Three Innate Cytokine Biomarkers Predict Presence of Acute Otitis Media and Relevant Otopathogens

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Abstract

Background: Diagnosis of Acute Otitis Media (AOM) is challenging, resulting in frequent over diagnosis and improper prescription of antibiotics. A serum biomarker of AOM would significantly improve pediatric care for this common illness.

Methods: Serum samples were studied from 197 children 6-36 months old during health, during viral Upper Respiratory Infection (URI) without middle ear involvement, and at the onset of AOM (confirmed by tympanocentesis). Serum concentrations of S100A12, IL-10, and ICAM-1 were measured by ELISA. Otopathogens were identified by culture of middle ear fluid. A predictive model for infection and causative otopathogen was developed based on density distributions of the measured cytokines.

Results: A biomarker score derived from subject age and serum concentrations of S100A12, IL-10, and ICAM-1 was significantly able to distinguish both between health and disease and between upper respiratory infections with and without middle ear involvement (AOM vs URI), and further predicted the specific causative bacterial pathogen. This biomarker could also identify recurrent OM-prone children.

Conclusions: For the first time we show that a biomarker risk score derived from serum cytokine levels can predict the presence of bacterial AOM, the likely Otopathogen, and the recurrent OM-prone child.

Clinical significance: (1) AOM is a widespread pediatric infection with a substantial economic burden. (2) Three serum cytokines can discriminate between URI and AOM, reducing over diagnosis. (3) Prediction of responsible pathogen enables targeted antibiotic prescription.

Keywords: Acute Otitis Media; Biomarker; *Haemophilus influenzae*; *Moraxella catarrhalis*; *Streptococcus pneumoniae*

Introduction

Accurate diagnosis of Acute Otitis Media (AOM) is difficult, as there are no unique clinical manifestations that can be relied upon to diagnose the infection. Otoscopic examination of the tympanic membrane in the context of a suspected AOM infection often results in misdiagnosis, leading to over-prescription of antibiotics [1-9]. The symptoms of AOM are virtually identical to those of a viral URI [2] and in the clinical setting of an irritable, perhaps febrile child, a parent seeking treatment to expedite recovery from illness is often advised without complete justification that an AOM is present and antibiotics should be prescribed. Video otoscopy tests of pediatricians from Italy, Greece, South Africa and the United States found that AOM is over diagnosed at rates of 30-50% [3,10]. Additional studies have demonstrated that over-diagnosis of AOM is commonplace [1-4]. In many developing country populations health care practitioners have neither the training nor the tools to accomplish otoscopy, sometimes using a speculum and a flashlight in an attempt to view the tympanic membrane. A low cost, simple blood-based test to identify biomarkers of inflammation detectable in serum at the time of clinical presentation compatible with AOM might prove useful. We previously identified mRNA transcripts differentially expressed (>2-fold) between the pre-infection healthy state and the AOM state in children experiencing AOM due

to the prominent Otopathogens *Streptococcus pneumoniae* (Spn), non-type able *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis* (Mcat) [11]. 6.7% of the differentiated transcripts could be grouped in the host immune defense category for Spn [11] and NTHi [12]. Among these signatures, 3 genes were identified with potential as biomarkers for AOM infection: S100A12, Intercellular Adhesion Molecule 1 (ICAM-1), and Interleukin 10 (IL-10). Those results led to studies of the serum concentrations for S100A12, ICAM-1, and IL-10 as biomarkers for the diagnosis of AOM and the identification of the causative Otopathogens, which are predominantly Spn, NTHi, and Mcat [12-14]. Levels of S100A12, a calcium-binding protein predominantly expressed by neutrophils in response to infection, significantly increased at the onset of AOM and returned to normal levels when children recovered [14].

Elevation of S100A12 correlated with AOM episodes caused by Spn or NTHi, but not with AOM caused by Mcat or upper respiratory viral infections in the absence of AOM. ICAM-1, which is involved in antigen presentation by innate immune cells and subsequent T-cell activation, was significantly higher in sera from Spn-, NTHi-, and Mcat-infected children during AOM, returning to pre-AOM levels during the convalescent stage after successful antimicrobial therapy [12]. The anti- inflammatory cytokine IL-10 was elevated in serum when children developed AOM due to Spn but not AOM caused by NTHi or Mcat [13]. Here we describe a biomarker risk score using a combination of the cytokines S100A12, IL-10, and ICAM-1 that could be used by clinicians as additional data to inform the clinical diagnosis of AOM and provide a probability estimate that the infection is caused by Spn, NTHi, or Mcat. S100A12 measurements can also identify otitis-prone children during health.

Methods

Subjects

The experimental human samples evaluated in this study were collected during a prospective study (funded by the U.S. National Institute for Deafness and Communication Disorders) between 2006 and 2015 as previously described [13,15,16]. Samples of blood, nasal wash, and Middle Ear Fluid (MEF) were collected from children 6 to 30 months of age according to a protocol approved by the Institutional Review Boards of Rochester General Hospital and the University of Rochester. Approximately four milliliters of heparinized peripheral venous blood were drawn from each subject at a study visit as previously described [12-16]. The diagnosis of AOM was based on symptoms of fever, irritability, or earache; signs of inflammation (red or yellow color or bulging) of the tympanic membrane; and the presence of MEF, as documented by tympanocentesis. Tympanocentesis was performed for all subjects as previously described [13,15-17]. After being diagnosed with AOM, children received various antibiotic treatments and returned

for a follow-up visit 3 weeks or later. All study children had a concurrent viral Upper Respiratory Infection (URI) at onset of AOM. Children who experienced at least 3 AOM episodes within 6 months or 4 episodes within a year were classified as stringently-defined Otitis Prone (sOP); all others were classified as Non-Otitis Prone (NOP). A virus infection was diagnosed on the basis of clinical symptoms and signs on physician examination, such as fever, rhinorrhea, and cough, and verified via multiplex PCR using a Seeplex RV12 detection kit (See gene, MD), following the manufacturer's instruction [12-15].

Microbiology

Routine bacteriologic detection of the three targets Otopathogens in Nasopharyngeal (NP) and MEF samples was accomplished as previously described using standard culture conditions [13,15,16]. Questionable pathogen identities were verified by multiplex PCR [17].

Biomarker assays: S100A12, IL-10, and ICAM-1 assays were performed as previously described [12-14]. For all three assays absorbance values of the unknown serum samples were run with appropriate controls and quantitated by comparison with a standard curve generated from known concentrations of each protein.

Bayes classifiers: A number of methodologies are commonly used to build classifiers. Because there is interest in elucidating the relationship between cytokines and immunological event, we use classifiers that estimate explicit analytical relationships, rather than nonparametric methods such as K-nearest neighbor classifiers or tree-based classifiers. Furthermore, we anticipate that correlation between cytokine abundance and immunological outcome will depend on the unobserved time sequence of immune process. Because of the time dependence of cytokine regulation, we anticipate that cytokine/response association will be a statistical mixture. For this reason, we use Bayesian classifiers. Because these are constructed by modeling distributions of cytokine levels conditional on immunological outcomes, the anticipated mixture structure can be modeled in that context. In contrast, while logistic regression can estimate quantitative relationships between cytokine levels and outcomes, it would be much more difficult to capture those anticipated forms of association in a linear model framework.

Let x be a k-dimensional biomarker vector, and let $j=1,...,m$ be m immunological classes. The premise is that the conditional distribution of the biomarker f(x | j) depends strongly on the class, which can therefore be predicted with controlled misclassification probability. We assume there are prior class probabilities $\pi = (\pi_1,...,\pi_m)$, and we can take J to be the class of a random selected observation. Then the conditional probability that a randomly selected biomarker observation x is from class $J = j$ is

$$P(J = j \mid x) = f(x \mid j) \, \pi_j \div f(x), \text{ where } f(x) = \Sigma_j f(x \mid j) \, \pi_j.$$

The Bayes classifier is a class prediction based on observation x taking the form $J(x) = \text{argmax}_j P(J = j \mid x)$. It is important to note that the Bayes classifier depends on the prior class distribution. It also minimizes misclassification probability among all predictors, assuming that the prior distribution is correctly specified (if $\pi_1 = 1$, $\pi_2 = \cdots = \pi_m = 0$, then $J(x) = 1$ for any x, as would be appropriate). However, the accuracy of the predictor can, to a large degree, be evaluated independently of the prior class distribution. To see this, suppose $m = 2$. Then

$$\text{Odds}(J = 1 \mid x) = \text{LR} \times \text{Odds}(J = 1), \text{where} \, \text{LR} = \frac{f(x \mid J = 1)}{f(x \mid J \neq 1)},$$

with LR referred to as the likelihood ratio. Thus, the predictive information of the conditional densities can be evaluated independently of the prior class probabilities. This example extends naturally to $m > 2$. Therefore, resolution of two classes A, B will be based on risk score

$$\eta_{A/B} = \log(\text{LR}) = \log(f(x \mid A)) - \log(f(x \mid B)),$$

where large values signify evidence of class A.

Density estimation: Conditional densities f(x | A) were modeled as finite Gaussian mixtures; fit using the R package **mclust** [18]. Data was found to be approximately Gaussian following a log transformation, so data was used in this form. The maximum number of components was bounded at three. The m clust package applies Bayesian information Criterion (BIC) to a hierarchical family of models generated by parametric constraints, ranging from multivariate Gaussian mixtures of maximum degree of freedom, to independent models, equivalent to a Naive Bayes classifier.

Biomarker evaluation: To define the set of candidate biomarkers first enumerate all 7 nonempty selections from 3 cytokines {S100,IL10,ICAM}. Seven additional biomarkers are defined by appending AGE to each, for a total of 14. Let b denote a biomarker. For any event A we may estimate density $f_b(x \mid A)$, yielding risk score

$$\eta_{A/B}^b = \log(\text{LR}) = \log(f_b(x \mid A)) - \log(f_b(x \mid B)),$$

Here x is a vector of log-transformed cytokine measurements, to which additional covariates such as AGE may be appended.

Prediction accuracy can be assessed in various ways. The commonly used area under ROC curve statistic (AUC) is equivalent to the probability that pair of risk scores randomly selected from each class is correctly ordered. Since the clinical objective is to reduce over-diagnosis of AOM, the appropriate goal is to maximize true negative rate λ_{TN} while controlling for false negative rate λ_{FN}, since this quantity is directly proportional to the reduction in over diagnosis. A cross validation procedure is employed. Test data was generated by selecting one observation from each class at random, the remaining data used for training. The procedure was repeated 1000 times.

Results

AOM and Otopathogen Prevalence

Three types of visits were analyzed: (1) Healthy visits, where the child did not present with AOM or viral URI symptoms (including some where children showed asymptomatic NP colonization with potential Otopathogens); (2) URI visits, where the child presented viral URI symptoms without middle ear involvement; and (3) AOM visits, where the child presented symptoms of a possible AOM and had the clinical diagnosis confirmed by tympanocentesis and microbiological culture demonstrating Spn, NTHi, or Mcat in the middle ear. Our study population as a whole comprises a total of 1544 visits where patients presented upper respiratory inflammatory symptoms: 721 URI (47%) and 823 AOM (53%) visits. 390 serum samples were analyzed: 95 collected during Healthy visits (49 visits without asymptomatic colonization by any of the three main Otopathogens, 22 colonized by Spn, 5 colonized by NTHi, and 19 colonized by Mcat); 149 collected during URI visits (41 without asymptomatic colonization, 21 colonized by Spn, 2 colonized by NTHi, 38 colonized by Mcat, and 47 colonized by multiple species); and 146 collected during AOM visits (19 Spn, 36 NTHi, 24 Mcat, and 67 with multiple Otopathogens). The makeup of the study population is presented in (Figure 1).
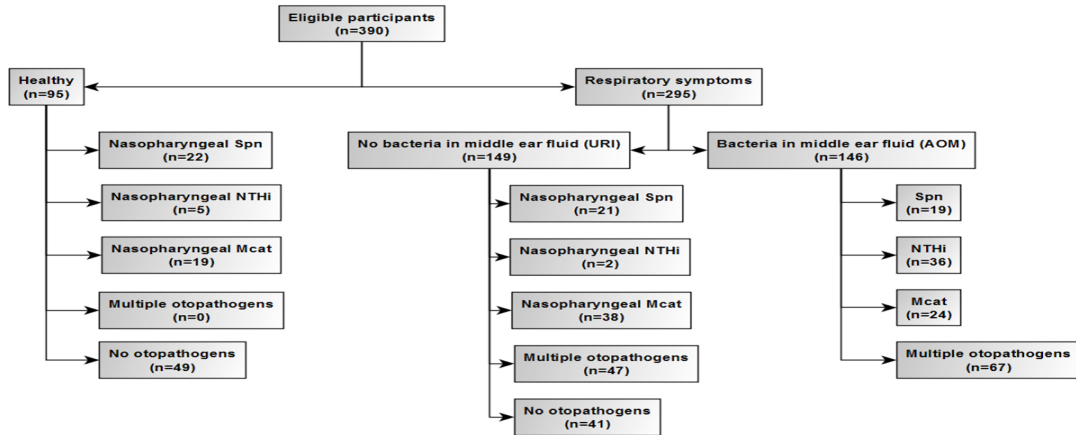
Figure 1: Diagnostic criteria and allocation of samples in this project.

Individual Cytokine Measurements Significantly Vary with Physiological Status

Concentrations of S100A12, ICAM-1, and IL-10 in samples collected from children during health, URI, and AOM as described above were measured by ELISA. Concentrations of all three cytokines were significantly affected by disease state. S100A12 concentrations were lowest during health, increased during URI, and peaked during AOM, with an additional significant effect of subject age ($p<0.01$). ICAM-1 concentrations were likewise lowest during health, but peaked during URI, while AOM samples displayed substantial variability. IL-10 concentrations were observed to be intermediate during health, highest during URI, and lowest during AOM (Figure 2).
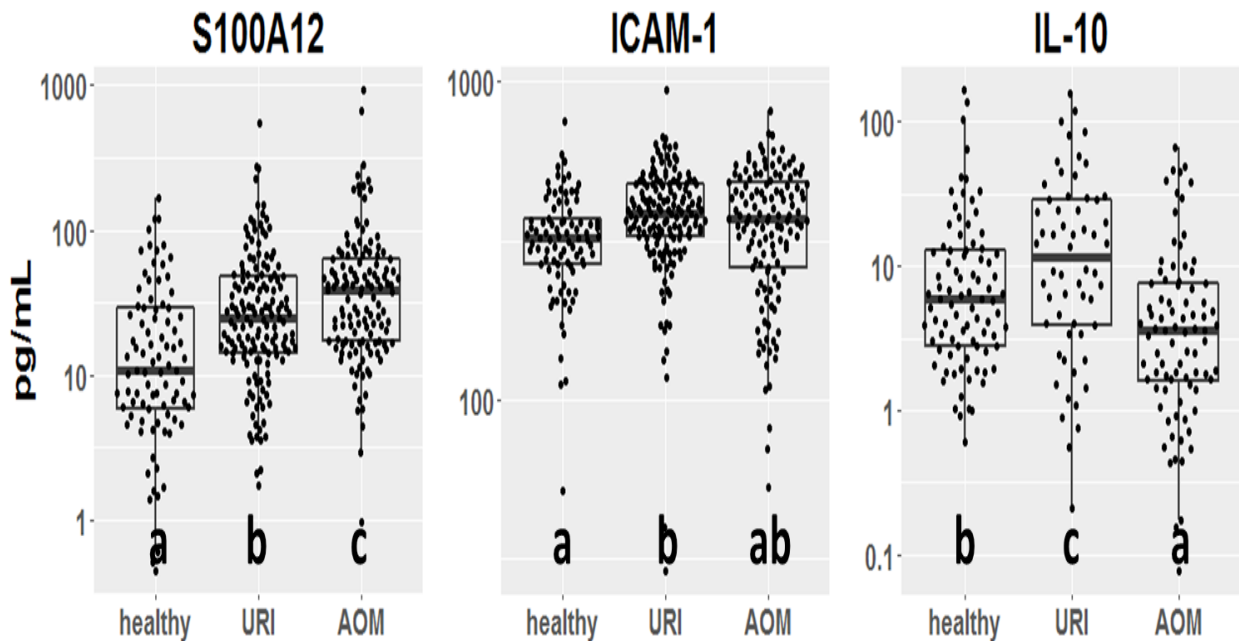

Figure 2: Variations in serum cytokine concentration during Health, URI, and AOM. Bars depict mean+95% confidence interval. Letters denote statistically significant differences between groups ($p<0.05$).

Biomarker Performance

(Table 1) gives the AUC statistic for each biomarker. The highest AUC model is in bold for each classification problem. It is important to note the decision theoretic aspect of the problem. A false negative means that the AOM hypothesis is rejected. Failure to

detect AOM is taken to be of higher cost than a false positive. For this reason, it is appropriate to minimize over-diagnosis of AOM while controlling for the false negative rate. Accordingly, (Table 2) shows estimates of λ_{TN} for fixed values of λ_{FN}=0.01,0.03,0.05 for the AOM/URI comparison, for pathogen specific and unspecified AOM classifiers (estimates in each table were obtained using cross validation). For example, accepting λ_{FN}=0.03 for the unspecified AOM classifier, we attain λ_{TN}=0.22 using the (S100,IL10,ICAM,Age) biomarker. In practical terms, 22% of AOM -ve children with URI can be correctly classified as AOM -ve, with 3% of AOM +ve children misclassified.

	Model	SPN	NTHi	MCAT	AOM
AOM/Healthy	S100	**0.787***	**0.801**	**0.685**	**0.738**
	IL10	0.340	0.627	0.460	0.590
	ICAM	0.624	0.633	0.648	0.633
	S100,IL10	**0.765**	**0.822***	**0.752**	**0.818***
	S100,ICAM	0.696	0.820	0.704	0.762
	IL10,ICAM	0.526	0.588	0.598	0.665
	S100,IL10,ICAM	**0.720**	**0.793**	**0.793***	**0.789**
	S100,Age	**0.770**	**0.794**	**0.633**	**0.716**
	IL10,Age	0.434	0.666	0.542	0.594
	ICAM,Age	0.569	0.650	0.527	0.648
	S100,IL10,Age	0.734	**0.816**	0.711	**0.778**
	S100,ICAM,Age	**0.787**	0.782	**0.725**	0.751
	IL10,ICAM,Age	0.389	0.662	0.561	0.673
	S100,IL10,ICAM,Age	**0.667**	**0.794**	**0.730**	**0.801**
AOM/URI	S100	**0.661**	0.674	0.484	0.592
	IL10	0.545	**0.724**	**0.566**	**0.684**
	ICAM	0.553	0.506	0.557	0.593
	S100,IL10	**0.675**	**0.750***	**0.683**	**0.701**
	S100,ICAM	0.488	0.642	0.476	0.548
	IL10,ICAM	0.579	0.601	0.585	0.647
	S100,IL10,ICAM	**0.677***	**0.640**	**0.664**	**0.646**
	S100,Age	**0.640**	0.682	0.502	0.564
	IL10,Age	0.541	**0.707**	**0.711***	**0.699**
	ICAM,Age	0.496	0.552	0.447	0.593
	S100,IL10,Age	0.615	**0.738**	0.650	**0.701**
	S100,ICAM,Age	**0.644**	0.596	0.554	0.559
	IL10,ICAM,Age	0.549	0.645	**0.652**	0.681
	S100,IL10,ICAM,Age	**0.598**	**0.679**	**0.644**	**0.720***

Table 1: Performance (AUC) of different candidate biomarker compositions for different classification problems. Highest AUC score is bold.

	$\lambda_{FN}=0.01$				$\lambda_{FN}=0.03$				$\lambda_{FN}=0.05$			
	SPN	NTHi	MCAT	AOM	SPN	NTHi	MCAT	AOM	SPN	NTHi	MCAT	AOM
S100	0.01	0.09	0.04	0.00	0.01	0.18	0.08	0.07	0.07	0.19	0.09	0.14
IL10	0.00	0.08	0.01	0.00	0.00	0.09	0.01	0.09	0.00	0.10	0.03	0.09
ICAM	0.02	0.00	0.01	0.01	0.02	0.00	0.02	0.01	0.07	0.00	0.02	0.01
S100,IL10	0.11	0.24	0.00	0.00	0.11	0.25	0.09	0.03	0.14	0.27	0.10	0.03
S100,ICAM	0.01	0.01	0.04	0.01	0.01	0.02	0.04	0.04	0.01	0.11	0.06	0.12
IL10,ICAM	0.07	0.04	0.03	0.09	0.07	0.06	0.03	0.10	0.15	0.07	0.04	0.17
S100,IL10,ICAM	0.08	0.03	0.01	0.01	0.08	0.05	0.01	0.11	0.11	0.19	0.13	0.14
S100,Age	0.01	0.01	0.04	0.00	0.04	0.04	0.10	0.05	0.07	0.04	0.10	0.08
IL10,Age	0.00	0.05	0.07	0.00	0.00	0.05	0.07	0.01	0.02	0.10	0.20	0.06
ICAM,Age	0.02	0.00	0.02	0.03	0.02	0.00	0.03	0.04	0.02	0.02	0.03	0.06
S100,IL10,Age	0.00	0.17	0.00	0.00	0.00	0.17	0.00	0.02	0.01	0.20	0.00	0.22
S100,ICAM,Age	0.18	0.00	0.03	0.01	0.18	0.02	0.08	0.03	0.20	0.02	0.08	0.04
IL10,ICAM,Age	0.06	0.01	0.01	0.02	0.06	0.02	0.01	0.13	0.15	0.12	0.02	0.27
S100,IL10,ICAM,Age	0.05	0.00	0.05	0.02	0.05	0.04	0.05	0.22	0.07	0.05	0.08	0.23

Table 2: True negative rates (λ_{TN}) for candidate biomarkers at selected false negative rates (λ_{FN}).

One notable feature of Tables 1 and 2 is the tendency for biomarkers including S100A12 and IL10 to have higher accuracy measures. This can be seen clearly in Figures 3 and 4, which superimpose estimated plots of λ_{TN} against λ_{FN} for each biomarker. For the AOM/ Healthy comparison, biomarkers containing S100A12 are indicated by black lines, while biomarkers without S100A12 are grey. Clearly, the set of biomarkers containing S100A12 almost uniformly outperforms the others, displaying more favorable relationships between λ_{TN} and λ_{FN}. The same tendency can be seen for the AOM/URI comparison, except that biomarkers containing IL-10 are favored. In addition, for both comparisons, biomarkers with both IL10 and S100A12 tend to be most accurate. However, if only one cytokine could be used, the best choices would be S100A12 and IL-10, respectively, for the AOM/Healthy and AOM/URI comparisons.
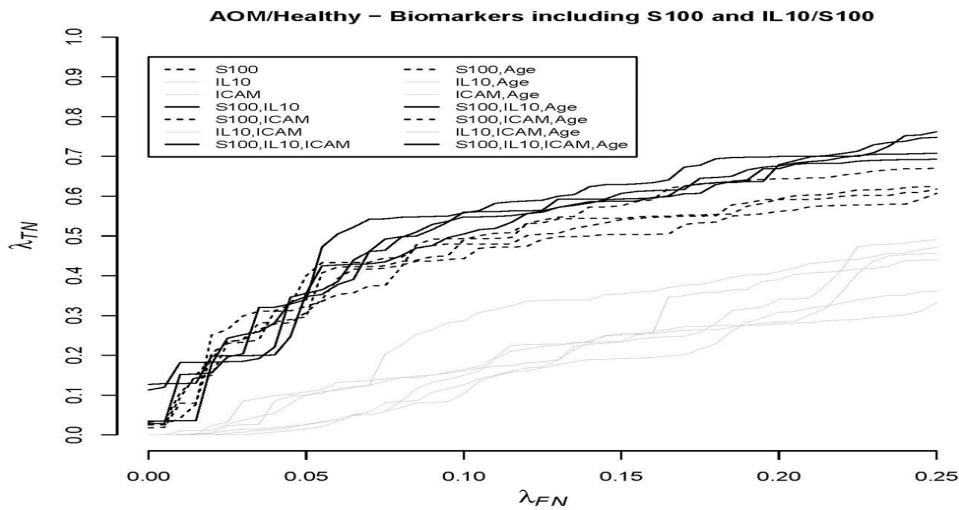
Figure 3: Biomarker performance ("Healthy" visits vs. "AOM" visits). Biomarkers containing S100A12 (black lines) consistently outperform biomarkers not containing S100A12 (grey lines).
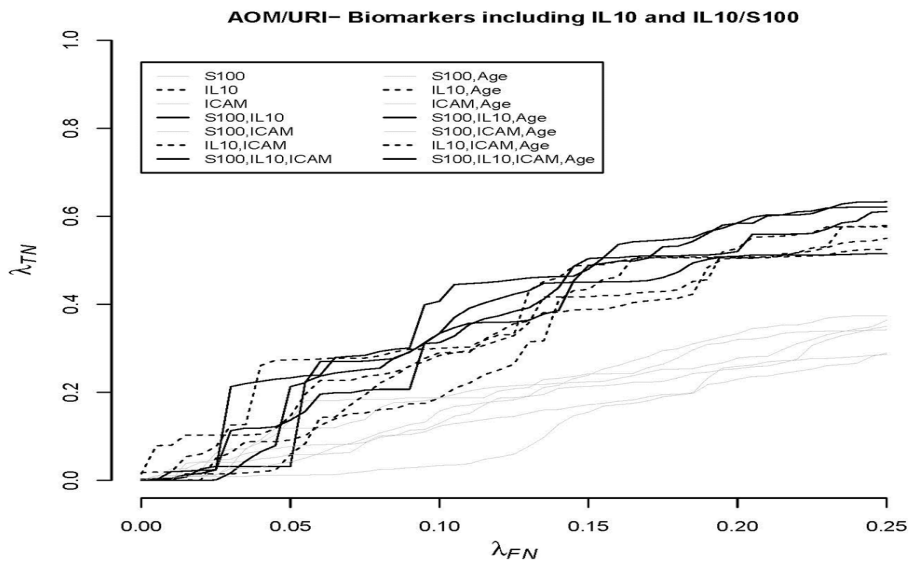

Figure 4: Biomarker performance ("URI" visits vs. "AOM" visits). Biomarkers containing IL-10 (black lines) consistently outperform biomarkers not containing IL-10 (grey lines).

Classification Boundary

To further examine the properties of the classifiers, classification boundaries using biomarker (S100, IL10), for pathogen specific and general AOM classifiers are shown for AOM/Healthy comparisons (Figure 5) and AOM/URI comparisons (Figure 6). For this analysis, the risk score was recalculated using the entire data set. The axes represent the log transformed cytokine values, and cases are superimposed with symbols distinguishing AOM +ve and -ve events (ie. URI or healthy as appropriate). Because the risk scores for SPN and MCAT were similar, they were combined into a single AOM event (SPN or MCAT). Contours for the posterior probability $P(\text{AOM}\,|x)$ are superimposed.
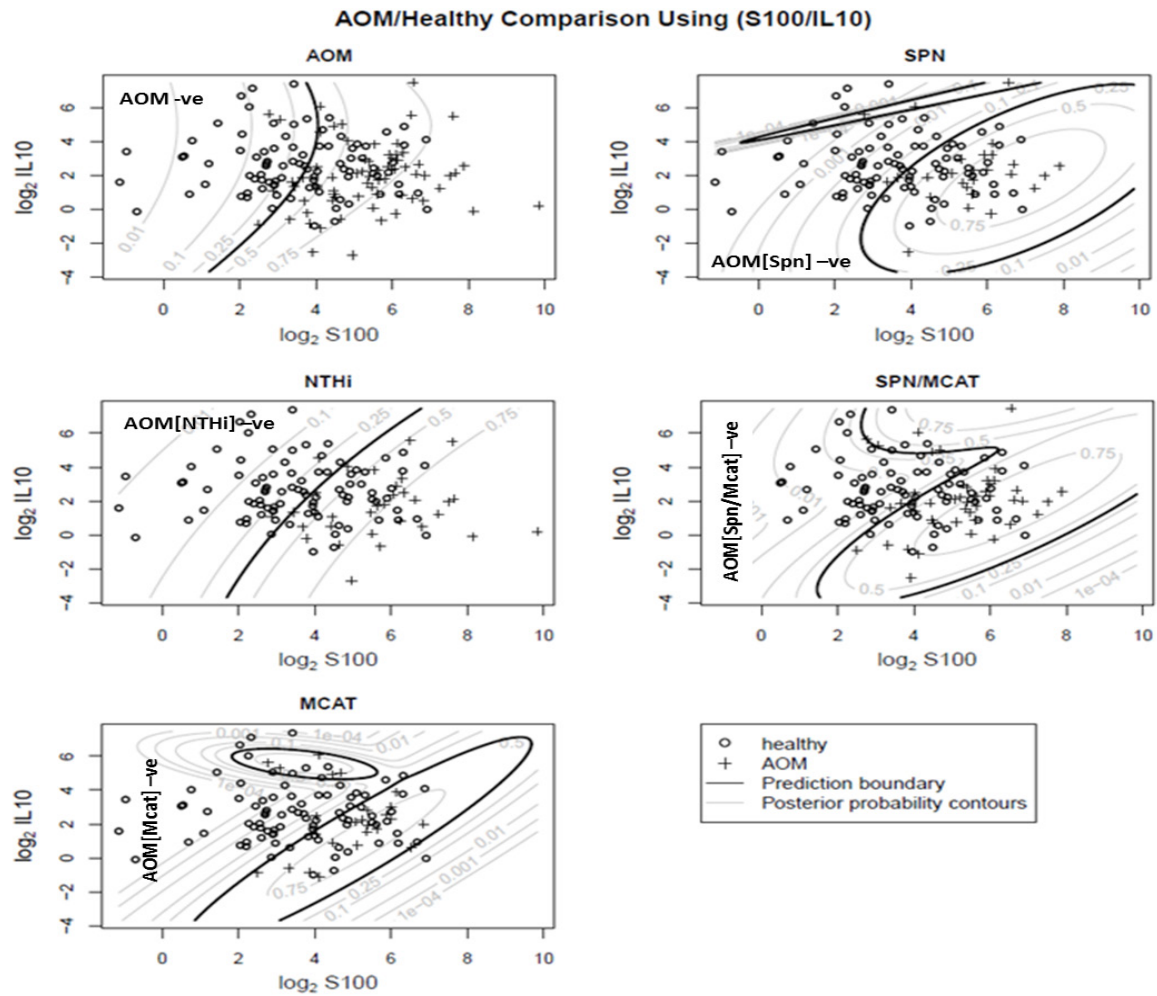
Figure 5: Classification boundaries using biomarker (S100, IL10), for pathogen specific and general AOM classifiers in AOM/Healthy cases: the same AOM and Healthy cases are depicted in each plot. The modes, or high density regions, of the biomarker joint densities are indicated by elliptical contours (grey lines) at levels 0.02, 0.04. Note that mixture densities possess multiple modes. A classification boundary defined by the nominal FN rate is superimposed (black lines), with prediction outcome represented by symbols (+) for AOM +ve and (o) for AOM -ve. AOM and/or pathogen-negative regions are labeled.
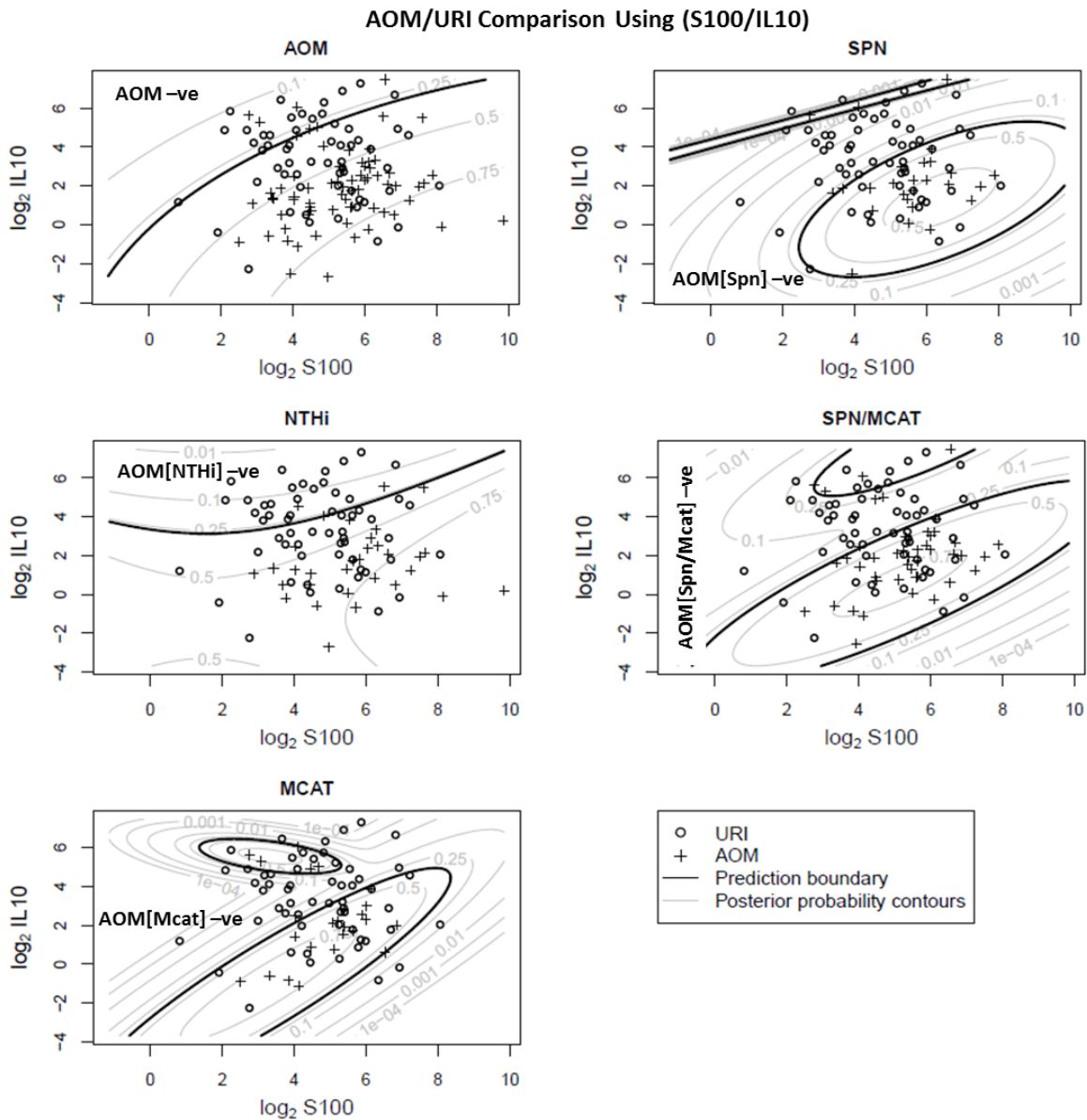
Figure 6: Classification boundaries using biomarker (S100, IL10), for pathogen specific and general AOM classifiers in AOM/URI cases: the same AOM and Healthy cases are depicted in each plot. The modes, or high density regions, of the biomarker joint densities are indicated by elliptical contours (grey lines) at levels 0.02, 0.04. Note that mixture densities possess multiple modes. A classification boundary defined by the nominal FN rate is superimposed (black lines), with prediction outcome represented by symbols (+) for AOM +ve and (o) for AOM -ve. AOM and/or pathogen-negative regions are labeled.

In addition, a single contour representing a prediction boundary of nominal value $\lambda_{FN}=0.1$ Is superimposed. Classification regions are labeled in each plot. The actual values of λ_{FN} and λ_{TN} are given in Table 3. Each pathogen-specific classification results in a different probability contour (Figures 5,6). Plotting individual cases on each classification contour may yield additional information regarding the likelihood of AOM. For example, a case in the AOM -ve region of the AOM plot (Figure 5, top left panel) may be located within the Spn +ve region of the Spn plot (Figure 5, top right panel). Conversely, a patient may be in an unlikely region for AOM caused by NTHi but high probability for AOM caused by Mcat. Thus, plotting S100A12 and IL-10 measurements for each case against the probability distribution for AOM with respect to every classification question can increase the power of the biomarker.

Note that the classifier is bimodal for the MCAT, SPN and combined MCAT/SPN responses, but not for the NTHi or combined AOM response. For the MCAT SPN responses the conditional densities $f(x \mid SPN)$ and $f(x \mid MCAT)$ are estimated to be a two component mixture, suggesting that for infections either S100 or IL10 are upregulated, but not both. In fact, the tendency is for one of these cytokines to be at levels lower than AOM -ve cases. This would be explained by a kinetic model predicting temporally separated upregulation, followed by significant down-regulation. In contrast, NTHI +ve cases are distinguished primarily by down regulation of IL10 for the AOM/URI comparison, and upregulated of S100A12 for the AOM/Healthy comparison.

	AOM/Healthy		AOM/URI	
	λ_{TN}	λ_{FN}	λ_{TN}	λ_{FN}
SPN	0.602	0.037	0.586	0.037
NTHi	0.602	0.061	0.397	0.061
MCAT	0.659	0.04	0.552	0.04
AOM	0.534	0.085	0.328	0.085
SPN/MCAT	0.591	0.067	0.466	0.067

Table 3: λ_{TN} and λ_{FN} associated with contour regions for pathogen-specific AOM

There is one other feature of note. For pathogen specific predictions, false negatives (AOM +ve cases in the AOM -ve prediction region) tend to be very close to the prediction boundary.

This means their posterior probabilities $P(AOM \mid x)$ would also be close to the boundary value. This information would be available in a clinical setting, so it would be known that the evidence against AOM is relatively weak. On the other hand, for many of the true AOM -ve cases, particularly for SPN and MCAT, the value of $P(AOM \mid x)$ is of an order of magnitude of 0.01 to 0.001, and so could be confidently classified, correctly, as $AOM - ve$. This is not the case for the compound AOM predictor, almost certainly the result of pooling cases with significantly different cytokine distributions.

Pathogen prediction

The problem of pathogen prediction is complicated by two factors. First, subjects may have multiple pathogens, so there are formally 8 exclusive classes. Second, false negatives are known to be present because microbiologic culture of middle ear fluid fails to identify the presence of Otopathogens in about 15% of cases when a more sensitive molecular detection method is used to identify Otopathogens [17].

To classify a specific pathogen a biomarker set b is selected. The conditional densities $f_b(x \mid SPN), f_b(x \mid NTHi), f_b(x \mid MCAT)$ are estimated. The pathogen risk score for SPN, for example, is then

$$\pi_b(SPN \mid x) = \frac{f_b(x \mid SPN)}{f_b(x \mid SPN)+f_b(x \mid NTHi)+f_b(x \mid MCAT)}, \qquad (2)$$

Note that despite the resemblance of (2) to a Bayesian classifier, it does not naturally define a probability distribution, since the pathogen +ve events are not mutually exclusive.

Cross validation was used to select biomarker set for each pathogen, with $b = (S100, IL10, ICAM)$ giving the highest AUC in each case (SPN: AUC = 0.64, P = 0.067; NTHi: AUC = 0.71, P = 0.005; MCAT: AUC = 0.68, P = 0.017).

Using CV estimates 25% of pathogens can be identified with a false positive rates 0.275 (SPN), 0.030 (NTHi) and 0.051 (MCAT). Using complete data the estimates are $P<0.01$ (SPN), $P<0.01$ (NTHi) and P = 0.026 (MCAT).

(Figure 7) shows boxplots summarizing posterior

probabilities of pathogen classification once AOM has been positively diagnosed. Estimates use complete data.
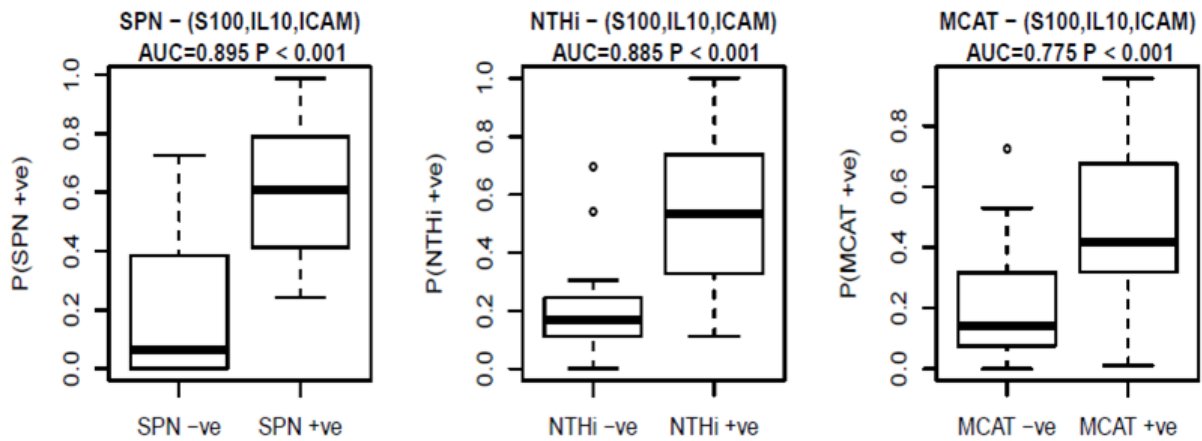

Figure 7: Posterior probabilities of pathogen classification for each pathogen contingent on AOM diagnosis. Estimates use complete data.

sOP prediction

Classifying sOP/NOP children was done using the same method as for pathogen classification. Classifiers were constructed for Healthy and AOM children (after deleting missing cytokine measurements only 3/58 URI children were sOP). Cross validation was used to select biomarker set for each pathogen, with $b = (S100)$ giving the highest AUC for Healthy children (AUC = 0.805, P = 0.001). The highest AUC for AOM children was only 0.669. For $b = (S100)$, among AOM children AUC = 0.402 (P = 0.122). In general, the classifiers were far more accurate for this classification problem among healthy children. (Figure 8) shows boxplots summarizing posterior probabilities of sOP classification for Healthy and AOM children using biomarker $b = (S100)$. Estimates use complete data.
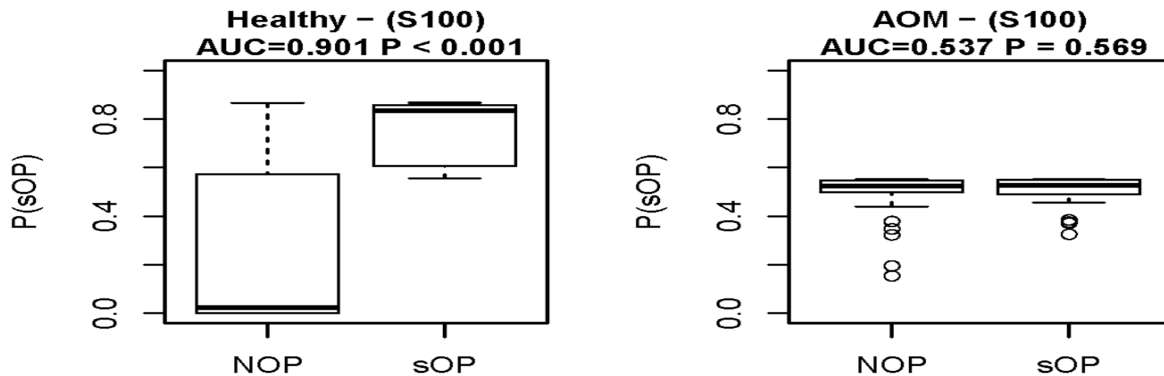

Figure 8: Posterior probabilities of sOP classification for Healthy and AOM children. Estimates use complete data.

Discussion

For the first time we show that a biomarker risk score based on serum levels of the innate immune molecules S100A12, IL-10, and ICAM-1 can be used to calculate a likelihood of the diagnosis AOM and probability regarding the responsible otopathogen (Spn, NTHi, or Mcat) in the clinical context of possible AOM.

Over diagnosis of AOM is commonplace [1,3,10], due to the difficulty of distinguishing AOM from URI visits without middle ear involvement. We found that URI visits made up some 47% of visits where upper respiratory inflammatory symptoms were apparent, supplying further evidence that existing AOM diagnoses are burdened by a high false positive rate. The specificity represents the percentage by which over-diagnosis would be reduced, provided the false negative rate is controlled. Therefore, a more objective

indication of the presence or absence of AOM in the clinical setting has great potential to reassure health care practitioners and parents.

The consequences of incorrectly diagnosing a URI when the patient in fact has AOM are much greater than incorrectly diagnosing AOM. Thus, this biomarker was designed to maximize the true negative rate while minimizing the false negative rate, and we found it was capable of distinguishing AOM from URI with a true negative rate of approximately 20% at a false negative rate of 3%. The value of this biomarker is increased since the prevalence of URI is almost equal to that of AOM in our study population: the more common URI is, the more likely it is to be misdiagnosed as AOM by a clinician, and thus the more valuable any reduction in false positives becomes. Our biomarker can reliably rule out AOM, with the additional benefit of identifying marginal cases. Potential AOM visits can generally be ruled in or out with high confidence, and the certainty or uncertainty of the prediction can help to inform a diagnostic decision even in marginal cases.

Predictive identification of an otopathogen in the clinical setting will favorably impact ineffective antibiotic use. Specific antibiotic treatment has been shown to result in significantly faster resolution of AOM [19,20]. The same measurements used to rule AOM in or out as a diagnosis can also return a probability that AOM is due to Spn, NTHi, or Mcat, along with indications of the relative certainty or uncertainty of this prediction. In fact, we found that the pathogen-specific classifiers were more accurate than the initial diagnosis of AOM (AUCs between 0.78 and 0.90). In other words, once a positive diagnosis of AOM is made, whether by the use of this biomarker or by other means, the responsible pathogen or pathogens can be identified with confidence. A pediatrician could use this information to better inform the choice of antibiotics to prescribe.

S100A12 serum level increases during an AOM episode are likely related to neutrophil activation in response to an increase in bacterial load in the nasopharynx during a viral URI that sets the stage for pathogenesis resulting in AOM. This observation is consistent with differences in pathogen-stimulated activation of neutrophils previously observed by others [21,22]. Also, the number of neutrophils in Middle Ear Fluid (MEF) of children with AOM caused by Spn has been shown to be significantly higher than that found in the MEF of children with AOM caused by NTHi [23].

Pairwise plots of the individual density distributions reveal regions of elevated AOM risk: in particular, S100A12 values characteristic of AOM visits tend to be associated with non-AOM IL-10 values, and vice versa. This was reflected in the analysis of candidate biomarker compositions, where biomarkers containing S100A12 were most effective in discriminating Healthy visits from AOM, while biomarkers containing IL-10 were most effective in

discriminating URI from AOM. This suggests opposing or cyclical regulation of innate immunity during AOM. A murine study of the transcriptome during AOM caused by *H. influenzae* found just such cyclical regulation, with neutrophil recruitment and activation appearing to peak approximately 24 hours, followed by proliferation of epithelial cells and the resolution of inflammation 3-5 days after infection [24]. Variability in cytokine measurements may be attributable to patients seeing a clinician at different time points during the progression of an AOM episode, with earlier or later time points associated with different cytokine patterns.

Identifying the child who is prone to AOM would offer a clinical opportunity to advise parents of a higher risk of recurrent infections and being able to test for this possibility when the child is healthy rather than waiting for an AOM to occur would be preferable. We found that measurement of elevated serum S100A12 when children in our study were healthy identified those who were AOM prone with an AUC of 0.9. This is consistent with a recent investigation by our group, which found that variations in nasopharyngeal innate immune gene transcription, cytokine secretion, and neutrophil recruitment during AOM and URI were largely attributable to variations in *S. pneumoniae* bacterial burden, rather than otitis proneness [25]. The levels of the measured cytokines in nasal washes are low due to dilution in the nasal wash. Therefore, we have not pursued the analysis of those samples. We have previously measured IL-10 in MEF and found it mirrored the levels in serum but were 10-fold higher (13). Since tympanocentesis would not be a procedure easily performed in clinical practice we did not pursue measurements of MEF levels of the cytokines in MEF."

In summary, we have identified 3 innate cytokines in serum-S100A12, IL-10, and ICAM-1-that can be used in conjunction with subject age to calculate a likelihood of AOM, demonstrating a systemic inflammatory profile associated with upper respiratory infection. This biomarker risk score also provides a probabilistic prediction of the specific bacteria causing AOM at the time of diagnosis, helping to suggest possible courses of treatment. Better-targeted antibiotic use associated with improved diagnosis of AOM will reduce antibiotic over-prescription and could lead to substantial cost savings by reducing over-diagnosis and increasing the efficacy of treatments, since the economic burden of AOM is estimated at over $6 billion annually in the United States alone [26]. Frequent diagnosis of AOM eventually leads to classification of a child as "otitis prone" [2,27] and these children often go on to receive tympanostomy tubes in a costly surgical procedure. We also found that a serum biomarker may be capable of identifying children at higher risk for recurrent OM infections with a consequently greater chance of benefitting from tympanostomy tube surgery. Thus, an accurate biomarker test would be of value

from a public health, individual, and health costs perspective.

This is a significant step toward the goal of using a small blood sample for rapid analysis of serum biomarkers in an outpatient clinical practice setting to predict the presence of AOM and the specific otopathogen responsible. Further studies to improve this approach appear justified. It will be especially important to distinguish between the single and multiple pathogen cases. This more complex model would almost certainly require larger sample sizes. Since the same bacteria and pathogenesis exists for acute sinusitis and many cases of community acquired pneumonia in children and adults, future studies using the same biomarker risk score derived from the same 3 serum cytokines to predict presence of those infections and identification of the bacterial pathogen should be considered.

Conclusion

We have developed a novel biomarker which uses serum concentrations of S100A12, ICAM-1, and IL-10 at the time of respiratory symptoms to determine whether a child is suffering from AOM or a viral infection without middle ear involvement. If AOM is present, the biomarker can also identify the responsible bacterial pathogen. Continued testing of this biomarker in a larger population of children will further refine its accuracy.

Funding

Declaration of Interest

Rochester General Hospital has received a patent regarding the use of cytokine biomarkers relating to diagnosis of acute otitis media, otopathogen identification and recovery from AOM and Dr. Pichichero is the inventor.

References

1. Pichichero ME, Casey JR (2008) Diagnostic inaccuracy and subject exclusions render placebo and observational studies of acute otitis media inconclusive. Pediatr Infect Dis J 27: 958-962

2. Pichichero ME (2013) Otitis media. Pediatr Clin North Am 60: 391-407

3. Spiro DM, Welker MA, Arnold DH, Meckler GD (2011) A proposal to limit otoscopy to reduce unnecessary use of antibiotics: a call for research. Expert Rev Anti Infect Ther 9: 177-181

4. Blomgren K, Pitkaranta A (2003) Is it possible to diagnose acute otitis media accurately in primary health care? Fam Pract 20: 524-527

5. Pichichero ME (2002) Diagnostic accuracy, tympanocentesis training performance, and antibiotic selection by pediatric residents in management of otitis media. Pediatrics 110: 1064-1070

6. Pichichero ME, Casey JR (2002) Otitis media. Expert opinion on pharmacotherapy 3: 1073-1090

7. Sorrento A, Pichichero ME (2001) Assessing diagnostic accuracy and tympanocentesis skills by nurse practitioners in management of otitis media. J Am Acad Nurse Pract 13: 524-529

8. Pichichero ME, Poole MD (2001) Assessing diagnostic accuracy and tympanocentesis skills in the management of otitis media. Arch Pediatr Adolesc Med 155: 1137-1142

9. Pichichero ME (2002) Assessing diagnostic accuracy and tympanocentesis skills of South African physicians in management of otitis media. S Afr Med J 92: 137-138

10. Pichichero ME (2003) Diagnostic accuracy of otitis media and tympanocentesis skills assessment among pediatricians. Eur J Clin Microbiol Infect Dis 22: 519-524

11. Liu K, Chen L, Kaur R, Pichichero ME (2013) Transcriptome signature in young children with acute otitis media due to non-typeable *Haemophilus influenzae*. Int Immunol 25: 353-361

12. Liu K, Casey J, Pichichero M (2010) Serum intercellular adhesion molecule 1 variations in young children with acute otitis media. Clinical and vaccine immunology: CVI 17: 1909-1916

13. Liu K, Kaur R, Almudevar A, Pichichero ME (2013) Higher serum levels of interleukin 10 occur at onset of acute otitis media caused by *Streptococcus pneumoniae* compared to *Haemophilus influenzae* and *Moraxella catarrhalis*. The Laryngoscope 123: 1500-1505

14. Liu K, Pichichero ME (2012) Clinical significance of serum S100A12 in acute otitis media in young children. Pediatr Infect Dis J 31: e56-58

15. Friedel V, Zilora S, Bogaard D, Casey JR, Pichichero ME (2014) Five-year prospective study of paediatric acute otitis media in Rochester, NY: modelling analysis of the risk of pneumococcal colonization in the nasopharynx and infection. Epidemiol Infect 142: 2186-2194

16. Casey JR, Kaur R, Friedel VC, Pichichero ME (2013) Acute otitis media otopathogens during 2008 to 2010 in Rochester, New York. Pediatr Infect Dis J 32: 805-809

17. Xu Q, Kaur R, Casey JR, Adlowitz DG, Pichichero ME, et al. (2011) Identification of *Streptococcus pneumoniae* and *Haemophilus influenzae* in culture-negative middle ear fluids from children with acute otitis media by combination of multiplex PCR and multi-locus sequencing typing. Int J Pediatr Otorhinolaryngol 75: 239-244

18. Fraley C, Raftery AE, Murphy TB, Scrucca L (2012) mclust Version 4 for R: Normal Mixture Modeling for Model-Based Clustering, Classification, and Density Estimation Technical Report No. 597, Department of Statistics, University of Washington

19. Tahtinen PA, Laine MK, Huovinen P, Jalava J, Ruuskanen O, et al. (2011) A placebo-controlled trial of antimicrobial treatment for acute otitis media. The New England journal of medicine 364: 116-126

20. Hoberman A, Paradise JL, Rockette HE, Shaikh N, Wald ER, et al. (2011) Treatment of acute otitis media in children under 2 years of age. N Engl J Med 364: 105-115

21. Qvarnberg Y, Holopainen E, Palva T (1984) Aspiration cytology in acute otitis media. Acta Otolaryngol 97: 443-449

22. Leibovitz E, Jacobs MR, Dagan R (2004) *Haemophilus influenzae*: a significant pathogen in acute otitis media. Pediatr Infect Dis J 23:1142-1152

23. Broides A, Leibovitz E, Dagan R, Press J, Raiz S, et al. (2002) Cytology of middle ear fluid during acute otitis media. Pediatr Infect Dis J 21: 57-61

24. Hernandez M, Leichtle A, Pak K, Webster NJ, Wasserman SI, et al. (2015) The transcriptome of a complete episode of acute otitis media. BMC genomics 16:259

25. Morris M, Pichichero ME (2017) *Streptococcus pneumoniae* burden and nasopharyngeal inflammation during acute otitis media. Innate Immun 23: 667-677.

26. Monasta L, Ronfani L, Marchetti F, Montico M, Vecchi Brumatti L, et al. (2012) Burden of disease caused by otitis media: systematic review and global estimates. PloS one 7: e36226

27. Stenstrom C, Ingvarsson L (1997) Otitis-prone children and controls: a study of possible predisposing factors. 1. Heredity, family background and perinatal period. Acta Otolaryngol 117: 87-93