy population, we found weak support for an association between pregnancy, birth, and early life characteristics and blood pressure in early adolescence. The relation between birthweight and offspring blood pressure seems to be different for low and high birthweight infants.

INTRODUCTION

High blood pressure have been found to be linked with obesity, insulin resistance and dyslipedemia in a condition described as the metabolic syndrome[1]. Individuals with this syndrome have an increase risk for cardiovascular disease[1, 2]. There is compelling evidence suggesting that the metabolic syndrome may originate in uterus, linking small size at birth in full-term pregnancies with the subsequent development later in life of the major features of this condition[3-5]. The commonest approach to test this hypothesis has been to use follow-up studies, which relate anthropometric measures in early life to subsequent disease[6]. The results from a number of these studies have been subject to criticisms because their conclusions are based on a small proportion of the subjects in the original cohorts, detailed information from pregnancy characteristics is usually not available or not reliable and newborn anthropometry is used as the only proxy of an adverse fetal environment[7, 8]. In the present study, we have prospective, detailed information on pregnancy, birth, and early life characteristics in a healthy, socio-economically and ethnically homogeneous population. A detailed children assessment was conducted 13 years after the index pregnancy. The aim of the present study was to assess the influence of intrauterine and early childhood factors, including indicator of gestational diabetes, pregnancy induced hypertension, newborn anthropometry and breastfeeding on offspring blood pressure at 13 years of age.

MATERIALS AND METHODS

Study population

Mothers of the children enrolled had participated in a randomized controlled trial of calcium supplementation during pregnancy[9]. Children born to these pregnancies were followed-up to the age 7 years and 13 years[10]. Women eligible for the trial were those seeking prenatal care in three clinics of Rosario, Argentina, before the 20th week of

gestation, with no past or present disease, with normal oral glucose-tolerance test and with blood pressure below 140/90 mmHg at enrolment. Only nulliparous women with singleton pregnancy were included into the trial. In all, 1194 nulliparous pregnant women were enrolled in two public hospitals (580 women) and one private hospital (614 women) affiliated to the Centro Rosarino de Estudios Perinatales (CREP), Rosario, Argentina, between August 1987 and November 1990. The randomization was stratified by hospital. Women in the two public hospitals were of low socioeconomic status, with a significant proportion living in "shanty towns" were houses do not have a formal address. The mobility of these populations is very high and it was assume that lost to follow-up among them would be too large and were not included in the study. All women from the private hospital had a known address at the time of enrollment in the trial. For these reasons the follow-up study was restricted to the 614 women and children born at the private hospital. The follow-up study was conducted between March 2000 to December 2000. Among the 614 live birth babies eligible, a total 554 children could be traced in the follow-up. These children constitute the population analyzed for the present study. The study population is described in full detail elsewhere[9-11].

Pregnancy and birth characteristics

Newborn anthropometric measurements used as independent variables were birth weight as a continuous variable, low birth weight defined as birth weight below 2,500g, small-for-gestational age defined as birth weight below the tenth percentile of expected weight for gestational age, head circumference and ponderal index (weight/height3). Gestational age at delivery was determined by the date of last menstrual period and confirmed or corrected by an early ultrasound examination. Maternal health and nutrition indicators included blood pressure after 30 weeks gestation (mmHg),

pregnancy induced hypertension, weight gain during pregnancy (kg/week), weight at 20 weeks gestation, fasting glycemia at 20 weeks gestation, lowest hemoglobin during pregnancy, maternal anemia defined as lowest hemoglobin during pregnancy < 10 g/dl, serum albumin at 20 weeks gestation, and smoking during pregnancy. Women were scheduled for clinical examination, collection of urine and blood samples and blood pressure measurements at 23, 25, 27, 31, and 35 weeks and then weekly until delivery. Hypertension during pregnancy was strictly monitored. During each visit blood pressure was measured five times with the patient seated after 10 minutes of rest using random-zero sphygmomanometer. The mean value of the five measurements was used in the analysis.

Measurement of parental and child's characteristics at follow-up

Child of the index pregnancy between 10 and 13 years of age and their mothers were invited to participate in the study. Examiners specifically trained for this follow-up study, who were blinded to the assignment group completed the health questionnaire, conducted the physical examination in mother and children and extracted the blood sample in the children. The procedures were conducted at the patient's home. Three consecutive measurements of the children's systolic and diastolic blood pressure (Korotkoff V sound) were taken at one minute intervals with an automated blood pressure measuring device (Omron HEM 705 CP). The examiners also took three consecutive blood pressure measurements in the mothers, following the same methodology as described for the children using the same device. The examiners measured height and weight with the children without shoes, and additional anthropometric measures including abdominal circumference, skinfold triceps and skinfold subscapular. Breastfeeding patterns were obtained by a questionnaire administered to the mother.

Rationale for adjusted analyses

There has been considerable debate on the best set of variables that should be included in regression models to estimate the effect of perinatal variables on systolic blood pressure later in life[12-15]. Children's age, sex, birth order, and socieconomic status are classical confounding factors. The population included in this report was very homogenous as the sample was restricted to healthy nuliparous women attending an expensive private hospital. For this reason only child sex and age were included in the model as confounders. It has been suggested that an inverse associations between birthweight and blood pressure in later life might be the consequence of inappropriate statistical adjustment for measures of current body size[12]. Birthweight is a predictor of current weight and adjusting might generate a spurious association with blood pressure, rather adjusting for confounding[12]. Simulations studies support this assertion[13]. Furthermore, there have been reports of a U-shape relationship between birthweight and blood pressure[16]. We are reporting estimates with and without adjusting for current weight and explored non linear trends. Maternal blood pressure outside pregnancy is a strong independent predictor of offspring blood pressure, being a surrogate for family genetic and environmental factors shared by mother and child[17, 18]. It has also being suggested that the correlation between low birthweight and subsequent high blood pressure is confounded by the influence of parental blood pressure[19]. To account for this potential confounder maternal systolic blood pressure outside pregnancy was included in regression models.

Statistical analysis

Multiple linear regression analyses were used to assess the association between independent variables and systolic offspring blood pressure at 13 years age. For regression analysis, all continuous independent variables were transformed to z-scores

(mean = 0, standard deviation = 1). Child's systolic pressure was not transformed. These transformations allowed the comparison of regression coefficients between variables with different scale of measurement since the coefficient represents the change in child's systolic pressure (in mmHg) for one standard deviation shift in the value of the independent variable. These regression coefficients computed using z-scores can be converted to regular regression coefficients by multiplying them by the standard deviation of the independent variable. To examine possible nonlinear associations, graphs of the relationships between exposures and outcomes were plotted, and quadratic terms, for continuous variables, were included in regression models. Continuous independent variables were also transformed to categorical indicators by quintiles and plot against the dependent variable to explore non-linear trends. Assessment of regression models included residual analyses (to check residual distribution, variance, non-linear trends and outliers) and Cook's distance to detect influential observations. Statistical analyses were performed using the Stata statistical package.

RESULTS

Out of 614 women randomized in the original randomized trial in the selected hospital, 602 were alive at last contact and eligible for the follow-up, 591 (98.0%) were found and contacted, 31 refused to participate, and systolic blood pressure was obtained for 554 (91.7%). Maternal characteristics at the time of enrollment in the trial were similar between children included in the study and those lost-to follow-up (data not shown). In crude analysis the only perinatal factors associated with children's systolic blood pressure were maternal weight at 20 weeks gestation, maternal diastolic blood pressure in the third trimester and newborn head circumference (see table 1). All children's anthropometric indicators at age 13 were associated with children's systolic blood pressure, but current weight was the strongest predictor (see table 1). Maternal systolic

and diastolic blood pressures 13 years after birth were strong predictor of children systolic blood pressure (see table 1).

Adjusted analyses are presented in table 2. Newborn head circumference was no longer significant after adjustment by age and sex. Maternal systolic blood pressure in the third trimester remained associated after adjustment by children's age, sex and weight, but further adjustment by maternal systolic blood pressure outside pregnancy removed such association.

A week negative association between birthweight and systolic blood pressure was observed only after adjustment by current weight (table 2). However, further inspection of this association revealed a non-linear, U-shape relationship (see figure 1). Mean children systolic blood pressure by quintiles of birthweight were 99.9 , 98.5, 97.8, 99.8 and 101.1 mm Hg. To incorporate this U-shape pattern into the linear regression analysis an interaction term was included in the model to obtain an estimate of the regression coefficient for birthweight values below and above the median (see figure 1). The regression coefficient between systolic blood pressure and birthweight was negative for birthweights below the median (beta: -1.95 mmHg, 95%CI = -7.22 to 3.31, p=0.47), and positive for birthweights above the median (beta: 5.35 mmHg, 95%CI = 0.04 to 10.7, p=0.043). The difference between the two coefficients was statistically significant (Interaction term p = 0.046).

DISCUSSION

This study could not support an association between perinatal factors or breastfeeding on blood pressure during childhood. Factors related to maternal nutrition and well being during pregnancy, including weight gain, glycemia, serum albumin, smoking and blood pressure in the third trimester showed weak and inconsistent relations with children's blood pressure. Furthermore, there was no consistent association between

neonatal anthropometric measures (birthweigh, low birth weight, small-for-gestational age, ponderal index and head circumference) and blood pressure. Our findings are consistent with previous published reports in adolescence [20, 21].

A weak negative association between birth weight and child's blood pressure was present only after adjustment for current weight. A more detailed analysis showed a U-shaped rather than a linear relation between birthweight and blood pressure, with a strong positive association for birthweights above the median. Other authors have found a similar pattern, and it has been proposed that influencing factors to the left and right side of the distribution are different [16, 22, 23]. The left side of the U-distribution, where low birthweight yielded higher blood pressure may be an effect of placental insufficiency, or inadequate maternal nutrition. The right side of the U-distribution, where higher birthweight is associated with higher blood pressure, may be an effect of an impaired maternal glucose tolerance or gestational diabetes resulting in larger babies, or the result of a stronger link between birthweight and current weight for newborns genetically programmed to have a larger body size[23].

In agreement with previous studies we found a positive association between maternal blood pressure during pregnancy and child's blood pressure[24-27]. Others found no relation between these two variables[28-30].The present study differs from previous research in one important aspects. In our study women with a history of hypertension or renal disease were not enrolled, and since maternal blood pressure outside pregnancy is a strong predictor of child's systolic pressure, chronic hypertension rather than pregnancy induced hypertension might have been responsible for the finding in previous studies. After adjusting for maternal blood pressure outside pregnancy the association disappeared, suggesting that genetic or environmental variables are responsible for the association, rather than fetal programming.

This study measured children abdominal circumference, skinfold subscapula, skinfold triceps and compute the BMI. Children weight was clearly the strongest predictor of blood pressure, suggesting that more sophisticated anthropometric measures are not necessary to assess cardiovascular risk in early adolescence.

This study had detailed and reliable information on pregnancy characteristics and maternal characteristics at follow-up not available in previous reports. Data was collected prospectively by trained personnel, and data quality was assured by routinely performed procedures that verify data consistency during patient's recruitment and follow-up. In comparison to other studies, lost to follow-up was small, and lost patients where very similar to included patients, making selection bias unlikely. Another strength of this study lies in the homogeneity of the study population. Enrolled women were healthy, nulliparous women, attending a single hospital covering a population homogeneous as regards socio-economic, ethnic and lifestyle variables. Women with chronic hypertension and diabetes were excluded from the study. Studying such population allowed minimizing the risk of spurious association resulting from residual confounding by unmeasured environmental or genetic variables, or by underlying diseases such as chronic hypertension. That being said, it should be noted that the results of this study could not be extrapolated to an underprivileged population. This is a "low risk" population and the proportion of newborns exposed to an adverse intrauterine environment, either because of pathologic conditions or maternal undernutrition, is expected to be small.

In summary, in this homogeneous, healthy population, we found weak support for an association between perinatal and systolic blood pressure in early adolescence. In this setting the relation between birthweight and offspring blood pressure seems to be different for low and high birthweight infants.

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Figure legends

Figure 1. Children systolic blood pressure by birthweight. Solid line is systolic blood pressure by birthweight quintiles. Dash lines are linear regression fits for birthweight below (beta 1) and above the median (beta 2).

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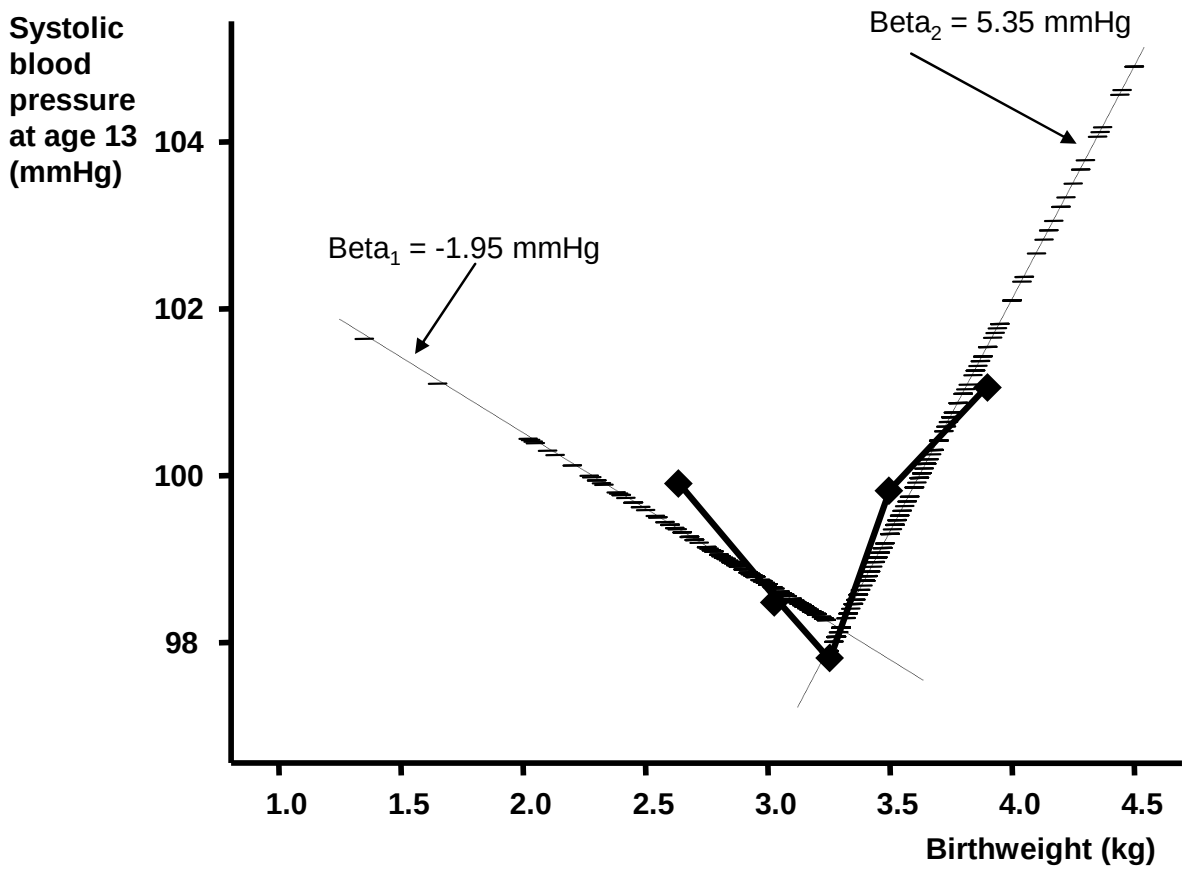

Systolic blood pressure at age 13 (mmHg)
104
102
100
98
Beta2 = 5.35 mmHg
Beta1 = -1.95 mmHg
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Birthweight (kg)

Table 1. Predictor of children systolic blood pressure at 13 years of age.

Independent characteristics*	Mean‡	Standard Deviation	Mean Change (95% CI) in Systolic Blood Pressure (mmHg) per Increase in 1 SD or per Category of Characteristic			
			Beta	95% CI		p
Maternal characteristics (during pregnancy)						
Anemia (yes/no)	14.4%		0.7	-2.3	3.8	0.642
Smoking (yes/no)	11.2%		1.8	-1.6	5.1	0.307
Glycemia (mg/dl)	77.17	8.28	-0.3	-1.5	0.9	0.641
Hematocrit (%)	36.10	2.49	0.5	-0.7	1.7	0.427
Hemoglobin (g/dl)	11.11	0.79	0.7	-0.6	1.9	0.281
DBP third trimester (mmHg)†	76.41	8.90	1.0	0.0	2.1	0.043
SBP third trimester (mmHg)†	116.36	10.22	0.9	-0.2	1.9	0.097
Weight at 20 weeks (kg)	57.35	8.13	2.1	1.0	3.1	0.000
Weight gain (g/week)	433.62	152.51	0.9	-0.2	2.0	0.093
Offspring characteristics (birth and infant)						
Head circumference (cm)	34.67	1.41	1.2	0.1	2.4	0.035
Low birth weight (yes/no)	6.0%		0.9	-3.5	5.3	0.681
Ponderal index (Kg/cm3)	26.04	2.81	-0.3	-1.4	0.8	0.588
Small for gestational age (yes/no)	10.2%		1.6	-2.0	5.2	0.386
Gestational age at birth (weeks)	39.33	1.30	0.6	-0.6	1.7	0.321
Birth weight (g)	3236.13	477.61	0.7	-0.3	1.8	0.181
Lenght at birth (cm)	499.30	22.05	0.8	-0.3	1.9	0.135
Brestfeeding >= 6 month (yes/no)	57.3%		0.3	-1.7	2.3	0.788
Breastfeeding (months)**	1.75	0.80	0.3	-0.7	1.3	0.532
Maternal Characteristics (13 years after delivery)						
Diastolic blood pressure (mmHg)	72.24	9.93	3.1	2.2	4.1	0.000
Systolic blood pressure (mmHg)	111.52	14.51	3.6	2.6	4.5	0.000
Weight (kg)	62.51	11.55	2.6	1.6	3.5	0.000
Maternal height (cm)	162.46	6.16	0.6	-0.4	1.5	0.278
Abdominal circumference (cm)	76.65	9.87	1.9	0.9	2.9	0.000
Offprint anthropometry (at 13 years of age)						
Sex (male)	53.4%		4.2	2.2	6.2	0.000
Age (years)	12.75	0.64	1.6	0.7	2.6	0.001
Weight (kg)	46.30	10.63	4.6	3.7	5.5	0.000
Body mass index (kg/cm2)	1.98	0.35	4.2	3.3	5.2	0.000
Skinfold subscapular (mm)	12.13	7.16	2.1	1.1	3.1	0.000
Skinfold triceps (mm)	17.19	7.67	2.2	1.2	3.2	0.000
Abdominal cirfunference (cm)	67.38	8.91	3.8	2.9	4.8	0.000

*Continuous independent variables were transformed to z-scores for regression analysis.

** Variable transformed to the log scale.

‡ Mean for continuos variables, or proportion for dichotomous (yes/no) variables.

† SBP, systolic blood pressure. DBP, diastolic blood pressure

Table 2. Adjusted associations between children systolic blood pressure at 13 years of age and maternal and children early life characteristics.

Independent characteristics*	Mean Change (95% CI) in Systolic Blood Pressure (mm Hg) per Increase in 1 SD or per Category of Characteristic											
	Adjusted by children age and sex				Adjusted by children age, sex and weight				Adjusted by children age, sex, weight and maternal systolic blood pressure outside pregnancy			
	Beta	95% CI		p	Beta	95% CI		p	Beta	95% CI		p
Maternal characteristics (during pregnancy)												
Anemia (yes/no)	0.7	-2.3	3.7	0.641	0.3	-2.5	3.0	0.858	-0.3	-2.9	2.4	0.826
Smoking (yes/no)	1.0	-2.3	4.3	0.563	-0.5	-3.6	2.6	0.758	0.5	-2.5	3.5	0.735
Glycemia (mg/dl)	-0.1	-1.3	1.1	0.830	-0.1	-1.2	0.9	0.842	-0.4	-1.4	0.6	0.434
Hematocrit (%)	0.3	-0.8	1.5	0.577	0.3	-0.8	1.3	0.590	0.0	-1.0	1.0	0.960
Hemoglobin (g/dl)	0.4	-0.8	1.6	0.523	0.5	-0.6	1.5	0.398	0.2	-0.9	1.2	0.741
DBP third trimester (mmHg)	1.3	0.2	2.3	0.017	1.1	0.1	2.0	0.025	0.2	-0.7	1.2	0.652
SBP third trimester (mmHg)	1.0	0.0	2.0	0.050	0.7	-0.3	1.6	0.149	-0.3	-1.3	0.7	0.564
Weight at 20 weeks (kg)	2.1	1.1	3.1	0.000	0.3	-0.7	1.4	0.530	-0.2	-1.3	0.8	0.642
Weight gain (g/week)	0.8	-0.3	1.8	0.163	0.5	-0.5	1.4	0.354	0.6	-0.4	1.6	0.218
Offspring characteristics (birth and infant)												
Head circumference (cm)	1.0	-0.1	2.1	0.077	0.3	-0.8	1.3	0.612	0.2	-0.8	1.2	0.700
Low birth weight (yes/no)	0.7	-3.6	5.0	0.746	0.9	-3.1	4.8	0.666	-0.9	-4.7	3.0	0.665
Ponderal index (g/cm3)	-0.1	-1.2	0.9	0.812	-0.4	-1.3	0.6	0.455	-0.3	-1.3	0.6	0.481
Small for gestational age (yes/no)	1.8	-1.8	5.3	0.327	0.3	-2.9	3.6	0.836	1.6	-1.5	4.8	0.306
Gestational age at birth (weeks)	0.7	-0.4	1.8	0.238	0.7	-0.3	1.7	0.164	0.9	0.0	1.9	0.063
Birth weight (g)	0.6	-0.4	1.6	0.255	-0.3	-1.3	0.7	0.515	0.0	-0.9	1.0	0.938
Lenght at birth (cm)	0.6	-0.5	1.7	0.288	-0.3	-1.3	0.7	0.587	0.1	-0.9	1.1	0.884
Brestfeeding >= 6 month (yes/no)	0.3	-1.7	2.3	0.757	0.1	-1.8	1.9	0.950	0.2	-1.6	2.0	0.819
Breastfeeding (months)**	0.2	-0.8	1.2	0.701	0.0	-0.9	0.9	0.961	-0.1	-1.0	0.8	0.799

*Continuous independent variables were transformed to z-scores for regression analysis.

** Variable transformed to the log scale.

8 PRESS-RELEASE

Mother's calcium intake during pregnancy has long term consequences for their children.

A research study conducted at the Centro de Pesquisas Epidemiológicas da Faculdade de Medicina da Universidade Federal de Pelotas and at the Centro Rosarino de Estudios Perinatales in Argentina have shown that mother calcium intake during pregnancy can have long lasting effects on their children. In the 80s a group of researchers in Argentina assigned healthy pregnant women to received dietary calcium supplements, or placebo. The original idea behind the study was to assess the effect of calcium on mother's health, in particular on hypertension during pregnancy. At that time, the study concluded that increasing calcium consumption during pregnancy was effective to prevent the development of hypertension during pregnancy. But an important question remains unanswered. What was the effect on the offspring? This question was very important because at the time there was increasing scientific evidence suggesting that problems during pregnancy can increase the risk for disease in the offspring later in life. Seven year after the original study the researchers contacted the mothers and children that participated in the original study to conduct a health assessment on the children. They found that the offspring of mother that receive calcium supplementation during pregnancy had a lower risk for high blood pressure at 7 years of age. Trying to understand the mechanisms behind the effect of calcium on children blood pressure a second follow-up of these children was conducted at age 13. This time they conducted a much more detailed evaluation, which included biochemical measurements to assess children calcium metabolism. The researchers found that in early adolescence the impact of maternal calcium intake during pregnancy on children blood

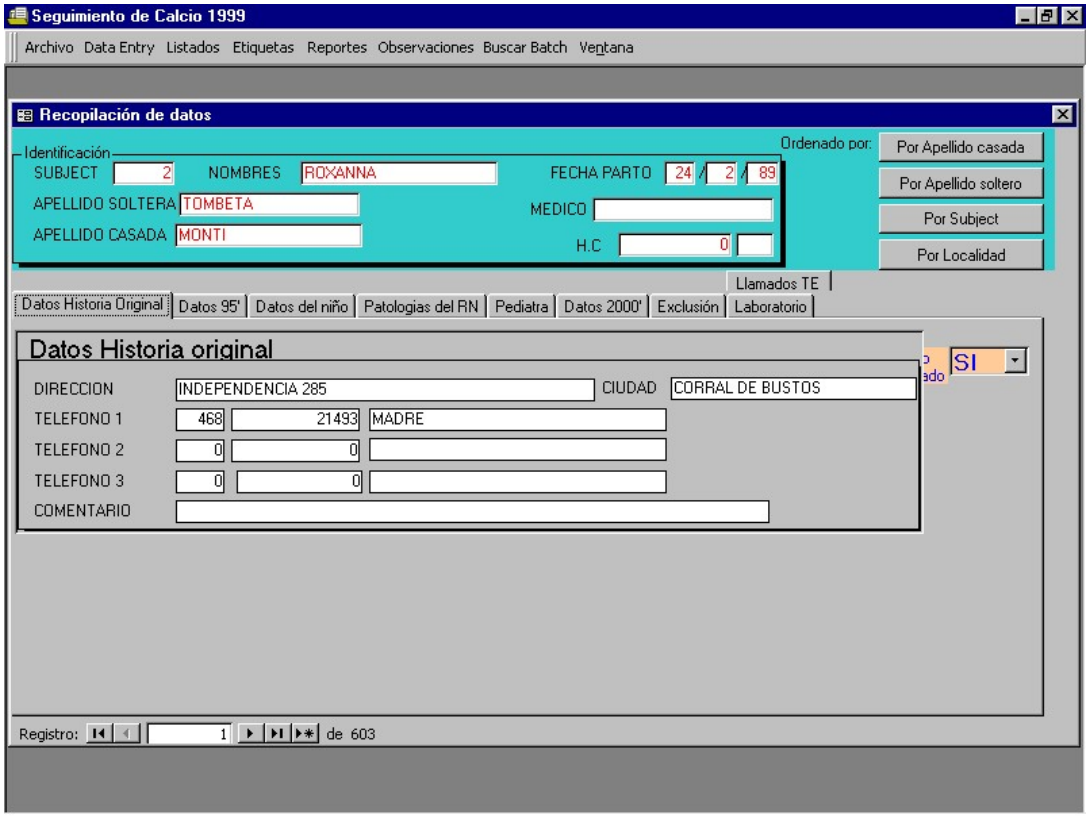
pressure was small. But they also found that vitamin D levels, commonly associated with bone strength, have an important role in blood pressure regulation. Higher blood vitamin D levels were associated with higher blood pressure, but only in children whose mother did not receive additional dietary calcium during pregnancy. This intriguing finding suggests that the effect of vitamin D on blood pressure is “programmed” during pregnancy, and that programming is affected by the amount of maternal calcium intake during pregnancy. These finding have important public health implications, as high blood pressure is a common problem in our countries, and this results can help scientists understand the origins of this disease, and can be an aid in the search of preventive measurements that can be implemented early in life, before the onset of the disease.

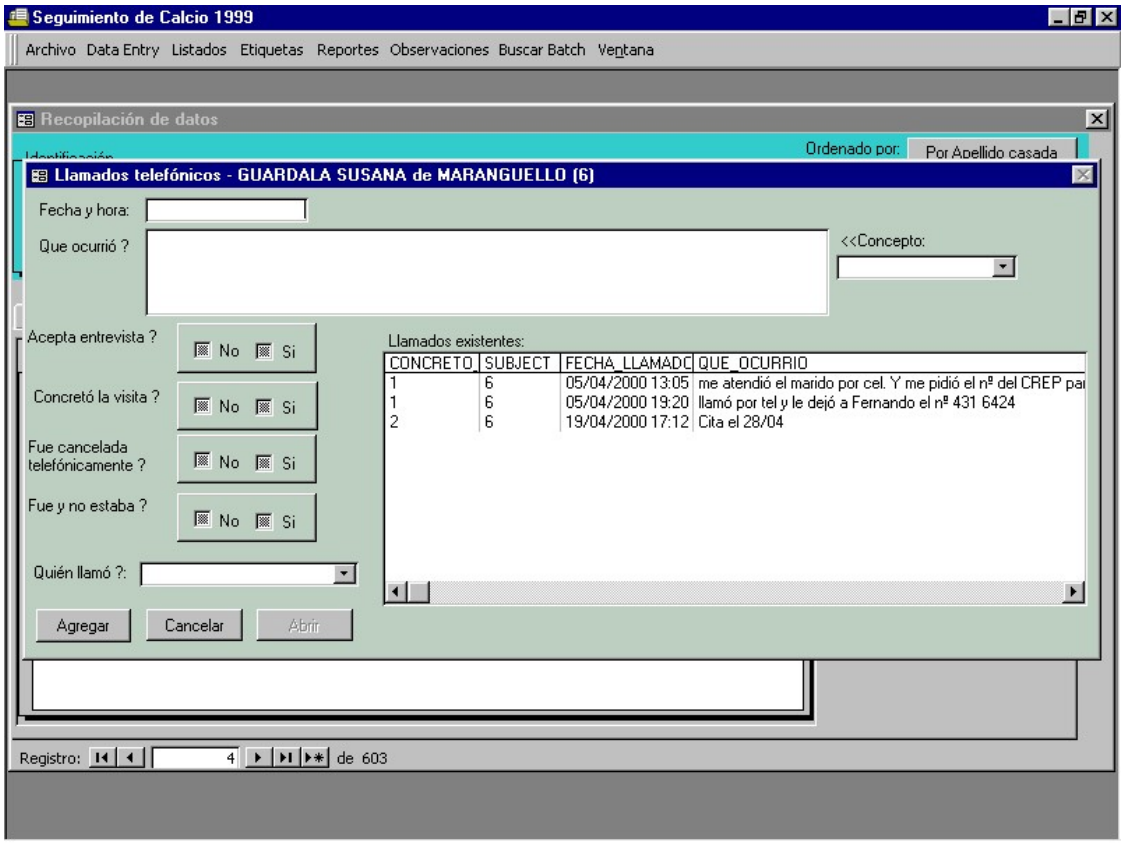
9 ANEXOS

9.1 LISTA DE ANEXOS

1. Screen shoots of the software developed to manage the study.
2. Information Brochures For Children (English Translation)
3. Information Brochures For Parents (English Translation)

9.2 APPENDIX 1. Software developed to manage the study.


Seguimiento de Calcio 1999
Archivo Data Entry Listados Etiquetas Reportes Observaciones Buscar Batch Ventana
Recopilación de datos
Identificación
SUBJECT 2
NOMBRES ROXANNA
APELLIDO SOLTERA TOMBETA
APELLIDO CASADA MONTI
FECHA PARTO 24 / 2 / 89
MEDICO
H.C 0
Ordenado por:
Por Apellido casada
Por Apellido soltero
Por Subject
Por Localidad
Llamados TE
Datos Historia Original
Datos 95'
Datos del niño
Patologias del RN
Pediatra
Datos 2000'
Exclusión
Laboratorio
Datos Historia original
SI
DIRECCION INDEPENDENCIA 285
CIUDAD CORRAL DE BUSTOS
TELEFONO 1 468 21493 MADRE
TELEFONO 2 0 0
TELEFONO 3 0 0
COMENTARIO
Registro: 1 de 603


Seguimiento de Calcio 1999
Archivo Data Entry Listados Etiquetas Reportes Observaciones Buscar Batch Ventana
Recopilación de datos
Ordenado por:
Por Apellido casada
Llamados telefónicos - GUARDALA SUSANA de MARANGUELLO (6)
Fecha y hora:
Que ocurrió ?
<<Concepto:
Acepta entrevista ? No Si
Concretó la visita ? No Si
Fue cancelada telefónicamente ? No Si
Fue y no estaba ? No Si
Quién llamó ?:
Llamados existentes:
CONCRETO SUBJECT FECHA_LLAMADO QUE_OCURRIO
1 6 05/04/2000 13:05 me atendió el marido por cel. Y me pidió el nº del CREP pa
1 6 05/04/2000 19:20 llamó por tel y le dejó a Fernando el nº 431 6424
2 6 19/04/2000 17:12 Cita el 28/04
Agregar
Cancelar
Abrir
Registro: 4 de 603

9.3 APPENDIX 2: Information Brochures For Children (English Translation)

THE CHILDREN WITH CALCIUM ARE HEALTHIER.

When your mother was pregnant, her doctor invited her to join a clinical research to know if calcium intake could prevent hypertension.

Calcium is a natural substance. An adequate calcium intake during pregnancy, in the foods or by pills, improves the health of mother and child.

Hypertension is a health problem, which affects a big proportion of the world population. Those people not suffering from hypertension have less probability of getting sick from the heart, and their veins and arteries are healthier.

You and your mother, together with another 599 children and their mothers, take part of a group that can help improve the health of millions.

CALCIUM PLAN I

The participation of your mother in the trial, when you were still in her womb, consisted in taking four pills of calcium every day.
This study showed that calcium helps on the improvement of the mother's health during pregnancy and delivery.

CALCIUM PLAN II

When you were between 6 and 8 years old we asked you and your mother again for some clinical measures, including a blood pressure measure.
Surprisingly, we found that those children whose mothers had calcium during pregnancy have less hypertensive problems.

CALCIUM PLAN III

In the third part of Calcium Plan we are proposed to know if the effect of calcium provided during pregnancy still benefits your health. In that case we could recommend that all mothers should take calcium for their children's health.

Now we want to invite you to keep on participating in this important study, not only for your health, but also for all the world's health.

We are asking for your contribution in measuring your weight, height, and blood pressure and taking a blood sample for biochemical measures.

Now you know you are part of The Calcium Plan. Thanks to your contribution we could improve the health of future generations.

9.4 APPENDIX 3: Information Brochures For Parents (English Translation)

CALCIUM PLAN

Calcium helps the mother during pregnancy
Her children are benefited for good.

Today we are inviting you to participate in a new step of the Calcium Plan.

CALCIUM AND PREGNANCY

Calcium is a natural substance present in the food, inexpensive and easily available. In the last few years important scientific studies, including the one carried out in our centre, in which you had participated, showed that an adequate calcium intake during pregnancy improves the health of the mothers and her children. These profitable effects in children could be extended and amplified for all their life.

CALCIUM PLAN I

When you were pregnant of your first baby you joined a clinical research aimed at preventing the development of hypertension during pregnancy and delivery.
So you started to be part of a collaborative group in the prevention of hypertension and the improvement of the quality of life.
Thanks to you and other families' contribution, it was demonstrated that an adequate calcium intake during pregnancy reduces the risk of development of hypertension during this stage.

CALCIUM PLAN II

Some years later, a group of English scientists showed that an adequate nutrition during pregnancy has long-term effects in children, modifying their predisposition for the development of illness not only during their childhood, but also during their adult life.
This evidence led us to check those children born during the Calcium Plan seven years later. Results of this second study were surprising: an increase in the calcium intake during pregnancy lowers the number of hypertensive children.

CALCIUM BENEFITS IN OFFSPRINGS

Recent studies suggest that the benefits of calcium intake during pregnancy increase as the child grows up.
Moreover, calcium taken at this stage could decrease the risk of hypertension during childhood.

Hypertension is a chronic illness, which can be hidden if pertinent studies are not performed for its identification. If hypertension is early recognised, it is possible to treat it and then prevent or minimise the damage that it causes. Nowadays there are some methods that can be used to detect the predisposition for the development of the disease, and then to take some preventive health measures.

These reasons led us to perform a new evaluation of those mothers and children that participated in the Calcium Plan I and II. Your contribution in this third stage – Calcium Plan III – will facilitate the evaluation of the benefits in children of an adequate calcium intake during pregnancy.

At the same time, it will be a unique opportunity for the present and future children health evaluation using biochemical measures, which are not usually available for the general population.

CALCIUM PLAN III

We are inviting you again to collaborate in the third stage of the Calcium Plan. Your participation consists in:

- ✓ A simple questionnaire,
- ✓ A weight and blood pressure measure from you.
- ✓ A weight, height, and blood pressure measure from your child.
- ✓ A blood sample from your child for biochemical analyses.

These biochemical results, will not only contribute to the science advancement and the improvement of the quality of life for the whole population, but also are a unique opportunity to know the health status of your child.

An adequate calcium intake during pregnancy could have preventive effects in the descendants: It could lower their risk for hypertensive diseases.

Children that participated in the Calcium Plan are today in their early teenage stage. It is interesting for us to observe if the fact of receiving calcium through their mothers during pregnancy still has beneficial effects in their health.

CREP

9.5 APPENDIX 4: Data Collection Form (English Version)

	CALCIUM PLAN III FOLLOW-UP 2000	Page 1

IDENTIFICATION

a) Form Code ☐☐☐☐

b) Study Number ☐☐☐

c) Subject Number in the original study ☐☐☐☐

GENERAL

-Mother's name and surname_____

Mother's address:_____

_____

Mother's phone number:_____

-Father's name and surname_____

Father's address:_____

_____

Father's phone number:_____

-Child's name and surname_____

Contact details: _____

_____

_____

-Paediatrician's name _____

Paediatrician's address:_____

_____

Paediatrician's phone number:_____

Date of Interview | Day ☐☐ | Month ☐☐ | Year ☐☐

DATA OF THE STUDY CHILD

1. Date of birth | Day ☐☐ | Month ☐☐ | Year ☐☐

2. Birthweight g. ☐☐☐☐

3. Sex ☐
1=Male 2= Female

4. a) ¿Did you breastfeed this child? ☐
1=NO 2=YES

If "YES",

b) ¿For how long? Months ☐☐

5. What was the last year that the child completed at school?

Year: ☐ Level:_____ ☐☐

6. Since birth, has the child had renal calculi? ☐
1= NO 2= YES

7. Since birth, has the child had gall-bladder calculi? ☐
1=NO 2=YES

8. a)Has the child had hypertension? ☐
1=NO 2=YES

If "YES",

b) ¿Since when? | Month ☐☐ | Year ☐☐

c) Treatment
- c.1) Diet ☐
- c.2) Antihypertensive drugs ☐
- c.3) Diuretics ☐
- c.4) Other, specify_____ ☐

9. a) Has the child had diabetes? ☐
1=NO 2=YES

If "YES",

b) ¿Since when? | Month ☐☐ | Year ☐☐

c) Treatment
- c.1) Diet ☐
- c.2) Insulin ☐
- c.3) Oral Hypoglicemic drugs ☐
- c.4) Other, specify_____ ☐

10.a)Since birth has child had any major illness, surgery or health problem?

1= NO 2=YES

If YES

b.1.1) First Illness_____

b.1.2) Age of beginning:

Years Months

Comments: _____

_____

b.2.1) Second Illness_____

b.2.2) Age of beginning:

Years Months

Comments: _____

_____

11. a)Is the child under any medical treatment?

1=NO 2= YES

If "YES",

b.1.1) First treatment_____

b.1.2) Since when?

Month	Year

Comments: _____

_____

b.2.1) Second treatment_____

b.2.2) Since when?

Month	Year

Comments: _____

_____

b.3.1) Third treatment_____

b.3.2) Since when?

Month	Year

Comments: _____

_____

12. a) In the last month before this interview was the child sick? (do not include diseases listed in questions 6 to 11)

1=NO 2= YES

If "YES"

b) Specify

b.1)_____

b.2) _____

b.3) _____

DATA FROM THE MOTHER

13.Maternal age years

14. Who lives with you in your home?
1= NO 2= YES

a) child's biological father

b) child's foster father

c.1) child's siblings

If "YES"

c.2) How many?

d.1) Others

If "YES"

d.2) How many?

15. How many years did you complete education?

Year: Level: _____

16. What is your occupation_____

17. Are you currently employed?
1=NO 2=YES

18. a) Have you had hypertension?
1= NO 2= YES

if "YES"

Month	Year

b) Since when?

c) Treatment

c.1) Diet

c.2) Antihypertensives drugs

c.3) Diuretics

c.4) Others

19. a) Have you had diabetes?
1= NO 2= YES

If "YES"

Month	Year

b) Since when?

c) Treatment

c.1) Diet

c.2) Insulin

c.3) Oral hipoglicemic drugs

c.4) Others

DATA FROM THE FATHER

20. How many years did the child's father complete of education?

Year: Level: _____

21. What is his occupation_____

22. Is he currently employed?
1=NO 2=YES

23. Father's height cm.

24. Father's weight Kg.

25. a) Has the child's father had hypertension?
1= NO 2= YES

if "YES"

Month	Year

b) Since when?

c) Treatment

c.1) Diet

c.2) Antihypertensives drugs

c.3) Diuretics

c.4) Others

26. a) Has the child's father had diabetes?
1= NO 2= YES

If "YES"

Month	Year

b) Since when?

c) Treatment

c.1) Diet

c.2) Insulin

c.3) Oral hipoglicemic drugs

c.4) Others

PHYSICAL EXAMINATION

27. MATERNAL examination:

MEASURE		Result 1	Result 2	Result 3
a) Weight	Kg.	☐☐☐.☐		
b) Triceps Skinfold	mm.	☐☐.☐		
c) Subscapular Skinfold	mm.	☐☐.☐		
d) Height	cm	☐☐☐.☐		
e) Blood pressure				
Systolic blood pressure		☐☐☐ mmHg	☐☐☐ mmHg	☐☐☐ mmHg
Diastolic blood pressure		☐☐☐ mmHg	☐☐☐ mmHg	☐☐☐ mmHg

28. CHILD examination

a) Weight	Kg.	☐☐☐.☐
b) Abdominal circumference	cm	☐☐.☐
c) Triceps Skinfold	mm.	☐☐.☐
d) Subscapular Skinfold	mm.	☐☐.☐
e) Height	cm	☐☐☐.☐
f) Blood pressure		
Systolic blood pressure		☐☐☐ mmHg
Diastolic blood pressure		☐☐☐ mmHg

	Result 2	Result 3
Systolic blood pressure	☐☐☐ mmHg	☐☐☐ mmHg
Diastolic blood pressure	☐☐☐ mmHg	☐☐☐ mmHg

Comments

BIOCHEMICAL MEASSURES

29) Biochemical meassures

a) Parathyroid Hormone (PTH): ☐☐☐ pg/ml

b) Calcitriol: ☐☐☐ ng/ml

c) Calcitonin Gene Related Peptide (CGRP): ☐☐☐ pg/ml

d) Parathyroid Hypertensive Factor (PHF): ☐☐☐☐ mmHg

e) Growth Hormone: ☐☐☐ ng/ml

f) Insulin-like Growth Factor I (IGF-I): ☐☐ ng/ml

g) Insulin-like Growth Factor Binding Protein-1 (IGFBP-1): ☐☐☐ ng/ml

h) Insulin-like Growth Factor Binding Protein-1 (IGFBP-3): ☐☐☐☐ ng/ml

i) Serum Total Calcium: ☐☐☐ mg/dl

j) Serum Ionised Calcium: ☐☐☐ mg/dl

k) Urinary Calcium: ☐☐☐☐ mg/dl

l) Cholesterol: ☐☐☐ mg/dl

m) Triglycerides: ☐☐☐ mg/dl

n) Glycemia: ☐☐☐ mg/dl

o) Urinary Creatinine: ☐☐ mg/dl

p) Gucose in Urine: ☐☐☐ mg/dl

q) Hemoglobin: ☐☐☐ g/dl

r) Hematocrit: ☐☐ %

s) Leucocyte: ☐☐☐☐☐☐ (per mm3)

t) Platelets: ☐☐☐☐☐☐ (per mm3)

u) Hemoglobin A1c: ☐☐☐ %