Supporting Information

Fiber-optic microsphere-based antibody array for the analysis of inflammatory cytokines in saliva

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Preliminary Cytokine Screening

Qualitative Array Screening

A panel of 79 inflammatory cytokines was initially screened in saliva samples from two asthmatics and two healthy control volunteers using a Human Cytokine Array V Kit from RayBiotech (Norcross, GA).[1] Several of the cytokines in the panel were associated with obstructive pulmonary diseases. This assay was used to qualitatively screen for a large number of cytokines in saliva, which could then be investigated further with follow-up screening using quantitative microtiter plate ELISAs.

The assay consists of nitrocellulose membranes that have been spotted with capture antibodies specific for cytokines of interest. Following incubation with sample, the membranes are incubated with a cocktail containing biotinylated detection antibodies for each of the cytokines on the array. Subsequently, the membranes are incubated with streptavidin-HRP conjugate and then a chemiluminescence substrate and exposed to film in a cassette.

The assays were completed following the instructions supplied by the manufacturer. Briefly, the membranes were blocked for 30 min at RT, then incubated

with 1 mL of undiluted saliva supernatant overnight at 4°C. Following three washes with 2 mL of Wash Buffer I and two washes with 2 mL of Wash Buffer II, the membranes were incubated with 1 mL of detection antibody solution for 2 h at RT. Following another set of washes as described above, the membranes were incubated in diluted HRP-streptavidin conjugate for 2 h at RT, then washed as described above. Finally, the membranes were incubated with a chemiluminescent substrate for 2 min at RT and exposed to Kodak Biomax Light film (Rochester, NY) for 20 min.

The results of the RayBio Array screening are shown in Figure S1. Each dark feature on the array indicates that a particular cytokine was present in the saliva sample at a concentration above the detection limit of the kit. The screening data are also summarized in Table S1, which includes a key that identifies each feature on the array. If the intensity of a feature on the array was observed to be comparable to the intensity of the positive control locations and more intense than the negative control locations, we determined that the saliva sample had an appreciable concentration of that cytokine; those proteins are indicated by red text in the table. Cytokines that are implicated with Th1 or Th2 inflammation are highlighted in yellow. The arrays utilized for preliminary screening were useful for gathering information about which cytokines were the most abundant in saliva and for producing qualitative comparisons between patient and control samples. From the array results, we chose several candidate cytokines associated with obstructive pulmonary diseases to be examined in subsequent quantitative ELISA screening.

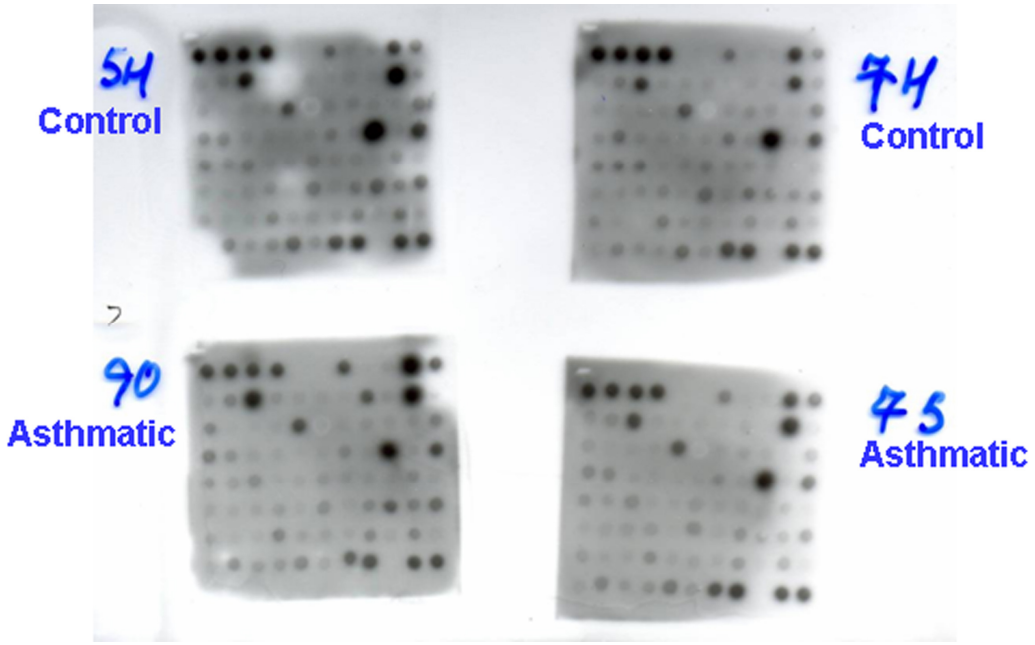

Figure S1. Digital scan of film developed from Raybio Cytokine Array V membranes following incubation with saliva supernatant samples, detection antibody cocktail, and chemiluminescence substrate.

Table S1. Human Cytokine Array V map and compiled results

	A	B	C	D	E	F	G	H	I	J	K
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-alpha
2	I-309	IL-1 alpha	IL-1 beta	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12p40p70	IL-13	IL-15	IFN-gamma	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1 beta
4	MIP-1 delta	RANTES	SCF	SDF-1	TARC	TGF-beta 1	TNF-alpha	TNF-beta	EGF	IGF-I	Angiogenin
5	Oncostatin M	Thrombopoietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ck beta 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Fit-3 Ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3 alpha	NAP-2	NT-3
8	NT-4	Osteoprotegerin	PARC	PIGF	TGF-beta 2	TGF-beta 3	TIMP-1	TIMP-2	Neg	Pos	Pos

*Note: Pos = Positive control, Neg = Negative control, yellow = Th1/Th2 cytokines, red = summary of cytokines detected by the kit. Cytokine abbreviations can be found at http://www.raybiotech.com/full_names.pdf.

Microtiter Plate Screening

Seventeen of the cytokines detected with the RayBio Cytokine Array V kit or having known implication with pulmonary inflammatory disease were examined in saliva supernatant samples using ELISAs.[1] Screening results for the ten cytokines selected for microsphere array development are shown in Figure S2 and summarized in Table S2. Saliva supernatant samples from patients suffering from asthma and/or COPD (shown in

red) and samples collected from healthy control individuals (shown in blue) were examined for each cytokine. The averages for each population are shown as green bars to the far right of each data set. The assays were completed using the instructions included by the manufacturer. An Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was used as a fluorescent substrate for analysis of each ELISA on a microplate spectrofluorimeter (SpectraMax Gemini, Molecular Devices or Infinite M200, Tecan). For the EGF and IP-10 ELISA results shown, the saliva samples were diluted in assay buffer prior to testing. Due to insufficient sample volumes, the entire panel of cytokines could not be examined for the same set of patients and controls in preliminary screening. With these results, however, we were able to compile a list of the preliminary biomarkers for the development of microsphere-based assays (described in the main text). All ten of the cytokines shown in Figure S2 were selected as candidates for further investigation with microsphere array testing.

Table S2. Summary of preliminary salivary biomarker screening results with ELISAs.

Preliminary Salivary Biomarker Screening Results				
Analyte	Concentration Range (pg/mL)		Median Concentration (pg/mL)	
	Control	Patient	Control	Patient
VEGF	224 – 1771	89 – 3384	558	472
EGF	6 – 206	27 – 242	35	125
IP-10	140 – 597	96 – 1130	290	193
IL-8	179 – 881	205 – 1863	442	595
MCP-1	15 – 294	5 – 531	57	78
TIMP-1	1328 – 13449	2620 – 17575	5590	9179
RANTES	4 – 10	6 – 32	7	11
MIP-1β	< 1 – 2	< 1 – 46	< 1	1
Eotaxin-2	< 1 – 5	< 1 – 9	< 1	1
IL-6	1 – 5	1 – 51	2	5

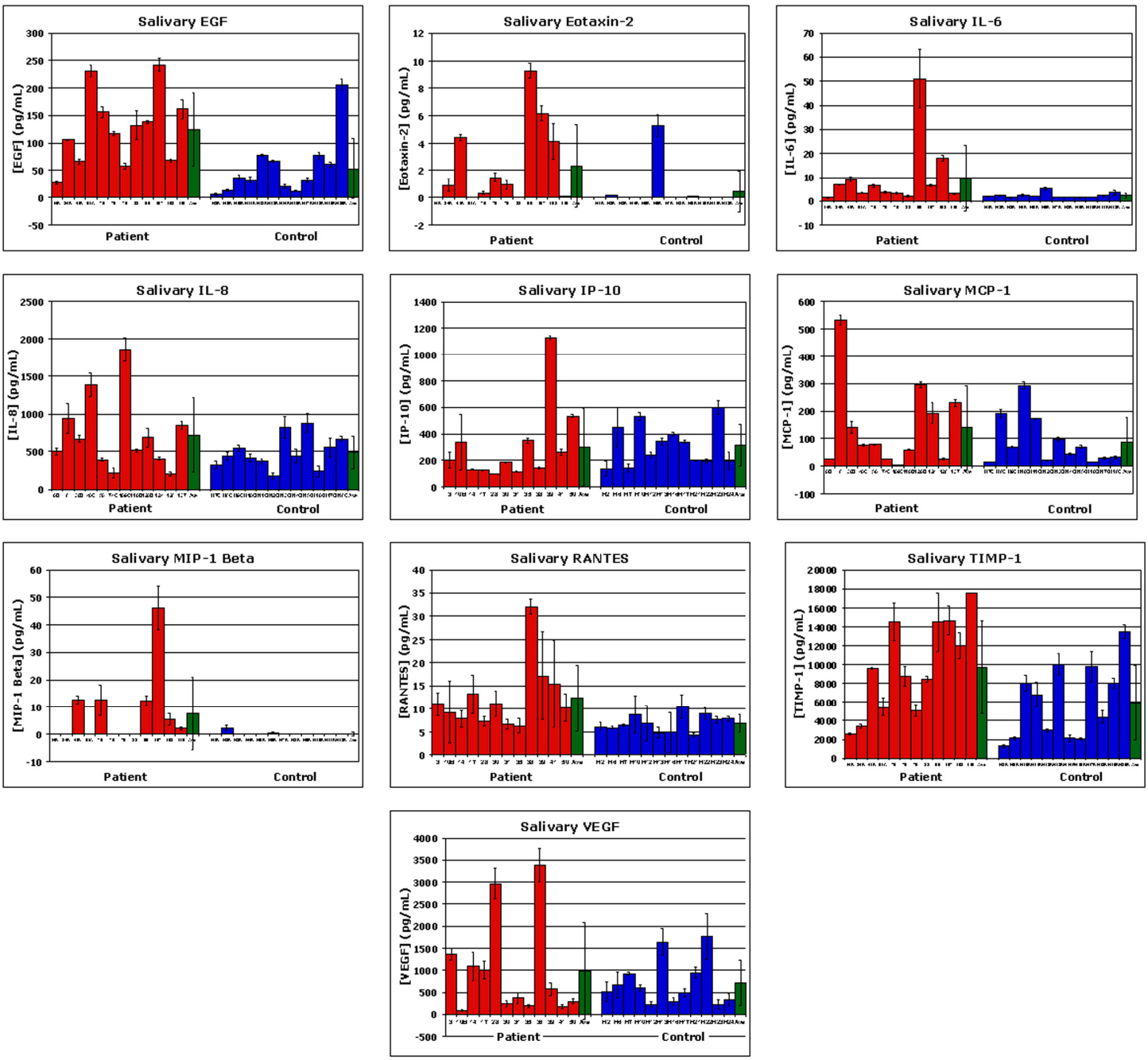

Figure S2. Secondary ELISA screening results for ten cytokines. Results for asthma/COPD patients are depicted in red and healthy control individuals in blue. The average for each population (patient or control) is represented by the green bars at the far right of each data set.

References

1. Walt, D. R.; Blicharz, T. M.; Hayman, R. B.; Rissin, D. M.; Bowden, M.; Siqueira, W. L.; Helmerhorst, E. J.; Grand-Pierre, N.; Oppenheim, F. G.; Bhatia, J. S.; Little, F. F.; Brody, J. S. Microsensor arrays for saliva diagnostics. *Annals of the New York Academy of Sciences* **2007**, *1098*, 389-400.