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Faecal volatile organic compounds in preterm babies at risk of necrotising enterocolitis

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Archives of Disease in Childhood

Faecal volatile organic compounds in preterm babies at risk of necrotising enterocolitis: the DOVE study

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3 **Faecal volatile organic compounds in preterm babies at risk of necrotising enterocolitis: the DOVE**
4 **study**
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Abstract

Background: early diagnosis of NEC may improve prognosis but there are no proven biomarkers.

Objective: to investigate changes in faecal volatile organic compounds (VOCs) as potential biomarkers for NEC.

Design: multicentre prospective study

Settings: 8 UK Neonatal Units

Patients: preterm infants <34 weeks gestation

Methods: daily faecal samples were collected prospectively from 1,326 babies of whom 49 subsequently developed definite NEC. Faecal samples from 32 NEC cases were compared to samples from frequency-matched controls without NEC. Headspace, solid phase micro-extraction gas chromatography/mass spectrometry was performed and VOCs identified from reference libraries. VOC samples from cases and controls were compared using both discriminant and factor analysis methods.

Results: VOCs were found to cluster into 9 groups (factors), three were associated with NEC and indicated the possibility of disease up to 3-4 days before the clinical diagnosis was established. For one factor, a one standard deviation increase increased the odds of developing NEC by 1.6 times; a similar decrease of the two other factors was associated with a reduced risk (OR 0.5 or 0.7, respectively). Discriminant analyses identified five individual VOCs, which are associated with NEC in babies at risk, each with an AUROC of 0.75-0.76, up to four days before the clinical diagnosis was made.

Conclusions: Faecal VOCs are altered in preterm infants with NEC. These data are insufficient to enable reliable cotside detection of babies at risk of developing NEC and further work is needed investigate the role of VOCs in clarifying the aetiology of NEC.

Keywords: necrotising enterocolitis; preterm infants; volatile organic compounds; longitudinal discriminant analysis.

Introduction

Necrotising enterocolitis (NEC) is the commonest gastrointestinal emergency in premature infants, occurring in up to 8% of admissions with to 40% mortality.¹⁻³ This has not significantly improved over recent decades^{3, 4} despite overall improvements in neonatal intensive care and survival³⁻⁵ and it is possible that the incidence and mortality from NEC are increasing as more preterm infants survive.³⁻⁵ In addition, NEC is associated with significant morbidity in up to 50% of survivors; either due to the disease itself^{6, 7} or the complications of prolonged parenteral nutrition, surgical procedures and short bowel syndrome.^{3, 8} Survivors of severe NEC are more likely to have neurodevelopmental impairment.^{3, 9, 10} Despite this, the pathogenesis is uncertain and there is no specific treatment. Conservative therapy is limited to discontinuing enteral feeding, antibiotics and supportive care. In advanced disease, surgery to remove affected bowel is a further option.³

Early detection of NEC is vital to limit progression to advanced fulminant disease. However, establishing the diagnosis in the prodrome is difficult because clinical features and investigations are often inconclusive. Diagnostic investigations, such as abdominal radiographs, only identify established disease and in fulminant NEC conservative management is often ineffective at preventing disease progression.

Potential biomarkers for NEC including CRP,¹¹ calprotectin,¹² breath hydrogen concentration,¹³ IL6 and IL8¹⁴ have been investigated, but have a limited role in detecting early disease.^{12, 15} The likely aetiology of NEC is a result of defective interactions between the intestinal microbiota and the host's response and it has been suggested that high-throughput sequencing technology may enable the detection of an early diagnosis biomarker.¹³ Detection of faecal biochemical changes that predict the onset of NEC would enable the instigation of early treatment measures which may reduce disease severity and limit morbidity and mortality.

Volatile organic compounds (VOCs) have a low boiling point (and high vapour pressure) and readily enter the vapour phase. VOCs are emitted from many bodily fluids and contribute to their smell (e.g. faeces).¹⁶ Faecal VOCs are produced by digestion and/or fermentation of intestinal luminal contents and are influenced by the microbiota and diet.¹⁷ Faecal VOCs have a similar, stable pattern in healthy adults,¹⁸ but there are characteristic changes in VOC patterns in some intestinal diseases.¹⁸⁻²¹ We have previously examined faecal VOCs in preterm infants²² and reported a pilot study that showed a characteristic change in VOC patterns in babies developing NEC.²³ De Meij *et al.* used an electronic nose to study faecal gases, and suggested the output could be used to diagnose NEC.²⁴

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3 We aimed to investigate further the pattern of faecal VOCs in a larger, prospective, multi-centre
4 study, comparing VOCs in apparently healthy preterm infants developing NEC with those without
5 NEC.
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11 **Methods**

12 *Participants*

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16 Between November 2011 and October 2013 all newborns <34 weeks gestation (and therefore at
17 greater risk of NEC), admitted to eight UK neonatal units [Birmingham Heartlands, Birmingham
18 Women's, Liverpool Women's, Royal Shrewsbury, Royal Wolverhampton, Sheffield Teaching,
19 University Hospital Coventry and Warwickshire, University Hospital Leicester], were considered for
20 recruitment. Babies were excluded if they were unlikely to survive or had significant
21 gastrointestinal anomalies. No centre used probiotics as routine prophylaxis at the time of study.
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27 Parents of potential recruits were approached by a research midwife who obtained written
28 informed consent. Daily faecal samples were collected, if available, from recruitment until NEC was
29 diagnosed or until discharge. Samples were placed in 7ml glass vials (Fisher, Loughborough, UK) and
30 immediately frozen at -20°C. We have previously shown that there is no significant change in VOCs
31 in samples stored for between 1 week, and 12 months at -20°C.²⁵ Batched samples were transported
32 on dry ice at regular intervals to the research laboratory.
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38 Demographic and clinical data on mode of delivery, feeding, antibiotic exposure, respiratory
39 support and episodes of illness were collected. Babies with possible NEC were identified by the
40 clinical team caring for the baby and the NEC grade was subsequently classified by consensus
41 between that team and the independent study team using modified Bell's criteria.²⁶ Controls did
42 not develop NEC of any grade. Approximately two control babies were frequency matched to each
43 case of NEC with Bell's criteria IIa or greater (i.e. definite NEC): matching was based on
44 demographic factors including mode of delivery, gestational age at birth, birth weight, sex, age at
45 sampling, mode of feeding and antibiotic exposure. Where possible we identified samples from six
46 days prior to the definitive diagnosis of NEC. For controls, a day equivalent to the NEC diagnosis
47 date was determined, and samples were analyzed from the previous 6 days.
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55 The study was sponsored by University of Birmingham and approved by West Midlands Research
56 Ethics Committee (11/WM/0078).
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Laboratory methods

We have previously reported the laboratory method used.²⁷ 50-100mg of faeces was transferred to 10 ml glass vials (Sigma-Aldrich, Dorset, UK) and analyzed by headspace solid phase micro-extraction (SPME). A PerkinElmer Clarus 500 gas chromatography/mass spectrometry (GC/MS) quadrupole benchtop system (Beaconsfield, UK) was used with a Combi PAL auto-sampler (CTC Analytics, Switzerland); a Zebron ZB-624 GC column with inner diameter 0.25 mm, length 60 m, film thickness 1.4 μm (Phenomenex, Macclesfield, UK); carrier gas was 99.996% pure helium (BOC, Sheffield, UK). Samples were pre-incubated for 30 minutes at 60°C prior to exposure to a pre-conditioned 85 μm Carboxen®/Polydimethylsiloxane SPME fiber (Sigma-Aldrich, Dorset, UK). GC oven initial temperature was 40°C, held for 1 minute before ramping to 220°C at a rate of 5°C/min and held for 13 min (total run time - 50 min). Solvent delay was set for the first 6 min. The MS was operated in electron impact ionization EI+ mode, scanning from ion mass fragments 10- 300 m/z with an inter-scan delay of 0.1 sec and a resolution of 1000 at full width at half maximum (FWHM). Helium gas flow rate was 1 ml/min. Instrument sensitivity was determined using 2-pentanone with limit of detection 3 times the signal/noise ratio. This laboratory analysis was conducted between November 2013 and August 2014.

Sample VOCs were identified using Automated Mass Spectral Deconvolution System (AMDIS- version 2.71, 2012) software and the NIST mass spectral library (version 2.0, 2011) in conjunction with the R package Metab.²⁸ IUPAC names were assigned to VOCs.

Statistical methods

Peak area values for VOCs, obtained from AMDIS, were log-transformed and normalized. There is no widely accepted approach for treatment of values below the detection threshold. In this study, VOC values below the detection threshold were given an abundance of -3 (equivalent to three standard deviations below the mean), which corresponds to <1% for normally distributed data. Within sample analysis of VOCs was performed independent of case/control status. Within the VOCs identified we established patterns using principal component analysis (PCA) to produce a smaller number of variables (factors) which captured most of the data variability. PCA uses the correlation matrix to identify compounds that are strongly correlated to one another and divide them into factors (Table s1-s3 for examples). Each factor can be expressed as a linear combination of the VOCs. Using factors rather than individual VOCs avoided the overfitting problems associated with fitting a model with more predictors than events. A similar approach was followed by de Meij *et al.*,²⁴ with the distinction that here the factors were allowed to be correlated with each other

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3 following a non-orthogonal rotation process (promax rotation power of 4, SPSS version 23). We
4 chose this method to reflect different unknown biological mechanisms by which these factors
5 might contribute to NEC and could be associated with each other. Factors were investigated for
6 biological plausibility by regressing against demographic variables. All regression analysis containing
7 multiple data points per infant used robust standard errors in Stata (version 14) and account for the
8 longitudinal nature of the data by clustering by infant. Logistic regression assessed the ability of
9 each factor to predict NEC.
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16 A recently-developed longitudinal discriminant analysis (LoDA) method²⁹⁻³¹ assessed the association
17 of individual VOCs with the risk of developing NEC. Linear mixed models were fit separately to each
18 VOC for both NEC and control samples adjusting for age, gestation, delivery type, sex and a random
19 effect intercept. Discriminant analysis was used for each VOC to determine whether the patient
20 should be classified as at risk of NEC: 1000 bootstrap datasets were generated to evaluate the
21 sensitivity, specificity and area under the curve (AUC). Mean lead time was calculated as the mean
22 time before diagnosis when the infant was correctly scored at risk of NEC.
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28 The classification scheme (Figure 1) was designed to predict the risk of NEC. The threshold chosen
29 was determined by receiver operating characteristic (ROC) curve analysis. When the data showed a
30 baby's risk of developing NEC was above the chosen threshold they were classified as an NEC case
31 (if this were conducted in real time this would allow a clinician to consider early intervention).
32 Whilst a baby's risk remained below the threshold, they were provisionally classified as not
33 developing NEC and their risk were reassessed with the addition of the next day's data.
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41 Results

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43 1,326 premature babies were recruited. 49 babies were diagnosed with NEC Bell's stage IIa or
44 greater and 32 of these had adequate sample collection for analysis; these were matched with 70
45 controls. There was no evidence of concomitant sepsis in any of the cases and all the controls were
46 deemed healthy at the time of sampling. Neonates did not defaecate daily: a median of 2 samples
47 were analyzed for both cases and controls. We collected 81 samples from cases and selected 163
48 from controls within the 6-day time window (Supplementary Table S4) following the matching
49 procedure described above. See Table 1 for demographic data.
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Table 1 Summary of demographic and clinical factors

	NEC	Control	Test	Probability
	32	70		
Sex			Chi-sq	0.05
Male (n [%])	13 [41]	43 [61]		
Birth weight (median [IQR], g)	795 [976]	1045 [541]	Mann Whitney	0.0002
Mode of delivery			Chi-sq	0.160
Vaginal (n [%])	15 [47]	27 [39]		
Missing data	0	1 [1.4]		
Feeding pattern (n[%])			Chi-sq	0.65
Breast+formula	25 [78]	52 [74]		
Exclusive breast	6 [19]	13 [19]		
Exclusive formula	1 [3]	5 [7]		
Postnatal age at first sample (median [IQR]), d	21 [22]	22 [27]	Mann Whitney	0.66
Duration of gestation (median,[IQR]), w	26.7[2.9]	28.4 [3.8]	Mann Whitney	0.002
Post conception at first sample (median,[IQR]), w	30.6[4.4]	32.4[3.7]	Mann Whitney	0.02
AB use before sampling	32	70		
Bell's grade				
2	14	NA		
3	18	NA		

Number of samples		
Days before diagnosis	Control	NEC
1	34	16
2	22	8
3	42	20
4	18	14
5	31	16
6	19	8

Number of samples		
Distribution	Control	NEC
Median	2	2
Q1	1.5	2
Q3	3.5	3
IQR	2	1

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3 50 headspace VOCs were identified (prevalence 45-99%). On average NEC cases had 35.8 VOCs
4 identified per sample and controls 36.4. Two VOCs with similar structures and retention times
5 (30.73 and 30.90 minutes, respectively) were difficult to resolve and their data were combined.
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9 We identified 9 different factors (groups of VOCs) that had correlated abundances and thus might
10 represent a common metabolic (examples in Supplementary Tables S1-3.) or demographic factor
11 (Table 2). They were investigated for biological plausibility and 7 of the 9 were associated with
12 demographic variables linked to NEC: birthweight (factors 1, 6 and 9), formula feed (factor 2 and 9),
13 postnatal age (factor 4), gender (factor 3) and postconception age (factors 5 and 8), biochemical
14 factors (factor 7). Multiple logistic regression identified three predictors of NEC (factors 4, 5 and
15 8), from which a ROC was generated. A one standard deviation increase in factor 4 increased the
16 chances of NEC (odds ratio (OR) 1.66 [95% confidence interval (CI) 1.13, 2.43]. Increases in factor 5
17 and 8 were associated with a reduced risk of NEC (OR 0.47 [95% CI 0.30, 0.78] and 0.73 [95% CI
18 0.53, 0.99]), respectively. Area under the ROC curve was 0.69 [95% CI 0.62, 0.76]. Results
19 separated by day are shown in Table 3.
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Table 2 Statistical summary variables (group) observed in the VOC profiles

Group	VOCs in the group (+)=positive direction (-)negative direction	Demographics associated with the group
1	(+) (E)-hex-2-enal (+) heptan-2-one (+) heptanal (+) 2-pentylfuran (+) (Z)-hept-2-enal (+) dec-1-en-3-one (+) octanal (+) 5-ethylcyclopentene-1-carbaldehyde (+) (E)-oct-2-enal (+) nonan-1-ol (+) prop-2-enal (+) propanal (+) 2-ethylfuran (+) pent-1-en-3-one (+) pentanal (+) (E)-pent-2-enal (+) hexanal	Birthweight
2	(+) ethanol (+) propan-1-ol (+) butane-2,3-dione (-) ethyl acetate (+) acetic acid (+) propyl acetate (+) propanoic acid (+) propyl propanoate	Post-conceptual age, breast feeding, gender
3	(+) 2,2,4,6,6-pentamethylheptane (+) 2,2,4,4-tetramethyloctane (+) 2,6,10-trimethyl dodecane (+) 3,3,4-trimethyl decane	Not associated with any recorded demographic variables
4	(+) 3-methylsulfanylpropanal (+) benzaldehyde (+) 2-phenylacetaldehyde (+) 2-methylpropanal (-) 3-methylbutanal (-) 2-methylbutanal	Formula milk, age
5	(-) heptan-4-one (+) methanedithione (+) butan-2-one	Post-conceptual age
6	(+) propan-2-one (-) propan-2-ol	Birthweight
7	(+) ethylbenzene (+) xylene (+)(4R)-1-methyl-4-prop-1-en-2-ylcyclohexene	Not associated with any recorded demographic variables
8	(+) 3-methylbutanoic acid (+) 2-methylbutanoic acid (+) butanoic acid	Post-conceptual age
9	(+) 6-methyl-5-hepten-2-one (+) 3,4-dimethylcyclohexan-1-ol (+) 4-acetyl-2,3,4,5,5-pentamethyl-2-cyclopenten-1-one	Formula use, birthweight

On average, LoDA was able to predict NEC for each VOC individually up to 4 days before the diagnosis of NEC (e.g., 95% CI for (Z)-hept-2-enal = (2.79, 4.42) days). 28 VOCs could classify infants reasonably with an AUC greater than 0.7 (Table 4). The best performing VOCs could identify approximately 70% of NEC cases, and also correctly identified approximately 70% of controls using the cut off which was chosen as the point on the ROC curve closest to the top left corner (the five VOCs with the best level of discrimination are shown in Figure 2).

Table 3 Mean values of the area under the ROC curve in the prodromal period

	Day -6	Day -5	Day -4	Day -3	Day -2	Day -1
AUC	0.70	0.76	0.45	0.70	0.74	0.74
	[0.47, 0.94]	[0.61, 0.91]	[0.24, 0.66]	[0.54, 0.85]	[0.56, 0.93]	[0.59, 0.90]
No. observations	27	47	31	59	30	50

Table 4 Prediction accuracy for VOCs with AUC >.7 using longitudinal discriminant analysis

	Cutoff	Sensitivity	Specificity	AUC	Mean Lead Time (d)
(Z)-hept-2-enal	0.37	0.78	0.68	0.76	3.68
pent-1-en-3-one	0.36	0.72	0.73	0.76	3.64
2-ethylfuran	0.38	0.71	0.73	0.76	3.59
pentanal	0.36	0.72	0.73	0.75	3.51
2-pentylfuran	0.37	0.73	0.71	0.75	3.47
dec-1-en-3-one	0.37	0.70	0.73	0.75	3.99
2,6,10-trimethyl dodecane	0.39	0.71	0.72	0.74	4.04
(E)-oct-2-enal	0.36	0.72	0.71	0.74	3.76
2-methylbutanoic acid	0.40	0.74	0.67	0.74	3.64
2,2,4,6,6-pentamethylheptane	0.40	0.69	0.74	0.74	3.91
nonan-1-ol	0.37	0.72	0.69	0.74	3.88
2-methylpropanal	0.35	0.71	0.70	0.73	3.70
3-methylbutanoic acid	0.39	0.71	0.67	0.73	3.61
3-methylsulfanylpropanal	0.38	0.68	0.71	0.73	3.90
butan-2-one	0.38	0.72	0.68	0.72	3.62
butanoic acid	0.35	0.71	0.70	0.72	3.94
propyl acetate	0.37	0.74	0.65	0.72	4.00
heptanal	0.36	0.70	0.69	0.72	3.58
propan-1-ol	0.36	0.69	0.70	0.72	3.96
2,2,4,4-tetramethyloctane	0.40	0.68	0.72	0.72	3.93
ethanol	0.37	0.67	0.73	0.72	4.09
(4R)-1-methyl-4-prop-1-en-ylcyclohexene	0.39	0.66	0.71	0.72	3.73
(E)-hex-2-enal	0.36	0.68	0.70	0.71	3.64
(E)-pent-2-enal	0.37	0.71	0.67	0.71	3.65
propan-2-one	0.35	0.71	0.66	0.71	3.48
octanal	0.35	0.70	0.67	0.71	3.55
propan-2-ol	0.37	0.68	0.66	0.71	3.54
2-phenylacetaldehyde	0.37	0.68	0.69	0.71	4.07

Discussion

We have undertaken GCMS analysis of faecal headspace gases obtained from premature infants at risk of NEC in the largest prospective study of its kind. Over 7000 samples were collected from 1326 babies and pre-diagnosis samples from the 32 babies who subsequently developed NEC were identified and produced 9 distinct groups of VOCs (factors). Comprehensive analysis of VOCs found three factors (4, 5 and 8) that were associated with NEC.

We have previously shown, in a much smaller study of only 6 NEC cases using a less sophisticated analysis, that changes in faecal VOC patterns may occur in the pre-clinical phase of NEC.²³ This finding has also been demonstrated by using an eNOSE system,²⁴ however the present study included almost three times as many cases of NEC than the study by de Meij *et al.*²⁴ and provided detailed analysis of individual VOCs (rather than just changes in the pattern) which allowed us to explore, for the first time, the biological plausibility of the changes in VOCs identified.

This biological plausibility, shown by the association of the different factors with demographic variables, may represent changes of VOCs produced by biological processes, such as the colonisation of the gut by specific bacteria. The association of three of the factors with post-conceptual age rather than gestational age or postnatal age alone, suggests an interaction between the maturation of the gut and bacteria colonisation, which may be protective.

Matching was carried out to take account of all demographic variables considered likely to confound the results of the analysis: thus, priority was given to gestation, mode of delivery, place of birth and feed type. Despite this adjustment there remained differences in birthweight, sex and gestation (Table 1) and perfect matching was not achievable as they were matched to available samples at the time that they were suspected of having NEC, to prevent excess frozen samples deteriorating prior to analysis. Nevertheless, the linear mixed models involved in the longitudinal discriminant analysis adjusted for a number of demographic and baseline factors, including age at sampling, gestational age at birth, mode of delivery and sex.

In the sample analysis of different factors identified within the compounds, we observed that of the 9 factors identified following PCA, at least three factors were associated with birthweight, but none of these was also associated with the diagnosis of NEC. Factor 4 contained six correlated VOCs which are associated with formula milk feeds and age (Table 2 and Table s1). The risk of NEC increases with the value of Factor 4, thus the VOCs may be biomarkers of NEC. Three of VOCs in Factor 4 are methyl-aldehydes: 3-methylbutanal and 2-methylbutanal, are specifically related to the metabolism of amino acids, leucine and isoleucine, respectively. Such aldehydes can be converted

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3 into their respective acids, 3-methyl and 2-methylbutanoic acid by aldehyde dehydrogenase. These
4 two acids are present in Factor 8 which is linked to a decreased risk of NEC. A change in the
5 abundance of bacteria or fungi that produce aldehyde dehydrogenase is a possible explanation for
6 these findings.
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10 Bifidobacteria reduce the likelihood of developing NEC.³² *In vitro* studies have shown that
11 Bifidobacteria produce 3-methyl and 2-methyl butanoic acid.³² Thus the occurrence of these VOCs,
12 present in Factor 8, might be a reflection of the abundance of Bifidobacteria in the controls.
13 Lactobacilli and Bifidobacteria are a source of butanoic acid present in Factor 8:³² thus, the greater
14 abundance of butanoic acid in control infants may be a result of the protective effect of these
15 bacteria.
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21 Both Bifidobacteria and Lactobacilli produce butan-2-one which was found in Factor 5 also
22 negatively associated with NEC. A further VOC in factor 5, methanedithione (carbon disulphide)³³
23 may also be produced by Bifidobacteria.³⁴ Carbon disulphide has numerous toxic properties, but
24 may be beneficial as it inhibits NfκB signalling by binding to IKKβ, NEMO and NfκB^{33, 34} and thus has
25 anti-inflammatory properties. Bifidobacteria 'secretions' have been shown to reduce NfκB in
26 transwell experiments.³⁴
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32 Univariate analysis showed several VOCs with abundances which differed between cases and
33 controls to such an extent that they appeared to act as biomarkers for NEC 3-4 days before
34 diagnosis. Some appeared in the factor analysis, but others were significant although not present in
35 Factors 4, 5 or 8. Notable were propanal, pentanal, hexanal: aldehydes identified in Factor 1.
36 Aldehydes are associated with Crohn's disease²⁰ and are thought to arise from lipid peroxidation as
37 a consequence of epithelial inflammation. The presence of these compounds may be an earlier
38 indicator of enterocolitis.
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45 Both the LoDA and the factor analysis highlight the fact that a number of VOCs are associated with
46 a high risk of developing NEC. The LoDA approach could be extended to consider multiple VOCs
47 simultaneously, although a greater sample size would be required. Validation in further datasets
48 would be required before the usefulness of individual VOCs as potential biomarkers for
49 classification of NEC could be determined.
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54 Although our study is the largest, multicentre, prospective study of its kind, the overall number of
55 NEC cases and the number of prodromal samples remained relatively small and interpretation of
56 our results needs to reflect this. However, we have demonstrated changes in VOCs between babies
57 developing NEC and those who did not, which may offer further insight into the pathophysiology of
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3 NEC, particularly if some of these are associated with microbial metabolic pathways. Understanding
4 these changes may later form the basis for a diagnostic tool.
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7 Further studies already underway intend to try to replicate our data (MAGPIE study:
8 ISRCTN12554594) and further investigate the role of VOCs in the aetiology of NEC.
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11 12 13 **What is already known on this topic?**

- 14 • NEC is a serious condition with a high mortality and morbidity. There are no known
15 biomarkers.
- 16 • Early diagnosis may reduce illness severity
- 17 • Volatile organic compounds (VOCs) have been suggested as potential biomarkers of NEC
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24 **What does this study add?**

- 25 • This is the largest prospective study investigating potential biomarkers for NEC
- 26 • VOCs have moderate sensitivity and specificity for identifying cases of NEC 3-4 days
27 before diagnosis
- 28 • The biological plausibility of the VOCs identified sheds further light on potential
29 mechanisms for the development of NEC
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3 **Contributions:** CSP and AE: study concept and design; acquisition of data; analysis and
4 interpretation of data; drafting of the manuscript; critical revision of the manuscript for important
5 intellectual content; obtained funding; study supervision. RG, DMH, MGF: statistical analysis;
6 analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript
7 for important intellectual content; AM, RA: acquisition of data; analysis and interpretation of data;
8 drafting of the manuscript; RJ, ES, HB: acquisition of data; drafting of the manuscript
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14 **Conflicts of interest:** none
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24 their parents of recruited babies.
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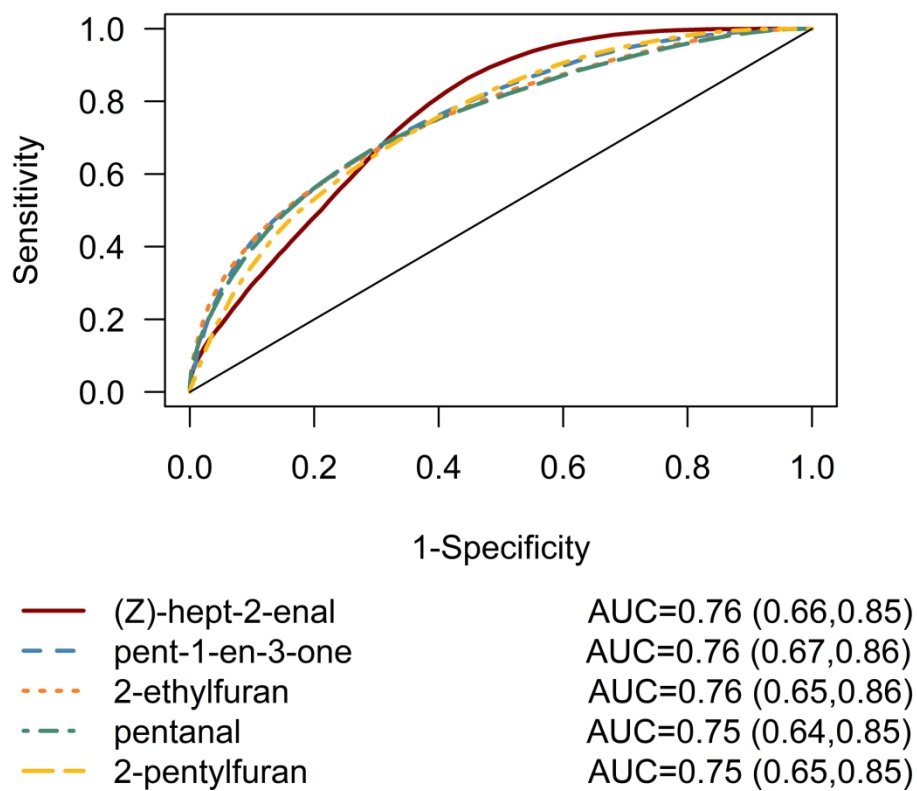
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46 Legends for Figures

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49 Figure 1: Classification scheme used to identify babies with increased risk of NEC

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53 Figure 2: ROC curves for the best five performing VOCs by AUC (95% CIs) using longitudinal
54 discriminant analysis
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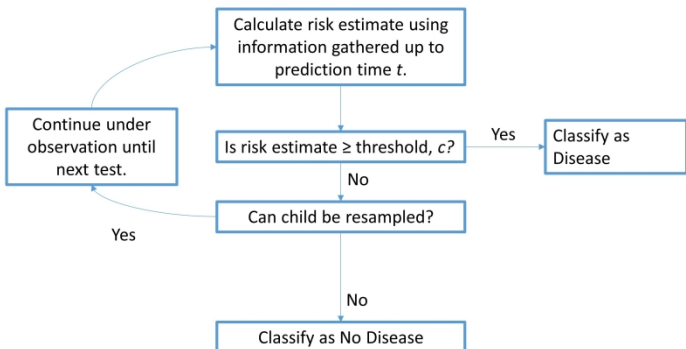


Figure 1: Classification scheme used to identify babies with increased risk of NEC

338x190mm (300 x 300 DPI)

Table S1 Spearman's rank correlation coefficients for compounds in group 4.

	3-methyl-sulfanyl-propanal	2-methyl propanal	Benzene-acetaldehyde	3-methyl butanal	Benz-aldehyde	2-methyl butanal
Methional	1.00					
2-methylpropanal	0.62	1.000				
Benzeneacetaldehyde	0.47	0.47	1.000			
3-methylbutanal	0.38	0.49	0.34	1.00		
Benzaldehyde	0.60	0.50	0.45	0.35	1.00	
2-methylbutanal	0.48	0.68	0.38	0.40	0.39	1.00

Table S2 Spearman's rank correlation coefficients for compounds in group 5.

	Butan-2-one	Methanedithione	Heptan-4-one
Butan-2-one	1.00		
Methanedithione	0.39	1.00	
Heptan-4-one	-0.14	-0.21	1.00

Table S3 Spearman's rank correlation coefficients for compounds in group 8.

	2-methylbutanoic acid	3-methylbutanoic acid	Butanoic acid
2-methylbutanoic acid	1.00		
3-methylbutanoic acid	0.68	1.00	
Butanoic acid	0.46	0.33	1.00

Table S4 Samples distribution along time line for analysis showing the number of samples available on each of the 6 days prior to the day of diagnosis

	Number of patients	Day -1	Day -2	Day -3	Day -4	Day -5	Day -6	Total samples
Healthy control	70	34	21	41	17	31	19	163
Confirmed NEC	34	17	9	19	15	16	8	84