mature and premature infants.[26] According to the present data, which were limited to surgical MRI in VLBW infants, twin pregnancy is significantly associated with the development of MRI. Recently, it has been reported that twin-to-twin transfusion syndrome (TTTS) is associated with intestinal complications, including FIP and NEC.[27,28] Thus, it is likely that circulatory impairment caused by TTTS is associated with postnatal intestinal dysmotility observed in MRI. The present data also showed that PROM is another risk factor for MRI. Siggers *et al.* reported that amniotic fluid has a protective effect on the immature intestines.[29] The lack of amniotic fluid due to PROM may be associated with the development of MRI. In contrast to these risk factors, maternal steroid treatment was found to be protective. Many previous studies reported that prenatal steroid therapy decreases the incidence of NEC by accelerating intestinal maturation.[18,19] Given that it has also been reported that immature bowel movement is crucial for the pathogenesis of MRI,[7] maternal steroid treatment may have a protective effect in enhancing intestinal motility in VLBW infants. A prospective study is required to clarify the effects of maternal steroid treatment on the development of MRI in premature infants.

Limitations

One limitation of this study is the fact that matched controls were chosen based on gestational age and birthweight. Because the comparison was made between age- and weight-matched groups, we were unable to evaluate the effects of gestational age and birthweight on the development of surgical intestinal disorders. Another limitation was that non-surgical NEC and non-surgical MRI were excluded. A prospective study based on unified diagnostic criteria for both surgical and non-surgical diseases is thus needed to identify risk factors for the entire spectrum of intestinal disorders observed in VLBW infants.

In conclusion, we have herein identified different risk factors for surgical NEC, FIP and MRI. The present results suggest that a different pathogenesis exists for each disease, thus indicating that different strategies are therefore needed for effective disease prevention of surgical intestinal disorders in VLBW infants.

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Original Article

Outcome in VLBW infants with surgical intestinal disorder at 18 months of corrected age

Masahiro Hayakawa,[1] Tomoaki Taguchi,[3] Naoto Urushihara,[4] Akiko Yokoi,[5] Hiroshi Take,[6] Jun Shiraishi,[7] Hideshi Fujinaga,[8] Kensuke Ohashi,[9] Makoto Oshiro,[2] Yuichi Kato,[10] Satoko Ohfuji[11] and Hiroomi Okuyama[12,13]

[1]*Division of Neonatology, Center for Maternal-Neonatal Care, Nagoya University Hospital,* [2]*Department of Neonatology, Japanese Red Cross Nagoya First Hospital, Nagoya,* [3]*Department of Pediatric Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka,* [4]*Department of Pediatric Surgery, Shizuoka Children's Hospital, Shizuoka,* [5]*Department of Pediatric Surgery, Hyogo Prefectural Kobe Children's Hospital, Kobe,* [6]*Department of Surgery, Kanagawa Children's Medical Center, Yokohama,* [7]*Department of Neonatology, Osaka Medical Center and Research Institute for Maternal and Child Health,* [8]*Division of Neonatology, Center for Maternal–Fetal and Neonatal Medicine, National Center for Child Health and Development,* [9]*Department of Pediatric Surgery, Nihon University School of Medicine, Tokyo,* [10]*Department of Pediatrics, Anjo Kosei Hospital, Anjo,* [11]*Department of Public Health, Osaka City University Faculty of Medicine, Osaka,* [12]*Department of Pediatric Surgery, Osaka University Graduate School of Medicine, Suita and* [13]*Department of Pediatric Surgery, Hyogo College of Medicine, Nishinomiya, Japan*

Abstract ***Background***: Surgical intestinal disorders, such as necrotizing enterocolitis (NEC), focal intestinal perforation (FIP), and meconium-related ileus (MRI), are serious morbidities in very low-birthweight infants (VLBWI). The aim of this study was to compare the composite outcomes of death or neurodevelopmental impairment (NDI) in VLBWI with surgical intestinal disorders and assess independent risk factors for death and NDI at 18 months of corrected age.

Methods: A retrospective matched-cohort study was conducted at 11 institutes. We included VLBWI who had undergone laparotomy for NEC, FIP, and MRI. Two control subjects were chosen for every surgical patient and matched for gestational age and birthweight to form the comparison group. Death and neurodevelopmental outcome at 18 months of corrected age were evaluated.

Results: The number of infants in the NEC, FIP, MRI, and control groups was 44, 47, 42, and 261, respectively. In-hospital mortality was higher in infants with NEC and MRI relative to those in the control group ($P < 0.001$). The incidence rate for NDI at 18 months of corrected age was higher in infants with MRI relative to those in the control group ($P = 0.021$). On logistic regression analysis, low gestational age, male sex, small for gestational age, intraventricular hemorrhage, and MRI were associated with increased risk of death or NDI at 18 months of corrected age.

Conclusions: NEC and MRI were associated with in-hospital mortality, and MRI was associated with NDI or death at 18 months of corrected age.

Key words focal intestinal perforation, meconium-related ileus, necrotizing enterocolitis, outcome, very low birth weight infants.

Recent advances in perinatal/neonatal medicine have reduced mortality in preterm infants,[1,2] but some preterm infants have either died during hospitalization or survived with neurological sequelae. Surgical intestinal disorders, such as necrotizing enterocolitis (NEC), focal intestinal perforation (FIP), and meconium-related ileus (MRI), are important diseases associated with serious morbidity and mortality in very low-birthweight infants (VLBWI).

Necrotizing enterocolitis is one of the most severe intestinal diseases affecting premature infants and leads to high mortality and morbidity.[2,3] The reported incidence of NEC ranges from 2.3% to 9%.[3–5] The pathogenesis of NEC could involve enteral feeding, gut immunity, and altered gut microbiota, but this has not been elucidated entirely. FIP is isolated, and the surrounding tissue appears otherwise normal.[6] The incidence of FIP ranges from 1.1% to 8% in extremely low-birthweight infants (ELBWI).[4,7,8] Because FIP is a localized disease, it is often assumed that it is less likely, relative to NEC, to be associated with a systemic inflammatory reaction, suggesting that infants with FIP may be at lower risk.[9,10] Other articles, however, have reported FIP outcomes similar to those of NEC.[11,12] Therefore, these outcomes have been controversial.

Meconium-related ileus is another intestinal disease that affects premature infants, as reported by Kubota *et al.*[13] There has been speculation that the pathogenesis of perforation in MRI involves immature or uncoordinated bowel movement, which

Correspondence: Masahiro Hayakawa, MD, PhD, Division of Neonatology, Center for Maternal-Neonatal Care, Nagoya University Hospital, 65 Tsurumai-cho Showa-ku, Nagoya 466-8560, Japan. Email: masahaya@med.nagoya-u.ac.jp

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causes a localized increase in intraluminal pressure and leads to focal perforation.[13] The morbidity and incidence of MRI in VLBWI have not been reported. In addition, there have been no articles published regarding outcomes in VLBWI with MRI.

Infants with such disorders are at high risk of both mortality and poor neurodevelopmental outcome.[7,11,12,14,15] Some studies have evaluated neurodevelopmental outcomes in infants with surgical intestinal disorders, but few publications have described comparative outcomes for VLBWI with NEC, FIP, and MRI. The aim of this study was to compare composite outcomes for death or neurodevelopmental impairment (NDI) in VLBWI with NEC, FIP, and MRI and to assess whether the presence of intestinal diseases is an independent risk factor for death or NDI, on regression analysis.

Methods

Participants

A retrospective matched cohort study was conducted at 11 tertiary institutes in Japan. We included VLBWI (birthweight ≤1500 g) who had undergone laparotomy for surgical intestinal disorder between January 2003 and December 2012. Two control subjects were chosen for every surgical patient and matched for gestational age (±1 week) and birthweight (±50 g) to form the comparison group.

Definition of surgical intestinal disorders

The surgical intestinal disorders observed in VLBWI were classified into the following three categories based on operative findings: NEC, an acquired condition of diffuse necrotic injury to the mucosal and submucosal layers of the bowel; FIP, intestinal perforation without mechanical obstruction or necrotic changes; and MRI, which, in operative cases, involves diagnosis of MRI with laparotomy performed for intractable ileus, indicating a microcolon or small colon extending to the distal ileum or a gradual change in the caliber of the ileum, with a dilated proximal ileum filled with sticky meconium. This condition is synonymous with "meconium disease" or "meconium ileus without mucoviscidosis".

Maternal, perinatal, and neonatal factors

In order to assess independent risk factors for death and NDI, information regarding maternal factors, perinatal factors, neonatal factors, and neonatal complications associated with death and/or NDI was collected from medical records. Maternal factors included age, placental abruption, umbilical collapse, pregnancy-induced hypertension (PIH), membrane rupture, and steroid treatment. Perinatal and neonatal factors included delivery mode, birth location (i.e. outborn, inborn), sex, gestational age, birthweight, number of fetuses, small for gestational age (SGA) and Apgar score (1 min and 5 min). Neonatal complications included delay in establishment of postnatal enteral feeding, sepsis, chronic lung disease (CLD), postnatal steroid treatment, intraventricular hemorrhage (IVH), and periventricular leukomalacia (PVL). PIH was diagnosed according to the definition established by the Japan Society of Obstetrics and Gynecology. Gestational age was determined on the basis of the last menstrual period and ultrasound findings during the early stage of pregnancy. SGA was defined as birthweight <10th percentile, according to Japanese reference data.[16] CLD was diagnosed if supplementary oxygen was necessary at 36 weeks of corrected age and accompanied by characteristic radiographic changes. Sepsis was diagnosed if an infant had severe deterioration, associated with positive blood culture, in his or her general condition. IVH and PVL were diagnosed on brain imaging (ultrasonography and/or magnetic resonance imaging). Establishment of enteral feeding was defined as achievement of enteral feeding consisting ≥100 mL/kg/day. Previous research has demonstrated adverse neurological outcome in infants for whom enteral feeding did not reach 100 mL/kg/day at 3 weeks of age.[17] Therefore, the cut-off for establishment of enteral feeding was 3 weeks of age.

Outcomes

The primary endpoint was death during hospitalization. The secondary endpoint was death or NDI at 18 months of corrected age. Information regarding death during hospitalization was collected from medical records. Neurodevelopmental outcome at 18 months of corrected age was evaluated by the attending physician using neurodevelopmental assessment tools via medical interview from parents. If the developmental quotient was <70, neurodevelopment was considered delayed. When a battery was not performed, the attending physician evaluated outcomes via physical examination. NDI was defined as the presence of neurological sequelae in infants at 18 months of corrected age.

Statistical analysis

All analyses were performed using SAS version 10.0.2 (SAS Institute, Cary, NC, USA). Categorical variables were evaluated using chi-squared or Fisher's exact tests. Numerical data were evaluated using the Kruskal–Wallis test. Bonferroni correction was used for post-hoc analysis.

An adjusted analysis was performed for primary and secondary endpoints, using multivariate logistic regression analysis to determine independent factors. The variables included in the regression analyses for death during hospitalization and NDI/death at 18 months of corrected age were the maternal, perinatal, and neonatal factors that had previously been shown to affect neurodevelopmental outcomes. $P < 0.05$ was regarded as statistically significant.

Ethics

This study was performed with the approval of the ethics committee of Hyogo College of Medicine (approval number: 1483) and the independent ethics committees of Nagoya University Hospital, Kyushu University, Shizuoka Children's Hospital, Hyogo Prefectural Kobe Children's Hospital, Kanagawa Children's Medical Center, Osaka Medical Center and Research Institute for Maternal and Child Health, National Center for Child Health and Development, Nihon University School of Medicine, Japanese Red Cross Nagoya First Hospital, and Anjo Kosei Hospital.

Table 1 Outcomes

	Control $n = 261$	NEC $n = 44$	FIP $n = 47$	MRI $n = 42$	P
Death in hospital					
Yes/No (Yes %)	18/243 (6.9)	17/27 (38.6)*	8/39 (17.0)	9/33 (21.4)†	<0.001
NDI at 18 months corrected age					
Yes/No (Yes %)	79/108 (42.3)	11/7 (61.1)	14/16 (46.7)	15/5 (75.0)‡	0.022

*Control vs NEC, $P < 0.001$; †control vs MRI, $P < 0.009$; ‡control vs MRI, $P < 0.21$. FIP, focal intestinal perforation; MRI, meconium-related ileus; NDI, neurodevelopmental impairment; NEC, necrotizing enterocolitis.

Results

The number of patients enrolled in the control, NEC, FIP, and MRI groups was 261, 44, 47, and 42, respectively. The number of deaths that occurred during hospitalization in the control, NEC, FIP and MRI groups was 18, 17, 8, and 9, respectively. Overall mortality at discharge was 13.2%. In-hospital mortality was higher in infants with NEC and MRI relative to those in the control group (control vs NEC, $P < 0.001$; control vs MRI, $P = 0.009$). The prevalence of NDI at 18 months of corrected age in the control, NEC, FIP, and MRI groups was 42.3%, 61.1%, 46.7%, and 75.0%, respectively. The incidence of NDI at 18 months of corrected age was higher in infants with MRI relative to that of the control group ($P = 0.021$; Table 1).

Maternal characteristics for the four groups are given in Table 2. The incidence of maternal PIH was significantly lower in the NEC group relative to the other groups (NEC vs control, $P = 0.014$; NEC vs FIP, $P = 0.007$; NEC vs MRI, $P = 0.034$).

Perinatal and neonatal characteristics of the four groups are listed in Table 3. The proportion of male infants in the NEC group was significantly higher than in the control group ($P = 0.034$). Gestational age in the NEC group was significantly lower relative to that observed in the MRI group ($P = 0.028$). The proportion of SGA infants was significantly higher in the MRI group relative to the control and NEC groups (MRI vs control, $P = 0.009$; MRI vs NEC, $P = 0.008$).

Neonatal complications are shown in Table 4. Establishment of enteral feeding was delayed in infants with NEC, FIP, and MRI relative to those in the control group (control vs NEC, $P < 0.001$; control vs FIP, $P < 0.001$; control vs MRI, $P < 0.001$). The proportion of infants with IVH was significantly higher in the NEC group relative to the control group.

On logistic regression analysis, after adjusting for potential confounders, NEC was associated with increased risk of in-hospital mortality (relative to the control group: OR, 2.87; 95%CI: 1.12–7.48). In addition, MRI was associated with increased risk of death or NDI at 18 months of corrected age (OR, 4.58; 95%CI: 1.25–19.0). Regarding risk factors other than the disease categories, SGA, establishment of enteral feeding at >3 weeks postnatally, and IVH were associated with an increased risk of in-hospital mortality (Table 5). Low gestational age, male sex, SGA, and IVH were associated with an increased risk of death or NDI at 18 months of corrected age (Table 6).

Discussion

In this retrospective study, we identified NEC and MRI as independent factors associated with in-hospital mortality and death or NDI at 18 months of corrected age, respectively.

Mortality

There was a high mortality rate in infants with NEC.[9,12] Hull *et al.* reported a mortality rate of 35% for VLBWI with surgical NEC.[4] In the present study, the mortality rate for infants with NEC was 38.6%, which was compatible with that of the previous study. In addition, NEC was an independent factor for in-hospital mortality on logistic regression analysis. The reason

Table 2 Maternal characteristics

	Control $n = 261$	NEC $n = 44$	FIP $n = 47$	MRI $n = 42$	P
Maternal age (years)					
Median (IQR)	31.5 (28–35)	32 (29–35)	31 (26–35)	32 (29–34)	0.720
Abruptio placenta					
Yes/No (Yes %)	14/246 (5.4)	1/42 (2.3)	2/44 (4.4)	2/39 (4.9)	0.823
Umbilical collapse					
Yes/No (Yes %)	12/247 (4.6)	1/42 (2.3)	1/45 (2.2)	2/39 (4.9)	0.761
Pregnant induced hypertension					
Yes/No (Yes %)	44/215 (16.7)	0/43 (0.0)*,†,‡	9/35 (20.5)	6/34 (14.2)	0.001
Rupture of membrane					
Yes/No (Yes %)	106/151 (41.3)	23/20 (53.5)	14/32 (30.4)	16/24 (40.0)	0.177
Maternal steroid treatment					
Yes/No (Yes %)	130/127 (50.6)	15/28 (46.7)	19/22 (46.3)	13/25 (34.2)	0.092

*NEC vs control, $P < 0.014$; †NEC vs FIP, $P < 0.007$; ‡NEC vs MRI, $P < 0.034$. FIP, focal intestinal perforation; IQR, interquartile range; MRI, meconium-related ileus; NEC, necrotizing enterocolitis.

Table 3 Perinatal and neonatal characteristics

	Control $n = 261$	NEC $n = 44$	FIP $n = 47$	MRI $n = 42$	*P*
Gender					
Male/Female (male %)	134/127 (51.3)	32/12 (72.3)*	30/17 (63.8)	25/17 (59.5)	0.030
Gestational age (weeks)					
Median (IQR)	26.1 (24.3–28.2)	25.4 (24.0–26.9)†	26.3 (24.6–28.4)	27.3 (25.2–29.9)	0.034
Birthweight (g)					
Median (IQR)	726 (594–952)	701 (579–842)	746 (644–988)	707 (508–942)	0.545
No. fetuses					
Multiple/singleton (multiple %)	44/217 (16.9)	14/30 (31.8)	14/33 (29.8)	13/29 (31.0)	0.020
Fetal growth					
SGA/non-SGA (SGA %)	75/186 (28.7)	9/35 (30.5)	14/33 (29.8)	22/20 (52.4)‡,§	0.010
Apgar score (1 min) Median (IQR)	4 (2–6)	4 (1–6)	4 (3–6)	4 (2–6)	0.800
Apgar score (5 min) Median (IQR)	7 (5–8)	6 (5–8)	7 (5–8)	7 (3–8)	0.577
Delivery					
CS/TV (CS %)	192/69 (73.6)	33/11 (75.0)	41/6 (87.2)	35/7 (83.3)	0.117
Birth location					
Outborn/inborn (outborn %)	42/219 (16.1)	13/31 (29.6)	14/33 (29.8)	7/35 (16.7)	0.055

*Control vs NEC, $P < 0.034$; †NEC vs MRI, $P < 0.028$; ‡Control vs MRI, $P = 0.009$; §NEC vs MRI, $P = 0.008$. CS, cesarean section; FIP, focal intestinal perforation; IQR, interquartile range; MRI, meconium-related ileus; NEC, necrotizing enterocolitis; SGA, small for gestational age; TV, transvaginal delivery.

for the higher mortality rate, relative to that for other intestinal diseases, is not clear but may involve a multitude of factors, including the extent of the disease,[9] varying pathogenesis,[18] and different organisms implicated in the etiology of peritonitis accompanying the disease process.[19] FIP is one of the most critical intestinal diseases observed in neonates. Attridge *et al.* reported a mortality rate of 29% in ELBWI with FIP.[7] In contrast, the mortality rate in the present study was 17.0%. The participant group in the Attridge *et al.* study was restricted to infants who weighed <1000 g.[7] The present subjects, however, also included infants who weighed ≥1000 g. This difference between the study populations may have led to the difference in results.

Neurological impairment

Some reports have been published in which neurodevelopmental outcome in infants with surgical intestinal diseases was poorer relative to that in infants without surgical intestinal diseases.[11,15,20] This was particularly evident in infants with surgical NEC.[11,12,15] In this study, the prevalence of NDI in infants with NEC at 18 months of corrected age was 61.1%. Several studies have compared neurodevelopmental outcome in infants with FIP and NEC;[10–12] some noted similar NDI in infants in both groups,[11,12] while Adesanya *et al.* noted lower Bayley score in survivors with NEC relative to infants with FIP.[10] In the present study, NDI in infants with either NEC or FIP at 18 months of corrected age did

Table 4 Neonatal complications

	Control $n = 261$	NEC $n = 44$	FIP $n = 47$	MRI $n = 42$	*P*
Postnatal day of enteral feeding establishment					
Median (IQR)	15 (12–23)	48.5 (33–89)*	33 (22–55)**	36 (26–53)†	<0.001
Enteral feeding establishment >3 weeks postnatally					
Yes/No (Yes %)	79/169 (64.9)	41/2 (95.3)††	8/35 (83.7)‡	36/2 (92.3)‡‡	<0.001
Sepsis					
Yes/No (Yes %)	42/219 (16.1)	7/37 (15.9)	8/39 (17.0)	9/32 (22.0)	0.839
Chronic lung disease					
Yes/No (Yes %)	96/158 (37.8)	18/20 (47.4)	17/26 (39.5)	15/21 (41.7)	0.719
Postnatal steroid treatment					
Yes/No (Yes %)	47/211 (18.2)	13/32 (27.3)	7/37 (15.9)	8/32 (20.0)	0.534
Intraventricular hemorrhage					
Yes/No (Yes %)	71/189 (27.3)	21/21 (50.0)§	13/30 (30.2)	16/25 (39.2)	0.024
Periventricular leukomalacia					
Yes/No (Yes %)	30/217 (12.2)	3/37 (7.5)	5/32 (13.5)	8/26 (23.5)	0.242

*Control vs NEC, $P < 0.001$; **Control vs FIP, $P < 0.001$; †Control vs MRI, $P < 0.001$; ††Control vs. NEC; $P < 0.001$, ‡Control vs FIP, $P < 0.001$; ‡‡Control vs MRI, $P < 0.001$; §Control vs NEC, $P = 0.012$. FIP, focal intestinal perforation; IQR, interquartile range; MRI, meconium-related ileus; NEC, necrotizing enterocolitis.

Table 5 Factors associated with death during hospitalization

	OR	95%CI	*P*
Gestational age (1 week increased)	0.84	0.68–1.01	0.064
Male	0.98	0.47–2.05	0.954
Small for gestational age	3.67	1.14–9.68	0.006
Multiple	1.52	0.66–3.41	0.320
Pregnancy-induced hypertension	0.65	0.16–2.25	0.507
Establishment of enteral feeding >3 weeks postnatally	6.10	2.17–20.1	<0.001
Intraventricular hemorrhage	3.08	1.44–6.81	0.004
NEC (vs control)	2.87	1.12–7.48	0.029
FIP (vs control)	1.16	0.36–3.39	0.797
MRI (vs control)	1.20	0.39–3.53	0.740

FIP, focal intestinal perforation; MRI, meconium-related ileus; NEC, necrotizing enterocolitis.

not differ from that of infants in the control group. In addition, on logistic regression analysis neither NEC nor FIP was associated with death or with NDI at 18 months of corrected age.

Mortality and neurological morbidity in MRI infants

Meconium-related ileus was proposed by Kubota *et al.*[13] The clinical features of the condition include intractable ileus due to functional obstruction, impaired meconium excretion, and microcolon or small colon. Cuenca *et al.* reported excellent outcomes for patients with meconium plug syndrome, which is on the MRI spectrum.[21] In the present study, the mortality rate for the MRI group was 21.4%. The incidence rate for NDI, however, was 75%, and MRI was an independent risk factor in infants at 18 months of corrected age. The discrepancy between the Cuenca *et al.* and the present findings may be explained by patient characteristics. Meconium plug syndrome is considered to be a relatively mild form of MRI, given that it can often be cured with an enema. In contrast, the majority of patients with MRI require surgical treatment. Therefore, it is necessary to conduct further research to examine the clinical features of MRI in VLBWI.

Table 6 Factors associated with death and NDI at 18 months of corrected age

	OR	95%CI	*P*
Gestational age (1 week increased)	0.76	0.65–0.88	<0.001
Male	1.91	1.05–3.53	0.035
Small for gestational age	2.40	1.07–5.60	0.034
Multiple	0.77	0.34–1.71	0.523
Pregnancy-induced hypertension	1.09	0.43–2.74	0.847
Establishment of enteral feeding >3 weeks postnatally	1.21	0.59–2.46	0.593
Intraventricular hemorrhage	4.55	2.24–9.63	<0.001
NEC (vs control)	1.36	0.40–4.78	0.620
FIP (vs control)	1.25	0.45–3.43	0.668
MRI (vs control)	4.58	1.25–19.0	0.021

FIP, focal intestinal perforation; MRI, meconium-related ileus; NDI, neurodevelopmental impairment; NEC, necrotizing enterocolitis.

Other risk factors for death or NDI at 18 months of corrected age

In the present study, SGA, delayed establishment of enteral feeding, and IVH were associated with an increased risk of in-hospital mortality, and low gestational age, male sex, SGA, and IVH were independent risk factors for death or NDI at 18 months of corrected age. These neonatal factors have been associated with outcome in low-birthweight infants with or without surgical intestinal disorders.[22,23] In contrast, none of the maternal factors were associated with in-hospital mortality, death, or NDI at 18 months of corrected age. This suggest that, relative to maternal factors, neonatal factors are more strongly related to outcome in VLBWI with surgical intestinal disorders. Improvement in perioperative management is necessary to reduce mortality and morbidity in these infants.

Limitations

This study had several limitations. First, data collection was retrospective. Therefore, there was inevitable data dropout due to loss of records. Finding an appropriate control group is always difficult in retrospective analysis. Second, the absolute number of cases was relatively small, even though the study was conducted in 11 Japanese tertiary centers. Third, the battery used to assess neurodevelopmental outcome was dependent upon the institution involved. To resolve these limitations, it is necessary to perform nationwide prospective surveillance.

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ORIGINAL ARTICLE

Surfactant protein-D attenuates the lipopolysaccharide-induced inflammation in human intestinal cells overexpressing toll-like receptor 4

Ryuta Saka[1,2,3] · Tetsu Wakimoto[3,4] · Fumiko Nishiumi[3] · Takashi Sasaki[1] · Satoko Nose[1] · Masahiro Fukuzawa[5] · Takaharu Oue[1] · Itaru Yanagihara[3] · Hiroomi Okuyama[2]

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Abstract
Purpose Necrotizing enterocolitis (NEC) is a devastating inflammatory disease of preterm infants that may depend on overexpression of toll-like receptor-4 (TLR4) in the immature intestine. Surfactant protein (SP)-D is a member of the collectin family and plays an important role in innate immunity, particularly in the airways. Although SP-D also exists in the intestines, little is known about its function. This study investigated whether SP-D can attenuate the inflammatory response of TLR4-overexpressing embryonal intestinal cells.
Methods All experimental procedures were performed using the human intestinal cell line INT407 originally derived from human embryonal intestines. Platelet-activating factor (PAF), reported to be elevated in NEC patients, was used to induce TLR4 overexpression in the human embryonal intestinal cell line INT407. TLR4 expression was measured using quantitative real-time PCR. Inflammatory responses to PAF (5 μM), the TLR4 agonist lipopolysaccharide (LPS, 100 ng/ml), PAF + LPS, and PAF + LPS following SP-D pretreatment (20 μg/ml) were assessed by enzyme-linked immunosorbent assay (ELISA) of interleukin-8 (IL-8) release (in pg/ml).
Results Expression of TLR4 mRNA (mean ± SD) was upregulated by PAF (369 % ± 28 %, $p < 0.001$). Stimulation with PAF + LPS resulted in higher IL-8 release (1959.3 ± 52.3) than control (141.2 ± 12.4), LPS (167.3 ± 65.8), or PAF (1527.2 ± 129.4) treatment ($p < 0.05$). Release in response to PAF + LPS (1590.1 ± 319.3) was attenuated by SP-D pretreatment (1161.6 ± 131.6; $p < 0.05$).
Conclusion SP-D attenuates LPS-induced IL-8 production in TLR4-overexpressing intestinal cells, suggesting that SP-D may have a protective effect in the development of NEC in preterm infants.

Keywords Necrotizing enterocolitis · Toll-like receptor-4 · Surfactant protein · Platelet-activating factor · Lipopolysaccharide

✉ Hiroomi Okuyama
okuyama@pedsurg.med.osaka-u.ac.jp

[1] Department of Pediatric Surgery, Hyogo College of Medicine, Nishinomiya, Japan

[2] Department of Pediatric Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita 5650871, Japan

[3] Department of Developmental Medicine, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Japan

[4] Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Suita, Japan

[5] Department of Pediatric Surgery, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Japan

Introduction

Necrotizing enterocolitis (NEC) remains one of the most common and life-threatening gastrointestinal diseases during early infancy, with approximately 7 % incidence in infants having a very low birth weight and 20–30 % mortality rate [1]. Recent studies have demonstrated that NEC is associated with hyperinflammatory responses and that toll-like receptor-4 (TLR4) overexpression is critical in NEC pathogenesis [2]. The fetal (immature) intestinal mucosa is characterized by elevated expression of TLR4 [3, 4], which regulates the proliferation and migration as well as apoptosis of epithelial cells [5]. Excessive TLR4

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activation in the immature intestine may stimulate enterocyte apoptosis, leading to impaired healing, reduced epithelial cell proliferation, and eventual NEC [6].

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine) also plays an important role in the pathogenesis of mucosal inflammation and intestinal injury [7]. PAF upregulates TLR4 expression in intestinal cell lines [8], and thus, it may also contribute to NEC onset [9]. Therefore, we employed PAF stimulation to induce TLR4 overexpression in immature intestinal cells as an in vitro NEC model.

Surfactant protein (SP)-D belongs to the collectin family, which is instrumental in innate immunity [10]. Although SP-D is the most abundant in the airways, extrapulmonary expression has also been documented [11, 12]. Previously, we reported that SP-A and SP-D are expressed on the luminal surface of the murine fetal and neonatal intestine [13]. SP-D enhances phagocytosis by opsonizing and aggregating microorganisms and acts as an activation ligand. In addition, it downregulates LPS-evoked inflammatory responses by altering LPS binding to TLR4/MD-2 [14].

To test the hypothesis that SP-D can attenuate the inflammatory response in immature intestinal cells, we investigated the effects of SP-D pretreatment on LPS-induced release of the inflammatory cytokine interleukin-8 (IL-8) in TLR4-overexpressing human embryonal intestinal cells.

Materials and methods

Cell culture

Human intestinal cells (INT407) were purchased from DS Pharma Biomedical (Osaka, Japan) and maintained in minimum essential medium (Life Technologies™) supplemented with 1 % nonessential amino acids, 2 mM L-glutamine, and 10 % fetal bovine serum (Sigma-Aldrich Co. LLC) at 37 °C in 5 % CO_2.

Reagents

Carbamyl PAF (BML-L120) (Enzo Life Science, New York, USA) dissolved in water was used for stimulation of TLR4 overexpression. Phenol-extracted *Escherichia coli* O55:B5 lipopolysaccharide (LPS) was obtained from Sigma (St. Louis, USA) and dissolved in water. Human recombinant SP-D (full-length) was obtained from Abcam (Cambridge, UK) and dissolved in PBS before use in experiments.

Stimulation with PAF and quantitative real-time PCR

Expression levels of TLR4 are very low in mature but high in immature/embryonic intestinal epithelial cells [3, 4]. PAF has been reported to upregulate TLR4 expression in a cancer cell line ($Caco_2$) [8]. TLR4 expression is also low in INT407 cells, so PAF was used to upregulate TLR4 expression as in the immature intestine. INT407 cells (3×10^5/well in six-well plates) were stimulated with PAF (5, 10, and 20 μM) for 6 h at 37 °C or left unstimulated (controls). Total RNA was isolated using the SV total RNA isolation system (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. cDNA was prepared via reverse transcription from INT407 cell RNA using the Prime Script RT reagent kit (TAKARA BIO INC., Otsu, Japan). TLR4 mRNA expression levels were measured using quantitative real-time (qRT)-PCR; transcript levels were normalized to that of GAPDH. The following primer sequences were used: TLR4, 5′-CAGAG TTTCCTGCAATGGATCA-3′ and 5′-GCTTATCTGAAG GTGTTGCACAT-3′; GAPDH, 5′-CGGGGAAGCTTGTC ATCAATGG-3′ and 5′-GGCAGTGATGGCATGGACT G-3′. Quantitative RT-PCR was performed using a CHROMO4 detector (Bio-Rad Laboratories, Hercules, CA, USA). Each 12.5 μl of the qRT-PCR reaction contained 1 μl of cDNA, 5 mM of each primer, and 6.25 μl of the STBR Premix Ex Taq II kit mix (TAKARA BIO INC., Otsu, Japan). The reaction conditions were 40 cycles of denaturing at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, respectively, and 40 cycles were performed. Each primer and probe set was analyzed and found to have a linear relationship with 2^{-CT}. The dilution factor for all reactions showed a CT value <38.

LPS stimulation of TLR4-overexpressing INT407cells, SP-D pretreatment, and measurement of IL-8

To establish an inflammation model of the immature intestine, INT407 cells were treated with PAF to induce TLR4 overexpression or with PAF plus LPS (Fig. 2a). To evaluate the effect of SP-D on this inflammation model, various concentrations of SP-D were added to the cells prior to stimulation with PAF + LPS (Fig. 3a). The magnitude of the inflammatory response was evaluated by measuring IL-8 release. Briefly, INT407 cells (2×10^4/well) were cultured in 96-well dishes, and the supernatants collected and stored at −80 °C after the various treatments (PAF, LPS, PAF + LPS, SP-D followed by PAF + LPS) for IL-8 measurement by enzyme-linked immunosorbent

assay (ELISA). The human IL-8 ELISA kit was obtained from BD Biosciences (Bedford, MA, USA). ELISA assays were performed in duplicate and quantified according to the manufacturer's instructions. The results are expressed in pg/ml.

Statistical analysis

The results of the qRT-PCR and ELISA analyses are expressed as mean ± SD. Treatment group means were compared by Tukey–Kramer HSD test or Dunnett's test. All statistical analyses were performed using the JMP®10 software package (SAS Institute Inc., Cary, NC). A $p < 0.05$ was considered significant.

Results

PAF upregulated TLR4 expression in the human intestinal cell line INT407

Following treatment with 5 μM PAF, TLR4 mRNA expression was significantly upregulated compared with untreated cells (3.69 ± 0.28 vs. 1.00 ± 0.10, $p < 0.001$) (Fig. 1). Increasing the PAF concentration above 5 μM did not result in any further enhancement of TLR4 mRNA expression (10 μM: 3.17 ± 0.31, 20 μM: 3.43 ± 0.22).

PAF + LPS increased IL-8 release from INT407 cells (Fig. 2)

The inflammatory responses of INT407 cells to stimulation by PAF, LPS, and PAF + LPS are shown in Fig. 2b. Expression of the pro-inflammatory cytokine IL-8 was not increased by LPS alone (167.3 ± 65.8 pg/ml) compared with controls (141.2 ± 12.4 pg/ml), while PAF alone induced a significant increase in IL-8 release (1527.2 ± 129.4 pg/ml, $p < 0.05$) that was further enhanced by subsequent stimulation with LPS (1959.3 ± 52.3 pg/ml, $p < 0.05$).

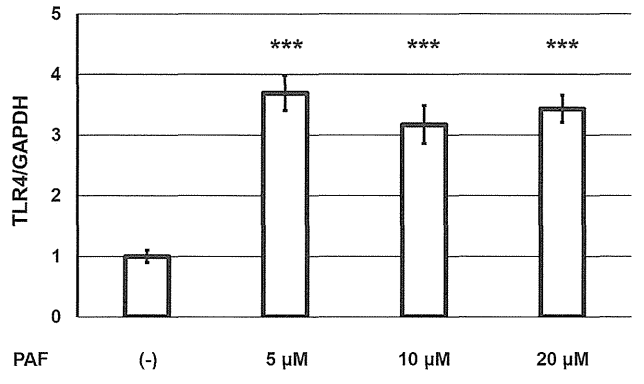

Fig. 1 PAF induces TLR4 expression in INT407 cells. Compared with untreated controls, INT407 cells exposed to 5 μM PAF exhibited markedly elevated TLR4 mRNA expression. There was no apparent dose-dependent increase above 10 μM PAF ($n = 5$, ***$p < 0.001$). *PAF* platelet-activating factor, *TLR4* toll-like receptor-4

Recombinant SP-D attenuated IL-8 expression induced by PAF + LPS in INT407 cells (Fig. 3)

Pretreatment with 20 ng/ml recombinant SP-D significantly attenuated IL-8 release evoked by LPS in PAF-treated cells (1161.6 ± 131.6 vs. 1527.2 ± 129.4 pg/ml, $p < 0.05$), while 5 and 10 mg/ml did not (5 ng/ml: 1372.3 ± 157.9 pg/ml, 10 ng/ml: 1292.0 ± 227.6 pg/ml).

Discussion

We used a human intestinal cell line, INT407, induced to overexpress TLR4 by PAF treatment and costimulated by the TLR4 agonist LPS, as an in vitro cell model of NEC to test the possible anti-inflammatory activity of SP-D. Indeed, a modest SP-D dose (20 ng/ml) markedly attenuated release of the pro-inflammatory cytokine IL-8 in response to combined PAF and TLR4 stimulation, seminal events in NEC pathogenesis [2–9], possibly by blocking TLR4-LPS binding [14]. We suggest that SP-D, which is widely expressed but normally low in the immature intestine, is a possible therapy for NEC.

The INT407 cell line is frequently used to model pathological responses in the small intestine, such gastroenteritis caused by food-borne bacteria [15]. In this study, however, IL-8 release was not significantly increased by LPS alone, possibly due to low expression levels of TRL4. Although the INT407 line was derived from human embryonic intestinal cells, the expression of TLR4 is suppressed under routine culture conditions. TLR4 expression was upregulated following treatment with PAF, a response akin to that observed under conditions of NEC. In addition, the TLR4-overexpressed INT 407 cells showed a strong reaction to LPS compared with that showed by the non-treated cells.

LPS alone, suggesting that TLR4 overexpression is necessary for the hyperinflammatory response characteristic of NEC. Indeed, the expression of TLR4 is higher in the embryonic/neonatal intestinal mucosa than in the adult mucosa [3], and human intestinal samples from NEC patients display significantly higher expression levels of TLR4 mRNA [16]. Moreover, TLR4 mutant mice were protected against the development of NEC [17].

In our model, TLR expression was elevated by PAF and LPS stimulation. The administration of PAF and LPS has been reported to cause NEC-like pathologies in vivo [18]. PAF is an endogenous phospholipid inflammatory mediator

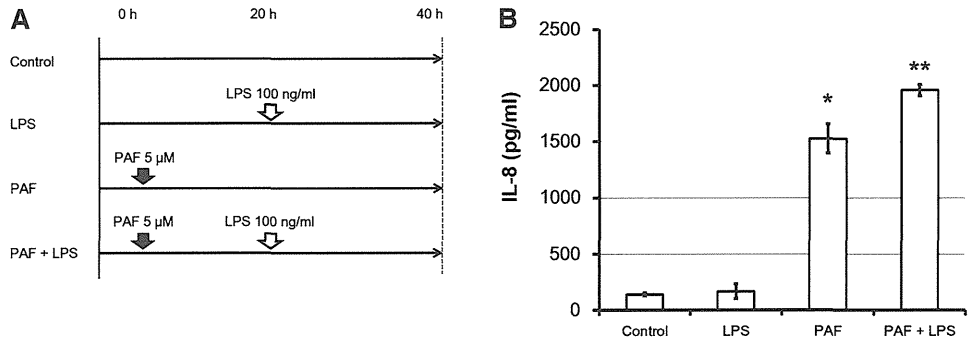

Fig. 2 LPS induces IL-8 release in cooperation with PAF stimulation. **a** The time course of stimulation. During PAF stimulation (*PAF* and *PAF + LPS*), PAF (5 µM) was added first. LPS (100 ng/ml) was added 20 h later in both LPS stimulation groups (*LPS* and *PAF + LPS*). **b** IL-8 release was not elevated following stimulation with LPS alone, but PAF caused a remarkable elevation of IL-8 and the combination of PAF and LPS led to even higher levels ($n = 4$, $*p < 0.05$ compared to "control" and "LPS", $**p < 0.05$ vs. the other three groups). *IL* interleukin, *LPS* lipopolysaccharide, *PAF* platelet-activating factor

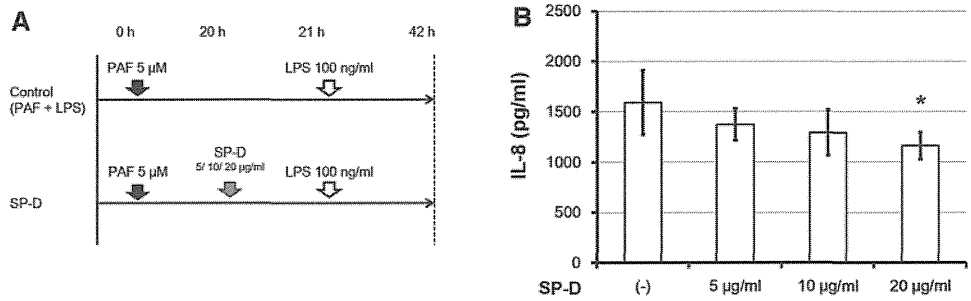

Fig. 3 SP-D attenuates IL-8 release in response to stimulation with PAF and LPS. **a** The time course of the experiments. SP-D was added 20 h after PAF (5 µM) and 1 h prior to LPS (100 ng/ml) stimulation at various concentrations. **b** Following treatment with 20 µg/ml SP-D, the release of IL-8 was significantly attenuated compared with that in cells treated with PAF and LPS only ($n = 4$, $*p < 0.05$ compared with controls, Dunnett's test). *IL* interleukin, *LPS* lipopolysaccharide, *PAF* platelet-activating factor, *SP-D* surfactant protein-D

found in most cells and tissues, and specific PAF receptors are abundantly expressed in the ileum and jejunum [19]. The activation of PAF receptors subsequently activates several signaling pathways, including NFκB and STAT-3 synthesis, resulting in their translocation into the nucleus and transcriptional activation of inflammatory molecules like IL-8 [20]. Intravenous administration of PAF causes ischemic intestinal necrosis [21]. Amer et al. reported that the stool concentrations of PAF are significantly higher in patients with NEC than in those without NEC [22]. PAF upregulation may lead to TLR4 overexpression and well as the synthesis of other pro-inflammatory cytokines, resulting in NEC induction and/or progression.

The LPS receptor TLR4/MD-2 is detectable in the fetal, neonatal, and adult intestine; however, LPS-induced inflammatory responses (NFκB activation and inflammatory cytokine production) are only detectable in fetal intestinal epithelial cells [23]. In contrast, mature intestinal epithelial cells express low levels of TLR4/MD2 and exhibit a poor response to LPS [8, 24]. Similarly, only cells overexpressing TLR4 (PAF treated) responded to LPS. Thus, LPS is a possible initiator of NEC [17] but requires other factors for upregulation of its receptor. IL-8 is a pro-inflammatory cytokine, the levels of which are elevated in patients with NEC [25], and a high IL-8 level may be associated with the severity of NEC [26]. IL-8 may also be a marker of the extent of affected intestinal necrosis [27]. Notably, the TLR4 agonist LPS [28] stimulates the production of IL-8 via NFκB [29].

SP-D is a member of the collectin family and is known to play an important role in innate immunity, especially in the airways [30]. Pulmonary SP-D directly binds to a variety of microorganisms and functions as an opsonin, thus enhancing bacterial uptake [31]. In addition, pulmonary SP-D binds to TLR4/MD-2 and inhibits LPS-induced inflammatory responses [14]. However, little is known about the function of SP-D in the intestines. Based on previous findings in the airways, we hypothesized that SP-D may attenuate the severity of NEC by reducing the inflammatory response in the intestines. Our data demonstrate that SP-D pretreatment attenuates IL-8 production induced by PAF and LPS. This observation suggests that a lack of SP-D in the premature intestine may be central to the pathogenesis of NEC. Moreover, our data suggest that

pretreatment with SP-D may prevent the development of NEC.

Several other cytokines are also elevated in NEC, including tumor necrosis factor-α, IL-1, and IL-6. Future investigations should evaluate the protective effects of intestinal SP-D against these components of the inflammatory response using in vivo NEC model.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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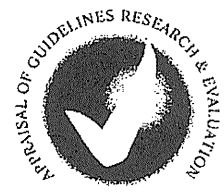
APPRAISAL OF GUIDELINES FOR RESEARCH & EVALUATION II



AGREE II

INSTRUMENT

The AGREE Next Steps Consortium
May 2009

UPDATE: September 2013

Advancing the science of practice guidelines

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FOR FURTHER INFORMATION ABOUT THE AGREE CONTACT:
AGREE II Project Office, agree@mcmaster.ca
AGREE Research Trust Web site, www.agreetrust.org

AGREE AGREE II

AGREE 10th Year Anniversary: 2003 - 2013

AGREE NEXT STEPS CONSORTIUM MEMBERSHIP

Dr. Melissa C. Brouwers
Principal Investigator, AGREE Next Steps Consortium
McMaster University, Hamilton, Ontario, Canada

Consortium Members:
Dr. GP. Browman, British Columbia Cancer Agency, Vancouver Island, Canada
Dr. JS. Burgers, Dutch Institute for Healthcare Improvement CBO, The Netherlands
Dr. F. Cluzeau, Chair of AGREE Research Trust; St. George's Hospital Medical School, London, UK
Dr. D. Davis, Association of American Medical Colleges, Washington, DC, USA
Dr. G. Feder, University of Bristol, UK
Dr. B. Fervers, Cancer et Environement, Centre Léon Bérard, France
Dr. I. Graham, Canadian Institutes of Health Research, Ottawa, Ontario, Canada
Dr. J. Grimshaw, Ottawa Health Research Institute, Ontario, Canada
Dr. SE. Hanna, McMaster University, Hamilton, Ontario, Canada
Ms. ME. Kho, McMaster University, Hamilton, Ontario Canada
Dr. P. Littlejohns, National Institute for Health and Clinical Excellence, London, UK
Ms. J. Makarski, McMaster University, Hamilton, Ontario, Canada
Dr. L. Zitzelsberger, Canadian Partnership Against Cancer, Ottawa, Ontario, Canada

NOTICE:

AGREE II Original Public Release and Publication Date: 2009/2010

AGREE II Update: September 2013

Please see the Update section, at the end of the Introduction, following the References section

Advancing the science of practice guidelines

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I. INTRODUCTION

I. OVERVIEW

i) Purpose of the AGREE II Instrument

Clinical practice guidelines ('guidelines') are systematically developed statements to assist practitioner and patient decisions about appropriate health care for specific clinical circumstances (1). In addition, guidelines can play an important role in health policy formation (2,3) and have evolved to cover topics across the health care continuum (e.g., health promotion, screening, diagnosis).

The potential benefits of guidelines are only as good as the quality of the guidelines themselves. Appropriate methodologies and rigorous strategies in the guideline development process are important for the successful implementation of the resulting recommendations (4-6). The quality of guidelines can be extremely variable and some often fall short of basic standards (7-9).

The *Appraisal of Guidelines for REsearch & Evaluation (AGREE) Instrument* (10) was developed to address the issue of variability in guideline quality. To that end, the AGREE instrument is a tool that assesses the methodological rigour and transparency in which a guideline is developed. The original AGREE instrument has been refined, which has resulted in the new AGREE II and includes a new User's Manual (11).

The purpose of the AGREE II, is to provide a framework to:

1. assess the quality of guidelines;
2. provide a methodological strategy for the development of guidelines; and
3. inform what information and how information ought to be reported in guidelines.

The AGREE II replaces the original instrument as the preferred tool and can be used as part of an overall quality mandate aimed to improve health care.

ii) History of the AGREE Project

The original AGREE Instrument was published in 2003 by a group of international guideline developers and researchers, the AGREE Collaboration (10). The objective of the Collaboration was to develop a tool to assess the quality of guidelines. The AGREE Collaboration defined quality of guidelines *as the confidence that the potential biases of guideline development have been addressed adequately and that the recommendations are both internally and externally valid, and are feasible for practice* (10). The assessment includes judgments about the methods used for developing the guidelines, the components of the final recommendations, and the factors that are linked to their uptake. The result of the Collaboration's effort was the original AGREE Instrument, a 23-item tool comprising 6 quality domains. The AGREE Instrument has been translated into many languages, has been cited in well over 100 publications, and is endorsed by several health care organizations. More details about the original instrument and related publications are available on the Web site of the AGREE Research Trust (http://www.agreetrust.org/), the official body managing the interests of the AGREE Instrument.

As with any new assessment tool, it was recognized that ongoing development was required to strengthen the measurement properties of the instrument and to ensure its usability and feasibility among intended users. This led several members of the original team to form the AGREE Next Steps Consortium (Consortium). The objectives of the Consortium were to further improve the measurement properties of the instrument, including its reliability and validity; to refine the instrument's items to better meet the needs of the intended users; and to improve the supporting documentation (i.e., original training manual and user's guide) to facilitate the ability of users to implement the instrument with confidence.

The result of these efforts is the AGREE II, which is comprised of the new User's Manual and 23 item tool organized into the same six domains, described here. The User's Manual is a significant modification of the original training manual and user's guide and provides explicit information for each of the 23 items. Table 1 compares the items of the original AGREE to the items in the AGREE II.

Table 1. Comparison of original AGREE and AGREE II items.

Original AGREE Item	AGREE II Item
Domain 1. Scope and Purpose	
1. The overall objective(s) of the guideline is (are) specifically described.	No change
2. The clinical question(s) covered by the guideline is (are) specifically described.	The health question(s) covered by the guideline is (are) specifically described.
3. The patients to whom the guideline is meant to apply are specifically described.	The population (patients, public, etc.) to whom the guideline is meant to apply is specifically described.
Domain 2. Stakeholder Involvement	
4. The guideline development group includes individuals from all the relevant professional groups.	No change
5. The patients' views and preferences have been sought.	The views and preferences of the target population (patients, public, etc.) have been sought.
6. The target users of the guideline are clearly defined.	No change
7. The guideline has been piloted among end users.	Delete item. Incorporated into user guide description of item 19.
Domain 3. Rigour of Development	
8. Systematic methods were used to search for evidence.	No change in item. Renumber to 7.
9. The criteria for selecting the evidence are clearly described.	No change in item. Renumber to 8.
	NEW Item 9. The strengths and limitations of the body of evidence are clearly described.
10. The methods for formulating the recommendations are clearly described.	No change
11. The health benefits, side effects, and risks have been considered in formulating the recommendations.	No change

Original AGREE Item	AGREE II Item
12. There is an explicit link between the recommendations and the supporting evidence.	No change
13. The guideline has been externally reviewed by experts prior to its publication.	No change
14. A procedure for updating the guideline is provided.	No change
Domain 4. Clarity of Presentation	
15. The recommendations are specific and unambiguous.	No change
16. The different options for management of the condition are clearly presented.	The different options for management of the condition or health issue are clearly presented.
17. Key recommendations are easily identifiable.	No change
Domain 5. Applicability	
18. The guideline is supported with tools for application.	The guideline provides advice and/or tools on how the recommendations can be put into practice. AND Change in domain (from Clarity of Presentation) AND renumber to 19
19. The potential organizational barriers in applying the recommendations have been discussed.	The guideline describes facilitators and barriers to its application. AND change in order – renumber to 18
20. The potential cost implications of applying the recommendations have been considered.	The potential resource implications of applying the recommendations have been considered.
21. The guideline presents key review criteria for monitoring and/ or audit purposes.	The guideline presents monitoring and/ or auditing criteria.
Domain 6. Editorial Independence	
22. The guideline is editorially independent from the funding body.	The views of the funding body have not influenced the content of the guideline.
23. Conflicts of interest of guideline development members have been recorded.	Competing interests of guideline development group members have been recorded and addressed.

II. APPLYING THE AGREE II

i) Which guidelines can be appraised with the AGREE II?

As with the original instrument, AGREE II is designed to assess guidelines developed by local, regional, national or international groups or affiliated governmental organizations. These include original versions of and updates of existing guidelines.

The AGREE II is generic and can be applied to guidelines in any disease area targeting any step in the health care continuum, including those for health promotion, public health, screening, diagnosis, treatment or interventions. It is suitable for guidelines presented in paper or electronic format. At this stage, the AGREE II has not been designed to assess the quality of guidance documents that address health care organizational issues. Its role in the assessment of health technology assessments has not yet been formally evaluated.

ii) Who can use the AGREE II?

The AGREE II is intended to be used by the following stakeholder groups:

- by **health care providers** who wish to undertake their own assessment of a guideline before adopting its recommendations into their practice;
- by **guideline developers** to follow a structured and rigorous development methodology, to conduct an internal assessment to ensure that their guidelines are sound, or to evaluate guidelines from other groups for potential adaptation to their own context;
- by **policy makers** to help them decide which guidelines could be recommended for use in practice or to inform policy decisions; and
- by **educators** to help enhance critical appraisal skills amongst health professionals and to teach core competencies in guideline development and reporting.

III. KEY RESOURCES AND REFERENCES

i) AGREE Research Trust

The AGREE Research Trust (ART) is an independent body established in 2004 at the conclusion of the activities of the original AGREE Collaboration. ART endorses the AGREE II and manages the interests of the AGREE enterprise, supports a research agenda regarding its development, and serves as the holder of its copyright.

The AGREE Research Trust web site http://www.agreetrust.org provides:

- free downloadable copies of AGREE II
- links to the AGREE II on-line training tool
- reference lists citing AGREE II and the original AGREE Instrument
- free downloadable copies of the original AGREE Instrument
- information about AGREE projects, the AGREE Next Steps Consortium and the original AGREE Collaboration

ii) How to cite the AGREE II

AGREE Next Steps Consortium (2009). *The AGREE II Instrument* [Electronic version]. Retrieved <Month, Day, Year>, from http://www.agreetrust.org .

iii) AGREE II On-Line Training Tool

For access to the AGREE II On-Line Training Tool, please visit http://www.agreetrust.org .

iv) References related to the AGREE II

AGREE II: Advancing guideline development, reporting and evaluation in healthcare. *Parallel publications in progress*

v) Primary reference related to the original AGREE Instrument

AGREE Collaboration. Development and validation of an international appraisal instrument for assessing the quality of clinical practice guidelines: the AGREE project. *Qual Saf Health Care.* 2003 Feb;12(1):18-23.

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11. AGREE II: Advancing the guideline development, reporting and evaluation in healthcare. Parallel publications in progress.

UPDATE: September 2013

In 2013, the AGREE marked its 10th anniversary since the original AGREE Instrument was first published and made available for use. To mark this anniversary, we provide a summary of activities that have taken place over the past 10 years and an update to the references originally noted in the AGREE II 2009 version.

UPTAKE AND USE OF AGREE II

As with the original AGREE Instrument, uptake and use of AGREE II has been significant. Between 2010 (publication of AGREE II) and July 2013, a cited reference search revealed a total of 590 articles referencing the four core AGREE articles. An analysis of the AGREE Enterprise Website (www.agreetrust.org) showed much activity with a total of 42,553 visits to the website over a seven month period (January – July 2013). In addition, to date nearly 2,000 users have registered accounts with the website. AGREE II continues to be used as the basis of PG development frameworks, academic course materials and requirements, and PG evaluation activities.

I. SUMMARY OF ACTIVITIES:

1. AGREE Website: Development Project www.agreetrust.org
   - Using a user-centred development strategy and working with a website development group specializing in building evidence-based healthcare sites (www.minervation.com), we redeveloped and redesigned the AGREE website.
   - The Website included various resources, including an online platform to complete and store individual AGREE II appraisals of PGs, "My AGREE".
   - www.agreetrust.org is the AGREE Enterprise's website and the home for all things AGREE.

2. *NEW* (2013) – "My AGREE PLUS"
   - In response to user feedback and in recognizing a service gap, we enhanced the original "My AGREE" online platform to include the functionality for completing group (multi-rater) AGREE II appraisals of practice guidelines.
   - New functions include the capacity to "Contribute" to a group appraisal and to "Coordinate" a group appraisal.
   - To facilitate use of "My AGREE PLUS", several "Help" videos are available.
   - Visit: www.agreetrust.org and click on top right tab, **"My AGREE PLUS"**

3. AGREE II Training Tools (online)
   - To facilitate the application and use of AGREE II, we developed two innovative, online training tools
     i. AGREE II Overview Tutorial
     ii. AGREE II Overview Tutorial + Practice Exercise
   - Visit the ***Resource Centre*** of the AGREE website: http://www.agreetrust.org/resource-centre/agree-ii-training-tools/

4. AGREE II Language Translations
   - As with the original AGREE Instrument, members of the international PG community have taken the initiative to translate the AGREE II in various languages. We extend our thanks to those members for undertaking and making available the translations.
   - Completed translations:
     i. Basques, Dutch, French, Italian, Korean, Portuguese, Portuguese (Brazilian), Slovakian, Spanish, Thai

- In progress translations:
    i. Arabic, Chinese (Traditional), Chinese (Mandarin), Czech, Farsi (Persian), German, Greek, Japanese, Romanian, Russian, Turkish
- If you would like to undertake a translation, please contact us by emailing agree@mcmaster.ca .
- Visit the ***Resource Centre*** of the AGREE website to access a translation: http://www.agreetrust.org/resource-centre/agree-ii-translations/

5. **Ongoing Program of Research**
    - As an assessment tool, AGREE II evaluates the methodological rigour used to develop a particular practice guideline. It does not assess the clinical validity of practice guideline recommendations.
    - To address the gap, the AGREE Enterprise is undertaking a program of research to develop a knowledge resource to direct the development, reporting and evaluation of practice guideline recommendation clinical credibility.
    - The knowledge resource will accompany the AGREE II.
    - Please visit the website for updates to ongoing research work: http://www.agreetrust.org/agree-research-projects/

As always, we welcome your feedback and suggestions. We enjoy hearing from our users and the PG community at large, so please contact us through our website or by emailing us directly via agree@mcmaster.ca .

II. AGREE II REFERENCES:

Listed below are the core references related to the AGREE II and its training tools:

AGREE II: Non-Technical Paper *(Main publication: Canadian Medical Association Journal; parallel publications in Journal of Clinical Epidemiology and Preventive Medicine)*
Brouwers M, Kho ME, Browman GP, Cluzeau F, feder G, Fervers B, Hanna S, Makarski J on behalf of the AGREE Next Steps Consortium. AGREE II: Advancing guideline development, reporting and evaluation in healthcare. Can Med Assoc J. Dec 2010, 182:E839-842; doi: 10.1503/cmaj.090449

Brouwers M, Kho ME, Browman GP, Cluzeau F, feder G, Fervers B, Hanna S, Makarski J on behalf of the AGREE Next Steps Consortium. AGREE II: Advancing guideline development, reporting and evaluation in healthcare. J Clin Epidemol. 2010, 63(12): 1308-1311

Brouwers M, Kho ME, Browman GP, Cluzeau F, feder G, Fervers B, Hanna S, Makarski J on behalf of the AGREE Next Steps Consortium. AGREE II: Advancing guideline development, reporting and evaluation in healthcare. Preventive Medicine, 2010, 51(5): 421-424

AGREE II: Technical Papers (Parts I and II)
Brouwers M, Kho ME, Browman GP, Burgers J, Cluzeau F, Feder G, Fevers B, Graham ID, Hanna SE, Makarski J, on behalf of the AGREE Next Steps Consortium. Performance, usefulness and areas for improvement: Development steps toward the AGREE II – Part 1. Can Med Assoc J. 2010, 182: 1045-52

Brouwers MC, Kho ME, Browman GP, Burgers J, Cluzeau F, Feder G, Fervers B, Graham ID, Hanna SE, Makarski J, on behalf of the AGREE Next Steps Consortium. Validity assessment of items and tools to support application: Development steps towards the AGREE II – Part 2. Can Med Assoc J. 2010, 182: E472-78

AGREE A3 Project, Stream 1 (Training Tools Development & Evaluation)
Brouwers MC, Makarski J, Levinson A. A randomized trial to evaluate e-learning interventions designed to improve learner's performance, satisfaction, and self-efficacy with the AGREE II. Implement Sci. 2010; 5:29

Brouwers MC, Makarski J, Durocher L, Levinson A. E-learning interventions are comparable to user's manual in a randomized trial of training strategies for the AGREE II. Implement Sci. 2011; 6:81

AGREE II:

USER'S MANUAL

II. USER'S MANUAL: INSTRUCTIONS FOR USING THE AGREE II

This User's Manual has been designed specifically to guide appraisers in the use of the instrument. We suggest reading the following instructions before using the instrument.

I. Preparing to Use the AGREE II

i) Accompanying Guideline Documents

Before applying the AGREE II, users should first carefully read the guideline document in full. In addition to the guideline document, users should attempt to identify all information about the guideline development process prior to the appraisal. This information may be contained in the same document as the guideline recommendations or it may be summarized in a separate technical report, methodological manual or guideline developer policy statement. These supporting documents may be published or may be available publicly on web sites. While it is the responsibility of the guideline authors to advise readers on the existence and location of relevant additional technical and supporting documents, every effort should be made by the AGREE II users to locate and include them as part of the materials appropriate for assessment.

ii) Number of Appraisers

We recommend that each guideline is assessed by at least 2 appraisers and preferably 4 as this will increase the reliability of the assessment. Reliability tests of the instrument are on-going.

II. Structure and Content of the AGREE II

The AGREE II consists of 23 key items organized within 6 domains followed by 2 global rating items ("Overall Assessment"). Each domain captures a unique dimension of guideline quality.

Domain 1. Scope and Purpose is concerned with the overall aim of the guideline, the specific health questions, and the target population (items 1-3).

Domain 2. Stakeholder Involvement focuses on the extent to which the guideline was developed by the appropriate stakeholders and represents the views of its intended users (items 4-6).

Domain 3. Rigour of Development relates to the process used to gather and synthesize the evidence, the methods to formulate the recommendations, and to update them (items 7-14).

Domain 4. Clarity of Presentation deals with the language, structure, and format of the guideline (items 15-17).

Domain 5. Applicability pertains to the likely barriers and facilitators to implementation, strategies to improve uptake, and resource implications of applying the guideline (items 18-21).

Domain 6. Editorial Independence is concerned with the formulation of recommendations not being unduly biased with competing interests (items 22-23).

Overall assessment includes the rating of the overall quality of the guideline and whether the guideline would be recommended for use in practice.