Leptin Predicts Bone and Fat Mass after Accounting for the Effects of Diet and Glucocorticoid Treatment in Piglets[1]

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The role of leptin in neonatal growth and bone metabolism has been investigated, but not simultaneously. The objectives of this study were to determine if leptin relates to bone mass during rapid growth; if consumption of maternal milk is related to elevated circulating concentrations of leptin resulting in higher fat mass; and if glucocorticoids result in higher fat mass and reduced bone mass due to elevated leptin. Thirty-two piglets were randomized to either a suckling or milk substitute plus either dexamethasone (DEX) or placebo injection for 15 days beginning at 5 days of age. Milk and blood samples were obtained at baseline, and after 15 days, blood was sampled again for measurement of leptin and bone biochemistry. Weight at baseline plus weight and length after 15 days were recorded, followed by measurement of whole body bone mineral content, bone area, and fat mass using dual energy x-ray absorptiometry. At baseline, plasma leptin was elevated in suckled piglets. Piglets that suckled had elevated fat mass as did those who received DEX. However, DEX resulted in suppressed weight and length, bone mass, and bone metabolism. Leptin was similar among groups after the 15 days. After accounting for body size and treatment effects, piglet plasma leptin was predictive of bone and fat mass. Leptin circulating early postnatally is linked to body composition, specifically fat and bone mass. Elevations in fat mass and reductions in bone mass observed after 15 days of DEX treatment are not related to leptin metabolism. Both human and porcine neonates share similar characteristics with respect to relationships of leptin with fat and bone mass.

[Exp Biol Med Vol. 227(8):639–644, 2002]

Key words: fat mass; bone mass; leptin; dexamethasone; piglet; growth

Mother's milk is set as the gold standard for nourishing the term-born infant, with the addition of multinutrient fortifiers recommended as the feed of choice for premature infants during hospitalization (1). Term-born infants fed mother's milk have higher fat mass within the first few months of life (2–5) than those fed formula, but this does not continue to 1 year of age (2, 3). Prematurely born infants are reported to have higher fat mass than expected by term age (6), and those fed mother's milk are reported to have higher fat mass by 1 year of age (7). In addition, prematurely born infants recovering from chronic lung disease treated with dexamethasone (DEX) and fed formula experience delay in growth to at least 3 months corrected age and have greater fat mass than expected (8). Similarly, in DEX-treated piglets fed formula, a delay in whole body growth is observed, as well as elevations in percentage of body fat mass after only 15 days (9). In both infants (10) and piglets (11, 12), bone metabolism is altered and bone mass is reduced by DEX treatment.

The physiological mechanisms behind infant growth patterns and bone mass resulting from feeding maternal or artificial milk substitutes (2–5) as well as treatment with glucocoricoids (8–12) are not clear, but may be related to leptin. Leptin, a hormone produced primarily in mature adipose tissue but also in placenta and mammary epithelial cells (13), is positively correlated with fat mass and percentage body fat (14) and is inversely related to gain in weight, length, and head circumference over the first 4 months of life, independent of birth weight (15). Leptin in mother's milk correlates to that in infant blood, suggesting a link between mother's milk and body composition (16). In addition, leptin is up regulated by glucocorticoid treatment whether studied *in vivo* (17, 18) or *in vitro* (19). Relationships among leptin, growth, and body fat mass in response to the glucocorticoid DEX and type of feeding has not been reported in human infants.

Recently, leptin has been studied for a role in regulating bone metabolism. A high bone mineral mass phenotype combined with whole body stunting is observed in *ob/ob*

[1] This research was funded by The Natural Sciences and Engineering Council of Canada.

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Received October 12, 2001.
Accepted April 11, 2002.

1535-3702/02/2278-0639$15.00

mice where leptin signaling is absent (20). Treatment with leptin reduces bone mass in older *ob/ob* mice (20), but normalizes bone growth and mineral mass in younger mice (21), suggesting a role in bone growth and modeling. In human neonates, bone metabolism is also linked to leptin whereby as leptin increases with gestation, formation of and breakdown of bone collagen matrix decreases (22). Because glucocorticoids elevate leptin (14–16), it is plausible that leptin may be involved in the mechanisms behind the observations of elevated body fat mass and reduced growth and bone mass caused by treatment with DEX.

Therefore, the objectives of this study were to determine if plasma leptin relates to bone mass during rapid growth; if consumption of maternal milk is related to elevated circulating concentrations of leptin resulting in higher fat mass; and if glucocorticoids such as DEX result in higher fat mass and reduced bone mass through elevation in circulating concentrations of leptin. The piglet model was selected to investigate the objectives based on the previously reported similarities in response of growth, and bone and fat mass to DEX in human infants (8, 10) and piglets (9, 11).

Methods

Animals and Care. At 3 days of age, 32 male piglets were randomized to receive 15 days of treatment to commence at 5 days of age. At 3 days postpartum, a sample of sow's milk was obtained from four of the eight sows and at 5 days postpartum in two sows to provide for measurement of leptin. Treatments were diet (suckling or substitute milk) and drug (placebo or DEX). Artificially fed piglets were transported from the Glenlea Swine Research Unit (University of Manitoba Research Station) to the Main Campus of the University of Manitoba on Day 3 of life. The suckling piglets remained at the Swine Research Unit. Both housing facilities used a 16:8-hr light:dark cycle. The artificially fed piglets were taught to lap the substitute milk between Days 3 and 5 of life and were permitted this amount of time to adapt to diluted formula (50% strength initially using distilled water), and then full-strength milk without symptoms of diarrhea. All procedures were in agreement with the *Guide for the Care and Use of Experimental Animals* (23) and were approved by the University of Manitoba Protocol Management and Review Committee. To control for stress during transportation (20 min between facilities) and changes in housing, the formula-fed piglets were transported at Day 3 in a warmed container maintained at 30°C and capable of transporting eight piglets (<3 kg each) and they were then permitted a 2-day adaptation period to the single housing, necessary to monitor feeding, prior to commencement of the treatments. Initially, the piglets are group housed, by litter, for 4–6 hr after transportation to observe the response to transportation and to begin feeding. The piglets maintained by the sow were housed in groups of eight to 10 until the evening of the 15th day of study, when they were transported in groups of two per container (previously used daily for attainment of body weight) followed by group housing, by litter, without the sow until necropsy.

Starting on Day 5 of life, the DEX dosing regimen was 0.5 mg kg^{-1} day^{-1} for the first 5 days, followed by 0.3 mg kg^{-1} day^{-1} and then 0.2 mg kg^{-1} day^{-1} for 5 days each. This regimen was designed to result in a cumulative dosage of DEX that mimics that delivered to preterm infants ~6 mg/kg (10, 24). The placebo drug was an equal volume of saline (0.9% by weight). Both treatments were delivered intramuscularly in half-dosages at 0900 and 2100 hr.

The substitute milk contained 60 g/l protein, 62 g/l carbohydrate, 62.5 g/l fat, 2.3 g/l calcium, and 1.8 g/l phosphorous. The energy (1005 kcal/l) and linoleic acid (11.7% of energy) were within recommended limits for healthy growing piglets between 3 and 10 kg as set by the National Research Council (25). The amount of fat in the substitute milk used in the present study was similar to sow's milk (62 g/l as measured in milk from the Glenlea Research Unit), and contained enough linoleic acid (13.1 g/l) to support growth. The milk was fed at 350 ml kg^{-1} day^{-1}, divided into equal amounts at 0900, 1500, and 2100 hr for the duration of the study. Artificially fed piglets were housed individually in stainless steel cages, but were group housed for 3–5 hr/day between feedings. Ambient temperature was maintained at 29° to 30°C.

On the evening of the 15th day of study, the suckling piglets were transported to the main campus of the University of Manitoba and were group housed under the same ambient conditions as the formula-fed piglets. At 2100 hr, all food and water was withdrawn from all housing. Piglets were then anesthetized using sodium pentobarbital (30 mg/kg of 65 mg/ml somnotol) on the 16th day at 0830 hr.

Growth. Weight was measured daily in the nonfed state to the nearest gram using a scale with an animal weighing program (Mettler-Toledo Inc., Hightstown, NJ) and average daily weight gain was calculated (g kg^{-1} day^{-1}). Snout to tail length was measured to the nearest millimeter using a nonstretchable measuring tape at the end of study while pigs were anesthetized.

Tissue Samples and Bone and Fat Mass. Blood was taken at 0900 hr into heparinized syringes on the first day of study just prior to treatment using the internal jugular blind stab technique to permit measurement of cortisol used as an index of stress induced by the study procedures. Blood was taken again at 0900 hr on the 16th day of study into heparinized syringes, followed by sodium pentobarbital overdose. Plasma was obtained by centrifugation at 2000*g* for 20 min at 4°C. Plasma was centrifuged again to remove any remaining red blood cells and was stored at −80°C until analysis of osteocalcin, cortisol, and leptin. Piglet carcasses were then transported to the dual energy x-ray absorptiometer (QDR4500W; Hologic Inc., Waltham, MA). Single scans were completed to determine whole body bone mineral content and area as well as whole body fat mass (software version V8.16a:5). All scans were performed with the piglet in the anterior-posterior position with limbs extended.

Biochemical Assessment of Bone Metabolism. Plasma osteocalcin was analyzed by radioimmunoassay (DiaSorin, Stillwater, MN). Plasma cortisol was measured using an enzyme-linked immunosorbent assay (ELISA; Cedarlane Laboratories, Hornby, Ontario, Canada). Urinary *N*-telopeptide was measured using an ELISA (Osteomark, Ostex, Seattle, WA). *N*-telopeptide was corrected to creatinine as determined by the Jaffe method (procedure no. 555; Sigma-Aldrich, Oakville, Canada). Plasma and milk leptin were measured using a radioimmunoassay (multi-species leptin; Linco Research Inc., St. Charles, MO).

Statistical Analysis. All data are the mean ± SD unless otherwise stated. Differences observed between groups were detected using two-way analysis of variance (ANOVA) with factors being drug (placebo or DEX) and feeding (suckling or standard formula) with values of $P < 0.05$ accepted as significantly different. *Post hoc* analysis with Student-Newman-Keuls all-pairwise test was used to identify differences between the treatment groups. Two-tailed Pearson correlation analysis was used to detect relationships among leptin and measures of fat mass and bone mass. To learn of the importance of leptin after accounting for other variables such as weight, length, feeding, and treatment group, multiple linear regression was conducted as well. To determine the best format to express treatment with DEX, the relationship of cumulative milligrams of DEX or treatment as categorical variables 0 (placebo) or 1 (DEX) to the main outcomes of bone mineral content, bone area, and body fat (%) were assessed using Pearson correlation coefficients. Despite the fact that both diet and DEX were categorical data, linear regression was used because the dependent variables were all continuous data.

Results

Piglets were of equal age, healthy, and of similar weight at baseline. Over the course of study, the median cumulative DEX dosage was 16.7 mg (interquartile range from 15.6 to 17.9 mg), resulting in significant effects on growth, bone and fat mass, as well as bone metabolism. Weight, average daily weight gain, and length were significantly reduced by DEX and fat mass was elevated by both DEX and consumption of sow's milk (Table I).

At baseline, piglets fed substitute milk ($n = 16$) had lower plasma leptin than those that suckled ($n = 8$, 107.9 ± 43.9 p*M* vs 175.9 ± 65.5 p*M*, $P = 0.006$). The lower values in the formula group did not likely resulting from stress because the cortisol values, although highly variable, were lower as well (90.2 ± 56.3 m*M* vs 151.5 ± 76.1 m*M*, $P = 0.034$). The average concentration of leptin in sows' milk ($n = 6$) 3–5 days postpartum was 732.5 p*M* ± 160 p*M*. No differences among feeding or treatment groups were detected in plasma leptin values obtained at the end of study, but cortisol was reduced by DEX (Table II).

In regard to bone, whole body bone mineral content and area were reduced by DEX treatment, but bone mineral content was elevated with consumption of sow's milk (Table II). Plasma osteocalcin was reduced by DEX treatment, but was elevated in the formula-placebo group, as was urinary *N*-telopeptide (Table II).

Correlation analysis (Table III) using sow's milk leptin obtained 3–5 days postpartum and piglets plasma leptin Day 0 or 15 of treatment with Day 15 measures of whole body bone mineral content, bone area, and fat (%) resulted in detection of equally strong relationships between sow's milk leptin and piglets plasma leptin Day 0 with bone mineral content. Bone area was only associated with sow's milk leptin, and fat (%) was only associated with baseline leptin (Day 0). Expression of DEX as categorical data or cumulative milligrams received demonstrated similar relationships to all outcome; however, the relationship was usually stronger when expressed as categorical data. Thus, categorical data was used in the regression analysis.

Whole body bone mineral content was best predicted by the regression line $y = 39.6 - 9.5$ (DEX = 1, placebo = 0) − 8.9 (sow = 0, formula = 1) + 11.4 (kilograms at baseline) + 0.08 (picomoles leptin at baseline), where all variables contributed significantly, and $P \leq 0.02$ and $R_{adjusted} = 0.72$, $n = 24$. In a suckled placebo piglet weighing 2 kg at baseline and using average plasma leptin of 176 p*M*, the equation for bone mineral content is: 39.6 − 9.5(0) − 8.9(0) + 11.4(2 kg) + 0.08(176 p*M*) = 76.5 g, which is very close to the average for the group at 78.6 g. The relative contribution of leptin is 14.08 g or 18% compared with 22.8 g or 29.8% due to body size. Whole body bone area was best predicted $y = 75.5 - 15.9$ (suckled = 0, formula

Table I. Weight, Weight Gain, and Length in Piglets That Were Suckled or Fed a Substitute Milk and Either Placebo or DEX Treatment for 15 Days

	Suckling		Substitute milk	
	Placebo	DEX	Placebo	DEX
Weight Day 15*† (kg)	5.8 ± 0.8^a	4.6 ± 0.8^b	6.5 ± 1.1^a	5.4 ± 0.5^b
Weight gain* (g kg^{-1} day^{-1})	57.0 ± 8.4^a	48.7 ± 6.1^b	60.0 ± 5.0^a	49.6 ± 3.7^b
Length Day 15* (cm)	55.8 ± 2.4^a	49.8 ± 2.2^b	57.4 ± 2.7^a	52.3 ± 1.4^c
Body fat Day 15 (% of weight)**γ	16.2 ± 3.4^a	19.0 ± 3.7^b	12.5 ± 1.1^c	14.7 ± 1.7^{ac}

Note. Values are mean ± SD, $n = 8$ per group. In rows, values with different superscripts are significantly different, $P < 0.05$.
* $P < 0.05$; ** $P < 0.01$, main effect of treatment.
γ $P < 0.01$, main effect of diet.
† $P < 0.05$, interaction between diet and treatment.

Table II. Bone Mineral Metabolism and Content in Piglets That Were Suckled or Fed a Substitute Milk and Either Placebo or DEX Treatment for 15 Days

	Suckling		Substitute milk	
	Placebo	DEX	Placebo	DEX
Bone mineral metabolism				
Plasma leptin (pM)	77.7 ± 17.8	71.8 ± 21.5	60.6 ± 24.2	73.9 ± 31.1
Plasma osteocalcin*† (n*M*)	18.4 ± 10.5[a]	11.7 ± 6.2[a]	29.1 ± 12.0[b]	14.8 ± 8.4[a]
Plasma cortisol** (m*M*)	161.7 ± 82.2	28.1 ± 21.5	172.7 ± 107.3	29.8 ± 21.2
Urinary *N*-telopeptide:creatinine*† (μ*M*:m*M*)	4.80 ± 1.26[a]	3.48 ± 2.15[a]	7.58 ± 1.13[b]	3.94 ± 0.64[a]
Bone				
Whole body bone mineral content*† (g)	78.6 ± 9.9[a]	61.4 ± 15.1[b]	66.3 ± 5.1[b]	58.3 ± 3.9[b]
Whole body bone area* (cm^2)	145.4 ± 20.5[a]	113.0 ± 20.5[b]	136.7 ± 16.0[a]	115.6 ± 9.0[b]

Note. Values are mean ± SD, $n = 8$ per group. In rows, values with different superscripts are significantly different, $P < 0.05$.
* $P < 0.05$; ** $P < 0.001$, main effect of treatment.
† $P < 0.05$, main effect of feeding.

Table III. Relationships Between Leptin and DEX with Whole Body Bone Mineral Content, Bone Area, and Percentage of Fat

Piglet	Leptin			DEX	
	Sows' milk	Piglet plasma			
		Day 0	Day 15	DEX = 1 and placebo = 0	Cumulative (mg)
Bone mineral content (g)	0.57, 0.004	0.58, 0.003	−0.06, 0.75	−0.52, 0.003	−0.42, 0.017
Bone area (g/cm^2)	0.60, 0.004	0.28, 0.21	−0.12, 0.52	−0.63, 0.001	−0.58, 0.001
Fat (%)	−0.32, 0.12	0.47, 0.022	0.15, 0.42	0.36, 0.04	0.36, 0.041

Note. $n = 32$ piglets with exception of 24 piglets for relationships to sows' milk leptin and pretreatment piglet plasma leptin. Values are Pearson correlation coefficients followed by *P* values.

= 1) + 13.8 (weight in kilograms on Day 15) − 0.2 (picomoles leptin on Day 15), where all variables contributed significantly, and $P < 0.05$ and $R_{adjusted} = 0.57$, $n = 24$. If a suckled placebo piglet weighing 5.8 kg on Day 15 with leptin of 78 pM is substituted into the equation, area = 75.5 15.9(0) + 13.8(5.8 kg) − 0.2 (78 pM) = 171.1 cm^2 in comparison with the average for this group of 145.4 cm^2. The relative contribution of weight is 80.0 cm^2 or 46.5%, and of leptin is 15.6 cm^2 or 9.1%. Whole body fat mass (%) was best predicted by $y = 14.7 + 1.5$ (DEX = 1, placebo = 0) − 3.7 (suckled = 0, formula = 1) + 0.02 (picomoles leptin Day 15), where all variables contributed significantly, and $P \leq 0.03$ and $R_{adjusted} = 0.68$, $n = 24$. If a suckled placebo piglet is substituted into the equation, fat (%) = 14.7 + 1.5(0) − 3.7(0) + 0.02 (78 pM) = 13.1% compared with the average of 16.2%. The relative contribution of leptin is 1.6% fat or 12.2% of the total value.

Discussion

The link between leptin and bone is relatively new in comparison with the more intensively investigated link to body fat. In this study, after accounting for the effects of body size and treatments, piglet plasma leptin at baseline was predictive of whole body bone mineral content, and bone area was predicted by leptin measured at the end of treatment. Thus, plasma leptin appears to relate to bone mass during growth and predicts 18% of bone mineral content in non-DEX-treated healthy suckled piglets.

All of the significant correlations between bone mass and leptin were positive. Steppan *et al.* (21) show in young *ob/ob* mice that leptin treatment elevates bone size, bone mineral content, and density, indicating that leptin is needed for bone growth and modeling. In contrast, Ducy *et al.* (20) describe the *ob/ob* mouse as having high bone mass phenotype. Treatment with leptin for 28 days in 3-month-old *ob/ob* mice results in bone loss thought to be due to inhibition of formation rather than stimulation of resorption. In human fetuses (22), leptin is negatively associated with bone metabolism (assessed using propeptide of type I collagen), suggesting a role in osteoblast metabolism or collagen formation. This relationship is also observed in postmenopausal women (26). Osteocalcin, another peptide produced by the osteoblast, is not related to leptin in postmenopausal women (27). The role of osteocalcin in bone metabolism is thought to reflect total osteoblast activity, including matrix synthesis and mineralization. In this study, propeptide of type I collagen was not measured as the assay was not linear after sample dilution. In the piglets, a negative relationship was observed at Day 15 between leptin and osteocalin ($r = -0.46$, $P = 0.01$, data not shown). This is consistent with the inhibition of formation by leptin reported by Ducy (20) in *ob/ob* mice treated with leptin. Thus, the young mice studied by Steppan (21), the human fetuses studied by Ogueh *et al.* (22), the women studied by Rauch *et al.* (26), and the piglets in this study support the thesis that leptin has a role in bone metabolism.

It is interesting that serum leptin concentrations in this study and also in prepubertal females (28) relate to bone area. Matkovic *et al.* (28) suggest that leptin causes larger bone area as a result of enhanced formation at the periosteal envelope. It is likely that the larger bone area results from widening of the bone because in the piglets, those that suckled tended to be shorter but had higher bone area than those fed formula. In healthy children of normal weight, bone area is enlarged in relation to body fatness when the effect of weight and height is accounted for (29). Because leptin is associated with body fat (28) as well as bone area, the elevation in leptin may be part of a mechanism to explain the enlarged bone area with elevated fat mass. Thus, it is speculated that when leptin is elevated, bone size advances and when leptin decreases, mineralization proceeds.

In human infants, plasma leptin is similar among those fed mother's milk or a substitute (30). However, the piglets fed formula in our study had lower plasma leptin at baseline than the suckled piglets. Because plasma leptin at baseline was predictive of subsequent bone mineral content, and artificial rearing resulted in lower leptin, it is reasonable to postulate that changes to diet early postpartum impact on subsequent bone development. Whether this is mediated by maternal leptin or some factor(s) related to the differences in maternal and substitute milk is unclear. It is possible that the higher plasma leptin in the suckled group was due to absorption of leptin from the sow's milk because Casabiell *et al.* (31) report that milk leptin is absorbed intact in suckling rats. In the piglets, those fed formula had lower values after only 2 days away from the sow. We speculate 2 days would have adequately exceeded the half-life for absorbed leptin. The fact that in humans, maternal milk feeding does not cause higher leptin may be time dependent.

In all piglets, plasma leptin declined with advancing age, as observed in human term and preterm infants (32). The concentration of leptin in our sow's milk is very similar to that reported in human milk [33] and sow's milk reported by Estienne *et al.* [34]. In contrast to serum leptin concentrations, sow's milk leptin is not related to body fatness (measured as backfat). Interestingly, sow milk leptin concentration declines after the first week of lactation [34]. Thus far, human milk leptin has not been measured earlier than the first 2 weeks of lactation [30, 33]). Human milk during mid-lactation (2–4 months) has a 10-fold lower concentration of leptin than infant plasma, and milk leptin is suggested to play a minor or nonexistent role in modulating growth (30). However, in our study, the values for milk are 1- to 5-fold higher than piglet plasma leptin measured at the same time of day and in early lactation, suggesting a potential role in modulating growth during the first week of life. It is possible that the role of leptin in swine physiology is different than human because in humans, maternal body mass index is associated with milk leptin (33) (note: authors did not state period of lactation at sampling). Resto *et al.* (35) report that at least by 2 weeks lactation, leptin remains constant, but reports of milk leptin throughout the transition period do not yet exist.

In human infants, leptin is similar in those breast fed or formula fed (30) at a time when body fat is typically higher in breast-fed infants (5). Thus, it is unlikely that the leptin was higher in the piglets at baseline due to higher fat mass. In fact, at the end of this study when fat mass was high, leptin was not greater with suckling or DEX treatment, but fat mass was. In contrast to evidence in pigs where maternal nutrition during gestation programs for leptin metabolism in the offspring (36), the effects of diet and DEX postnatally do not. Evidence for the concept of dietary programming of physiology and growth is expanding (37).

The piglets in this study grew rapidly when consuming either sow's milk or the substitute milk. Treatment with DEX lead to elevated body fat mass and reduced growth and whole body bone mineral content as expected (9), but did not affect leptin 15 days later. Post-treatment, blood was sampled at a time when leptin would be lowest in the day (38), and thus, an elevation in leptin may only have been detected if measured later in the day. Recently, Russell *et al.* (19) provide evidence that DEX, in culture, stimulates release of leptin from subcutaneous adipose cells but not of omental adipose cells. This may explain lack of elevation in leptin post-DEX treatment in the piglets because previously, the majority of fat responsible for elevation in body fat was located in the abdomen (9) where omental adipose is deposited. It is also possible that the stimulatory effect of DEX was not observed as a result of adaptation. Elevation in leptin is observed after 1 to 7 days of glucocorticoid treatment in healthy human adults (17, 39) and growing rats (40), but the effect is not prolonged with chronic treatment (39, 40). Thus, the mechanisms behind the piglets' greater fat mass with suckling and DEX are not likely due sustained elevations in leptin.

In summary, leptin circulating in neonatal piglets is linked to body fat and bone mass. Elevated fat mass, resulting from DEX treatment, was not due to altered leptin metabolism, but rather was with linked with maternal milk just as in human infants (2, 5). The pig, like the human infant, has elevated leptin near birth that declines thereafter (32, 41). Thus, the piglet appears to be a good model for further understanding of the relationships among infant nutrition, growth, and body composition.

This study was funded by the National Science and Engineering Research Council (NSERC) of Canada. Ms. J. Adolphe was awarded a Summer NSERC Scholarship and Ms. C. Murdock and H. Kovacs both were awarded Summer Research Scholarships by the Children's Hospital Foundation of Manitoba. Dr. Weiler is in receipt of a New Investigator Salary Award from the Canadian Institutes of Health Research.

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