Association between Retinal Neovascularization and Serial Weight Measurements in Murine and Human Newborns

Short title: Weight gain and retinal neovascularization

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The murine study was approved by the Institutional Review Board, Centre for Molecular and Vascular Biology, University of Leuven. Experiments were performed in compliance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

For the human study, the protocol was approved by the Institutional Review Boards of each study center (EudraCT number, 2004-002170-34). Written informed consent of at least one of the parents was an inclusion criterion.

Abstract

Introduction

Retinopathy of prematurity (ROP) is a leading cause of preventable blindness throughout the world. Several risk factors have been studied, but most studies remain inconclusive. Over the last years, evidence is accumulating that one of the strongest predictors of ROP, in addition to oxygen use and low gestational age, is poor weight gain during the first postnatal weeks.

Methods

In a prospective study, we sought to determine the importance of serial weight measurements to help predict neovascularization (NV). In a first stage, a summary of the response in each case is identified and calculated as area under the curve (AUC). In a second stage these different AUC's are analyzed by non- parametric Mann-Whitney U test. For the murine study, pups were redistributed in smaller and larger litters. On postnatal day (P)7-12, the oxygen-induced retinopathy (OIR) model was applied. Body weight was measured on P7, P14 and P17. Retinal NV was assessed on P17. For the human study, the subjects were part of the control arm of the NIRTURE trial. Ophthalmologists screened for ROP. Birth weight was recorded. Weekly weight measurements were performed for the first 4 weeks.

Results

AUC of serial weight (gain) measurements was significantly lower in those murine (14 vs. 17 g; $p=0.01$) and human (140g/wk vs. 240g/wk; $p=0.0001$) newborns developing retinal NV.

Conclusion

This prospective study supports previous findings - however by using a new way of statistical analysis - that early postnatal weight gain is an important indicator in the development of neovascular disease.

Keywords

retinal – neovascularization – weight – mice – newborns

Introduction

Retinopathy of prematurity (ROP) is a neovascular eye disease affecting the retina in premature infants and is the leading cause of preventable blindness in children throughout the world. Most cases are mild and regress spontaneously without treatment, however they may lead to lifelong and irreversible cyto-architectural and functional alterations. Severe cases of ROP can lead to retinal detachment and consecutive blindness. Timely detection and early treatment significantly reduces the incidence of blindness, but does not normalize the ophthalmological status as reduced visual acuity, visual fields, contrast sensitivity, refractive errors and strabismus are common in these children. Therefore, prevention is sorely needed (1).

Several risk factors that might contribute to ROP development have been studied over the past years, but most studies remain inconclusive. At present, oxygen use and low gestational age still remain the major risk factors for developing ROP. Over the last years however, evidence is accumulating that one of the strongest predictors of ROP, in addition to oxygen use and low gestational age, is poor weight gain during the first weeks of life (2-15). A few reports have shown a relationship between poor postnatal weight gain and an increased risk of developing ROP, as judged by absolute or relative weight gain during the first 6 weeks of life (3, 4, 7). A new diagnostic tool based on weekly neonatal measurements of body weight and serum IGF-I levels, was shown to be highly predictive of ROP development (6, 8, 10, 12, 14).

In this prospective study, we sought to determine the importance of serial weight measurements to help predict neovascularization (NV) in a strictly controlled animal experimental setting as well as in a clinical setting with more interfering variables.

Materials & methods

In a prospective study, we sought to determine the importance of serial weight measurements to help predict NV in murine as well as in human newborns. In a first stage, a summary of the response in each case is identified and calculated as area under the curve (AUC). In a second stage these different AUC's are analyzed by non-parametric Mann-Whitney U test. The design of both murine and human study will be explained below.

1. Murine study

The murine oxygen-induced retinopathy (OIR) model of Smith was applied (16). In brief, 7-day-old C57BL/6J WT mice with their dams were exposed to 75% oxygen for 5 days; on postnatal day (P)12 the mice were returned to room air. Pups were nursed by their dams; food (standard mouse chow) and water were given ad libitum. Pups were sacrificed on P17, after 5 days of room air recovery, when NV is known to peak.

Pups from concomitantly born litters were on P1 redistributed among dams, so that there were smaller litters (median 6 pups; range 4-9) and larger litters (median 15 pups; range 14-16). On P7-12, the OIR model was applied. Body weight was measured on P7, P14 and P17.

Retinal NV was assessed on P17. Retinal analyses were conducted blind to every study group by one investigator according to the published international guidelines. On P17, eyes were removed, fixed in 4% paraformaldehyde for 24 hr. and embedded in paraffin. Serial sections of 6 μ m were cut through the cornea, parallel to the optic

nerve, and were either stained with hematoxylin and eosin to assess retinal vasculature by light microscopy (x400). Oxygen-induced retinopathy was quantified by number of NV nuclei per 10 retinal sections.

The predictive accuracy of the number of NV nuclei was assessed by comparing the area under the receiver operating characteristic (ROC) curve that plots sensitivity vs. (1-specificity). This curve is useful in evaluating the discriminatory ability of a test to correctly pick up diseased and non-diseased subjects and finding the optimal cut-off point to least misclassify diseased and non-diseased subjects. Cut-off point with the highest sensitivity and specificity in this analysis was 77, meaning that those mice with > 77 NV nuclei were highly sensitive for developing OIR.

Serial weight measurements (P7, P14 and P21) were compared between the two subgroups; NV nuclei ≤ 77 versus NV nuclei > 77 .

2. Human study

This prospective study was performed from February 2005 till August 2007 in all very low-birth weight (VLBW) infants admitted in two neonatal intensive care units. The subjects were part of the control arm of the NIRTURE trial (ISRCTN78428828) (17, 18). Only those with conclusive ROP assessments who all received standard clinical care were included in this analysis.

Oxygen administration aimed for an oxygen saturation of $\sim 93\%$ with lower and upper alarm settings at 89-94% and all studied infants were continuously monitored for this variable. During the first 3 days, parenteral nutrition consisted of IV glucose

10% infusion (4-6 mg/kg.min on day 1; 6 mg/kg.min on day 2; and 7 mg/kg.min on day 3) with electrolytes. Subsequently, standard PN solutions were scheduled to deliver 64 kcal/kg.day of glucose, 2.8 g/kg.day of proteins, and 27 kcal/kg.day of lipids. PN was lowered with the initiation of enteral feedings, aiming for a total intake of ~120 kcal/kg.day (19). Virtually all infants ended up receiving a combination of human milk and formula feedings.

Senior ophthalmologists screened for ROP with indirect fundoscopy. First screening was performed 4 weeks after birth, and findings were scored according to the International Classification of Retinopathy of Prematurity (ICROP) (20). In this study, we only considered any stage of ROP vs. no ROP as numbers of patients would otherwise be too small to analyze.

Birth weight was recorded. Weekly weight measurements were performed for the first 4 weeks. Absolute (g/wk) en relative weight gain (g/kg/d) was calculated for each week until the age of 28 days. Serial weight gain measurements (day (D) 7, D14, D21 and D28) were compared between the two subgroups; no ROP versus ROP.

3. Ethics & Statistics

The murine study was approved by the Institutional Review Board, Centre for Molecular and Vascular Biology, University of Leuven. Experiments were performed in compliance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

For the human study, the protocol was approved by the Institutional Review Boards of each study center (EudraCT number, 2004-002170-34). Written informed consent of at least one of the parents was an inclusion criterion.

Cut-off value with the highest sensitivity and specificity to distinguish between two diagnostic groups in the murine study was set by ROC-analysis (21). Difference in serial weight measurements in the subgroups was calculated by determining the AUC (22) using a Mann-Whitney U test. The cut-off for statistical significance was set at $P < 0.05$, two-tailed.

Results

AUC of serial weight (gain) measurements was significantly lower in those murine and human newborns developing the neovascular disease OIR or ROP.

A total of 27 mice were analyzed. 14 mice had a number of NV nuclei ≤ 77 and 13 mice had >77 NV nuclei. Median weight gain in the subgroup NV ≤ 77 was 17g (14-18; 95% CI) versus 14g (7-17; 95% CI) in the subgroup NV > 77 ($p=0.01$) (figure 1, left panel).

A total of 101 premature infants with a median gestational age of 29 (27-31) weeks and a median birth weight of 1053g (888-1324) were included. Twenty-three of them developed any stage of ROP, whereas 78 did not. Median absolute weight gain was 240g/wk (187-272; 95% coincidence interval (CI)) in those without ROP and 140g/wk (83-188; 95% CI) in those developing ROP ($p=0.0001$) (figure 1, right panel). Median relative weight gain was 25g/kg/d (21-28; 95% CI) in those without ROP and 16g/kg/d (12-25; 95% CI) in those developing ROP ($p=0.04$).

Discussion

This prospective study performed in both animals and humans showed – by using a new way of analysis by comparing AUC of serial weight measurements (21)– that poor weight gain and severe ROP are highly associated. In a first stage, a summary of the response in each individual case is identified and calculated as AUC. In a second stage these different AUC's are analyzed by non- parametric Mann-Whitney U test. As this is a valid statistical technique, it is a very relevant method to analyze these serial weight gain measurements (21). The results of this analysis are in line with the results of previous studies and emphasize once more the importance of this postnatal factor.

As mice and rats are born with incomplete retinal vasculature and exposure to high levels of oxygen results in OIR, they exhibit an excellent model for studying the pathophysiology of ROP (16, 23). Already in 1994, Holmes reported that NV was more pronounced in growth retarded rat pups who were raised in larger litters and therefore were less nourished (24,25). Our own group, reported that neonatal mice from larger litters had lower weight, lower levels of circulating IGF-I and developed more OIR. Treatment with rhIGF-I resulted in greater weight gain and less OIR, indicating that postnatal supplementation with IGF-I may help to prevent ROP (26).

We are aware that the controlled animal experimental setting with nutritional deprivation is not similar to the postnatal growth restriction in the NICU graduate. However, poor postnatal weight gain has also been shown to be associated with ROP humans (2-15). Based on these findings, ROP prediction models such as WINROP, PINT-ROP and ROPScore have been developed (27-30). Only very

recently, the WINROP system was assessed in a large geographically and racially diverse study population and proved to be a useful adjunct for ROP screening in optimizing care and potentially reducing the number of ROP examinations (28). Poor postnatal weight gain is associated with a poor postnatal course, as well as with immaturity of the gastro-intestinal system, loss of growth factors and loss of poly-unsaturated fatty acids. Optimization of postnatal weight development should be undertaken by focusing on the above points (improving early enteral intake, composition of parenteral nutrition, supplementation with growth factors such as insulin-like growth factor 1, ...) (10,13).

Limitations of this study are mainly the important differences between animal and human subjects.

This study supports previous findings that early postnatal weight gain is an important indicator in the development of neovascular disease. Therefore, we urge that serial weight measurements are taken into account evaluating premature infants. This might help us not only in early detection of neovascular disease, but also in the development of preventive strategies focusing on optimisation of early growth.

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Figure legends

Figure 1. Serial weight measurements in mice (upper panel) and humans (lower panel) in the subgroups comparing OIR (NV>77)/ROP versus no OIR (NV≤77)/ROP. Each case is plotted. AUC (mice: baseline = first value; men: baseline=0) was calculated non-parametrically using a Mann-Whitney U test.