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# Pilot feasibility study to detect mesenchymal stem cell biomarkers of bronchopulmonary dysplasia in the tracheal aspirate fluid of preterm infants

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**Abstract**.
**OBJECTIVE**: This study aimed to detect novel mesenchymal stem cell peptides/biomarkers of bronchopulmonary dysplasia (BPD) in the tracheal aspirate fluid (TAF) of preterm infants.
**STUDY DESIGN**: Participants included infants less than 32 weeks' gestational age or birth weight under 1500 grams who required endotracheal intubation and mechanical ventilation within first 24 hours of life. TAF sample collection was performed at the time of the first clinically indicated routine suctioning. Standardization curves for human levels of osteopontin (Opn), macrophage colony stimulating factor 1 (Csf1), transforming growth factor beta 1 (TGF-β1), and secretory immunoglobulin A (sIgA) were generated for 15 enrolled participants.
**RESULTS**:We demonstrated that stem cell biomarkers are secreted into the TAF of preterm infants and their concentrations can be easily measured during the first week of life.
**CONCLUSION**: Further studies are warranted to determine a causal relationship between these biomarkers and BPD development and severity.

**Abbreviations**

| | |
|---|---|
| BPD | Bronchopulmonary dysplasia |
| TAF | Tracheal aspirate fluid |
| Opn | Osteopontin |
| CSF1 | Macrophage colony stimulating factor 1 |
| TGF- β | Transforming growth factor beta |
| sIgA | Secretory Immunoglobulin A |

## 1. Introduction

Bronchopulmonary dysplasia (BPD) is a significant chronic lung disease affecting prematurely born infants resulting from an arrest in lung vascular and alveolar development [1]. Clinically, BPD is defined as persistent oxygen requirement at 36 weeks' postmenstrual age [2, 3]. BPD is a multifactorial disease caused by both antenatal and postnatal factors, including pre-eclampsia, oxygen- and mechanical ventilation-associated lung injury, infection, and the inherent pulmonary under development due to prematurity. All these factors lead to increased cytokine signaling, inflammation, and ultimately, to dysmorphic vasculogenesis and arrested alveolar development [4, 5].

BPD is associated with multiple morbidities including decreased pulmonary function, reactive and obstructive airway disease, pulmonary hypertension, cor pulmonale, failure to thrive, and increased mortality. Those born at the most premature gestational ages and smallest birth weight are at the highest risk of being affected; and as we see continued advances in neonatal care, a greater number of these infants are surviving to be affected by BPD [6]. Additionally, BPD has far-reaching negative financial and health effects, including increased utilization of pediatric care, increased hospitalizations, and poorer neurodevelopmental and cognitive outcomes during early childhood [7, 8–10].

Currently, there are no effective biomarkers available to predict BPD. Similarly, there is a lack of clinical or pharmacologic treatments for prevention or reversal of BPD [11, 12]. These challenges remain a significantly important area of study for BPD and investigators are working to bring animal data to clinical investigations in an effort to formulate disease targets for directed therapy against BPD. One promising

approach has been to screen for biomarkers that could suggest novel mechanisms underlying BPD. Several investigators have reported perturbations in the levels of plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6), interleukin- 8 (IL-8), interleukin-10 (IL-10), interleukin-1β (IL-1 β), pulmonary trypsin-2, keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), soluble intracellular adhesion molecule-1 (sICAM-1), and pepsin in the TAF of infants at risk to develop BPD [13–23].

In 1998, Madtes et al., (1998) observed elevated transforming growth factor-β (TGF-β) levels in the TAF of patients with acute respiratory distress syndrome and suggested that this mediator might contribute to the pathogenesis of that disease [24]. Several other studies suggest that transforming growth factor beta (TGF-β) may act as a biomarker for arrested alveolar development in BPD [22, 23, 25, 26]. TGF-β is a superfamily of numerous proteins overexpressed in animal models of neonatal lung injury. Various TGF-β proteins have completely different functions in normal and disrupted lung development and inflammation. While the exact role of TGF-β in the disruption of terminal lung development is not completely understood, it has been associated with increased fibrosis and may cause proliferation of cells and inflammatory cytokines leading to dysfunctional repair. In 2012, Popova et al., demonstrated that TGF- β1 plays a role in the pathogenesis of BPD by disturbing MSC signaling pathways [27].

However, these studies did not provide any conclusive evidence that these specific markers are part of signaling cascade systems that modify newborn lung disease or predict which premature newborns would develop BPD.

Other studies have sought to define the potential role of mesenchymal stem cells (MSCs), which have shown promise in the treatment and prevention of chronic pulmonary diseases in animal models. MSCs have the potential to differentiate into a multitude of different cell types. They are thought to act in a paracrine fashion via the release of immunomodulatory and vasoprotective factors to reduce the parenchymal and vascular injury of BPD. Popova et al., (2010) linked the presence and functional impairment of tracheal aspirate MSCs to the later development of BPD [28, 29]. They later described that tracheal aspirate-derived proteins secreted by MSCs may predict BPD [30]. Mesenchymal stem cell conditioned media (MSC-CM) has been found to work similarly or better than MSCs themselves [31]. Furthermore, multiple animal studies from our group and others using a hyperoxia-induced neonatal lung injury model have shown MSCs or MSC-CM to reverse or attenuate the injurious cascade leading to BPD [32–36].

Our prior animal work using proteomics analysis of the MSC-CM to identify factors that might play a role in the protection against BPD has identified two proteins of interest – osteopontin (Opn) and macrophage colony stimulating factor 1 (Csf1) [37]. Opn plays an important functional role in both physiologic and pathologic states and is implicated in cancer and metastatic disease in several organ systems [38, 39]. In addition, it functions by inhibiting free radical production, stimulating signal transduction pathways, regulating cytokine production, mediating chemotaxis and adhesion of macrophages and other immune cells, enhancing immunoglobulin production, inhibiting apoptosis, and can promote cell survival. Opn can have either a pro- or anti-inflammatory effect depending on other cellular inputs [40–47]. Csf1 functions primarily on macrophages and monocytes and can act as a differentiation, growth, and survival factor on these cells. It serves an immunomodulatory role by enhancing cytotoxicity, superoxide production, phagocytosis, chemotaxis, and secondary cytokine production [48, 49].

These two peptides (Opn and Csf1) were present in highest concentration in the MSC-CM and absent in control media (mouse lung fibroblasts, mouse pulmonary artery smooth muscle cells). In addition, heat treatment of the MSC-CM abrogated the protective effect in mouse BPD which points towards the peptide source as the potential paracrine pathway. The challenge remains as how these two peptides/biomarkers are regulated in to human newborn infant lung injury or portend the development of BPD.

The application of MSC therapy for the prevention of BPD is an art, though it remains a considerable, but worthwhile challenge. In this study, we postulated that both Opn and Csf1 can serve as biomarkers which can predict BPD and help generate a targeted therapy against this disease. We hypothesized that MSC biomarkers, specifically Opn and Csf1, are secreted into the TAF of preterm infants at risk to develop BPD and their levels can be measured during the first week of life using standard tests. The objectives of the current study were to measure the levels of these peptides, TGF-β1 and secretory IgA (sIgA) in the TAF of the enrolled preterm study population as a pilot project which will help guide further multi-center studies to study these stem cell biomarkers in relationship to BPD.

## 2. Participants and methods

### *2.1. Participants*

Infants less than 32 weeks' gestational age or birth weight under 1500 grams and less than 24 hours old were identified as eligible participants. Those who required endotracheal intubation as deter determined by the medical team were enrolled into the University of California Irvine, CA Institutional Review Board (IRB)-approved study (#2014-1081). Participant enrollment followed IRB guidelines of informed consent and all samples were assigned research numbers before transport to the laboratory to protect patient privacy. Infants with neuromuscular disease, congenital anomalies, or pulmonary hemorrhage were excluded from the study. Limitations of our pilot study included the study population enrolled 15 infants with a gestational age range of 24.3 weeks to 32.5 weeks and a birth weight range of 425 grams to 2510 grams. We are currently in the process of conducting a larger study to determine how these early biomarkers correlate with BPD severity as this would require a longer follow-up period for this to be determined at 36 weeks gestational age (or 4 weeks of life if born at >32 weeks PCA).

### *2.2. Tracheal aspirate fluid (TAF) collection*

At our institution, each infant with an endotracheal tube in place receives tracheal suctioning every four to six hours or sooner as needed. The first TAF sample collection was performed at the time of the first clinically-indicated routine suctioning (before the administration of exogenous surfactant) by the method previously described [13]. Briefly, the bedside registered nurse and respiratory therapist collected the TAF samples during routine endotracheal tube care using a Leukens trap and standard suctioning technique. A small amount of sterile normal saline (0.5–1.0 mL) was instilled into the endotracheal tube and the returned fluids were aspirated using regulated suction, with a goal volume of 1mL per sample. An attempt was made to collect a second TAF sample just prior to extubation. The second samples were collected randomly during the first week of life. If the subject had received exogenous surfactant, a minimum of six hours elapsed before obtaining the second TAF sample. This particular time frame was chosen in order to avoid inadvertently depriving the patient of surfactant and to avoid contaminating samples with exogenous surfactant. TAF samples were immediately placed on ice and stored within 30 minutes of collection in a –20◦C freezer located within the NICU prior to transport to the research laboratory for processing. Samples were transported in a frozen state on ice, and subsequently were placed in an –80◦C freezer located in the processing laboratory.

Table 1
Demographic profile of participants

| Subject | Age (hours) at sample 1 | Age (hours) at sample 2 | GA (wks) | BW (gms) | Sex | Antenatal steroids (course completed) | Duration of ROM | Latency antibiotics |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.3 | | 26.2 | 796 | F | Y | 0 | N |
| 2 | 3.5 | | 25 | 640 | F | N | 12 hrs | N |
| 3 | 2.25 | | 30.5 | 1310 | M | Y | 12 hrs | N |
| 4 | 0.7 | 40.25 | 27.4 | 1028 | F | Y | 20 days | Y |
| 5 | 4.5 | | 30.5 | 1620 | F | Y | 0 | N |
| 6 | 1.2 | 10 | 27.4 | 1100 | M | Y | 20 days | Y |
| 7 | 2.7 | | 27 | 940 | M | N | 19 days | Y |
| 8 | 6.5 | | 29.5 | 1050 | M | Y | 0 | N |
| 9 | 1 | 8.3 | 30.5 | 890 | M | Y | 0 | N |
| 10 | 0.25 | | 28.1 | 1320 | F | Y | 0 | N |
| 11 | 57 | | 24.3 | 735 | M | N | 0 | N |
| 12 | 1 | 11.5 | 27.5 | 890 | M | N | 0 | N |
| 13 | 11.5 | | 29.6 | 1490 | F | N | 3 hrs | N |
| 14 | 0.3 | 96.3 | 24.4 | 457 | M | Y | 4 days | Y |
| 15 | 0.5 | 170 | 25.3 | 425 | M | N | 0 | N |

Gestational age (GA); Birth weight (BW); Rupture of membranes (ROM).

### *2.3. Biomarker analysis using ELISAs*

For the biomarker analyses, the samples were first thawed at room temperature, and subsequently were transferred to ice to keep cold during processing. Two sets of 1.7mL vials were pre-labeled with the sample ID. The first vial was for transferring the original aspirate from the large Leukens trap containers. The second vial was to use after spinning the sample to store the supernatant. Once the samples were thawed, the TAF was transferred to the first pre-labeled 1.7mL vial for each sample, the volume was estimated, and the vials were placed on ice. Once all the samples were transferred to 1.7mL vials, they were spun in a microfuge at 10,000 g for five minutes. A pellet made of mucin and cells was separated out from the supernatant. The clear supernatant was then transferred to the second pre-labeled new 1.7mL vial for each sample by pipette without touching the pellet. Additional 1.7mL vials were used as needed for participants with larger sample volumes. Processed samples were stored in the 1.7mL vials at –80◦C and thawed for each ELISA assay.

For this pilot feasibility study, standardization curves for human levels of osteopontin (Opn), macrophage colony stimulating factor 1 (Csf1), transforming growth factor beta 1 (TGF-β1), and secretory immunoglobulin A (sIgA) were generated for 15 enrolled participants. The TAF samples were assayed using commercially purchased human ELISA kits (Opn – DOST00, Csf1 – DMC00B, and TGF- β 1 – DB100B, R&D Systems, Minneapolis, MN; sIgA – 1-1602 Salimetrics, Carlsbad, CA). The ELISA assays were performed according to the kit instructions and read using an optical density microplate reader using logistic curve fitting model.

### *2.4. Data collection and statistical analysis*

Demographic and clinical data were compared by t-test and Wilcoxon-Mann-Whitney tests for continuous variables. A standardization curve was generated for each assay using logistical curve-fitting by the microplate reader software. All samples were assayed in duplicate and blinded without knowledge of the clinical condition of the infants. All data were described as mean ± SD. P values were considered statically significant if $< 0.05$.

## 3. Results

### *3.1. Patient characteristics and data*

Table 1 shows the demographic data of the patients. We successfully enrolled 15 infants with a gestational age range of 24.3 weeks to 32.5 weeks and a birth weight range of 425 grams to 2510 grams. Out of 15 infants, six were females and the rest males. Nine out of 15 infants completed at least one prenatal steroid course (two doses). None of the infants had histologic chorioamnionitis, but four out of 15mothers received latency antibiotics for prolonged preterm rupture of membranes. Duration of rupture of membranes ranged from immediately at delivery to 20 days prior to delivery.

### *3.2. TAF sample collection*

We successfully obtained TAF samples from all enrolled participants within the first week of life. The first sample was obtained before the first dose of exogenous surfactant for 14 out of 15 infants. Six out of 15 infants have a second sample, which was obtained between days of life 1–7. Table 1 demonstrates the number of samples and the age in hours at which the samples were collected.

### *3.3. Biomarker analysis of TAF samples*

Using the commercially available ELISA kits for Opn, Csf1, TGF-β1 and sIgA, we analyzed all the samples and generated data after volume and concentration correction (Table 2). Biomarker levels were corrected for volume

with a correction factor of 1 mL. The sample values were also corrected for dilution using sIgA. Standard curves were generated for each biomarker to calculate reference values for these assays (Figs. 1–4). Our results in this initial study showed that the Opn levels ranged between 0.3–20 ng/mL, Csf1 levels ranged between 75–5000 pg/mL, and TGF-β1 levels ranged between 30–2000 pg/mL (Table 2).

## 4. Discussion

Despite advancements in the care of extremely preterm infants, BPD continues to remain a major public healthcare problem. Currently, little is known of our understanding of the exact pathogenesis of the BPD, which is hindering the development of a targeted therapy against this chronic debilitating disease. Factors implicated in BPD include hyperoxia, mechanical ventilation and maternal chorioamnionitis. Evidence in the literature has shown that in preterm infants who develop BPD, there is increased Interleukin (IL)-1β, IL-8, and tumor necrosis factor-α (TNF α) in tracheal aspirates and bronchoalveolar lavage fluid [30, 39].

**Table 2**

Levels of Opn, Csf1, and TGF-β1 in 15 participants (after sIgA and volume correction)

| Subject | Age (hours) at Sample 1 and 2 | Opn (ng/mL) | Csf1 (pg/mL) | TGF-b1 (pg/mL) |
|---|---|---|---|---|
| 1 | 0.3 | 20 | 4630 | 30 |
| 2 | 3.5 | 0.8 | 102 | 2000 |
| 3 | 2.25 | 18 | 4780 | 266 |
| 4 | 0.7 | 17 | 5000 | 134 |
| | 40.25 | 20 | 3212 | 1890 |
| 5 | 4.5 | 14 | 868 | 716 |
| 6 | 1.2 | 20 | 3031 | 101 |
| | 10 | 15 | 3784 | 94 |
| 7 | 2.7 | 0.5 | 150 | 1670 |
| 8 | 6.5 | 2 | 75 | 1530 |
| 9 | 1 | 19 | 4442 | 89 |
| | 8.3 | 9 | 779 | 1103 |
| 10 | 0.25 | 6 | 219 | 260 |
| 11 | 57 | 20 | 911 | 323 |
| 12 | 1 | 7 | 98 | 1430 |
| | 11.5 | 17 | 393 | 1011 |
| 13 | 11.5 | 20 | 5587 | 248 |
| 14 | 0.3 | 5 | 371 | 870 |
| | 96.3 | 17 | 1136 | 560 |
| 15 | 0.5 | 19 | 470 | 270 |
| | 170 | 18 | 2024 | 176 |

**Fig. 1.** Macrophage colony stimulating factor 1 (Csf 1) standard curve, values in pg/mL.

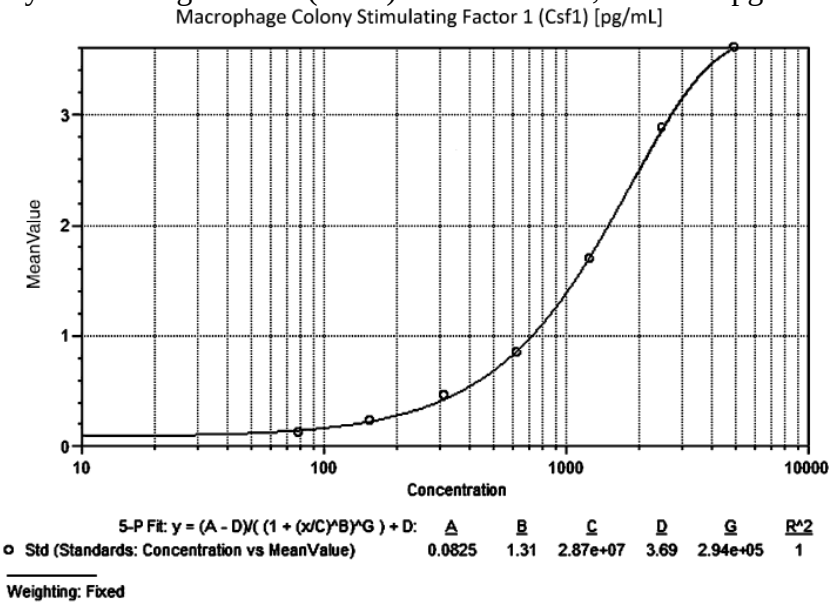


**Fig. 2.** Osteopontin (Opn) standard curve, values in ng/mL.

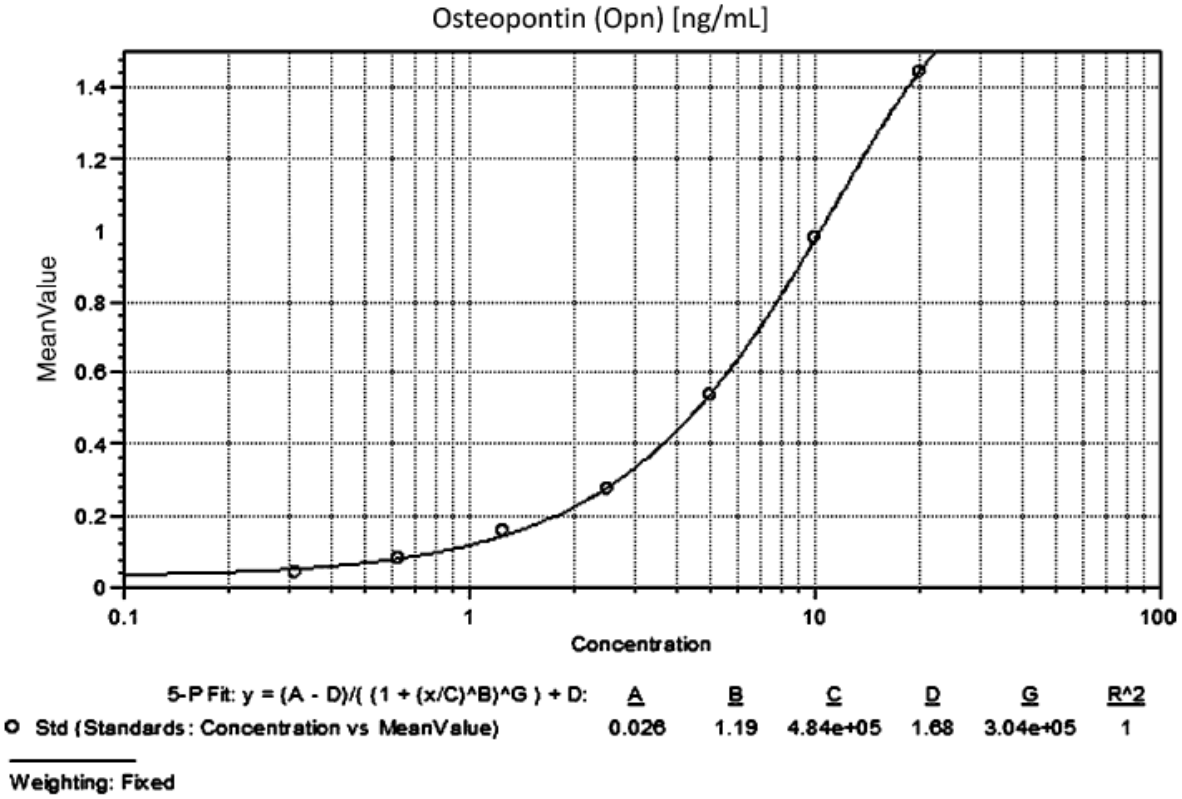



**Fig. 3.** Transforming growth factor – beta1 (TGF-□1) standard curve, values in pg/mL.

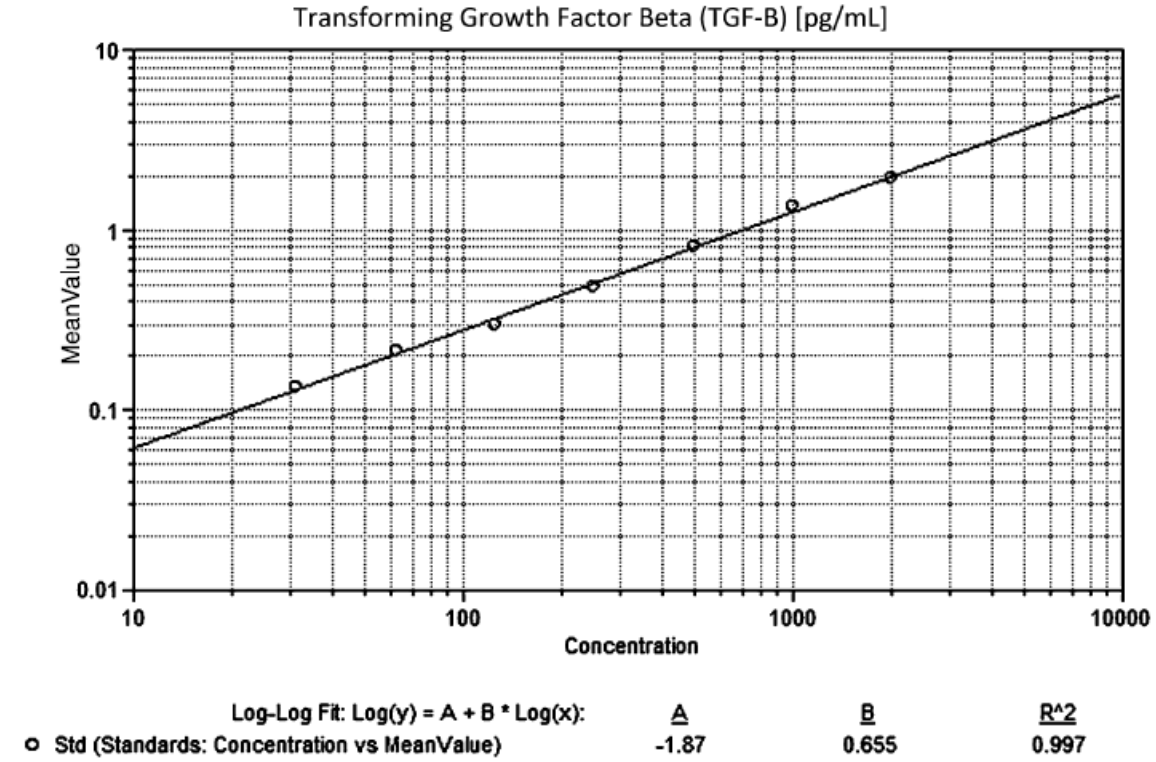
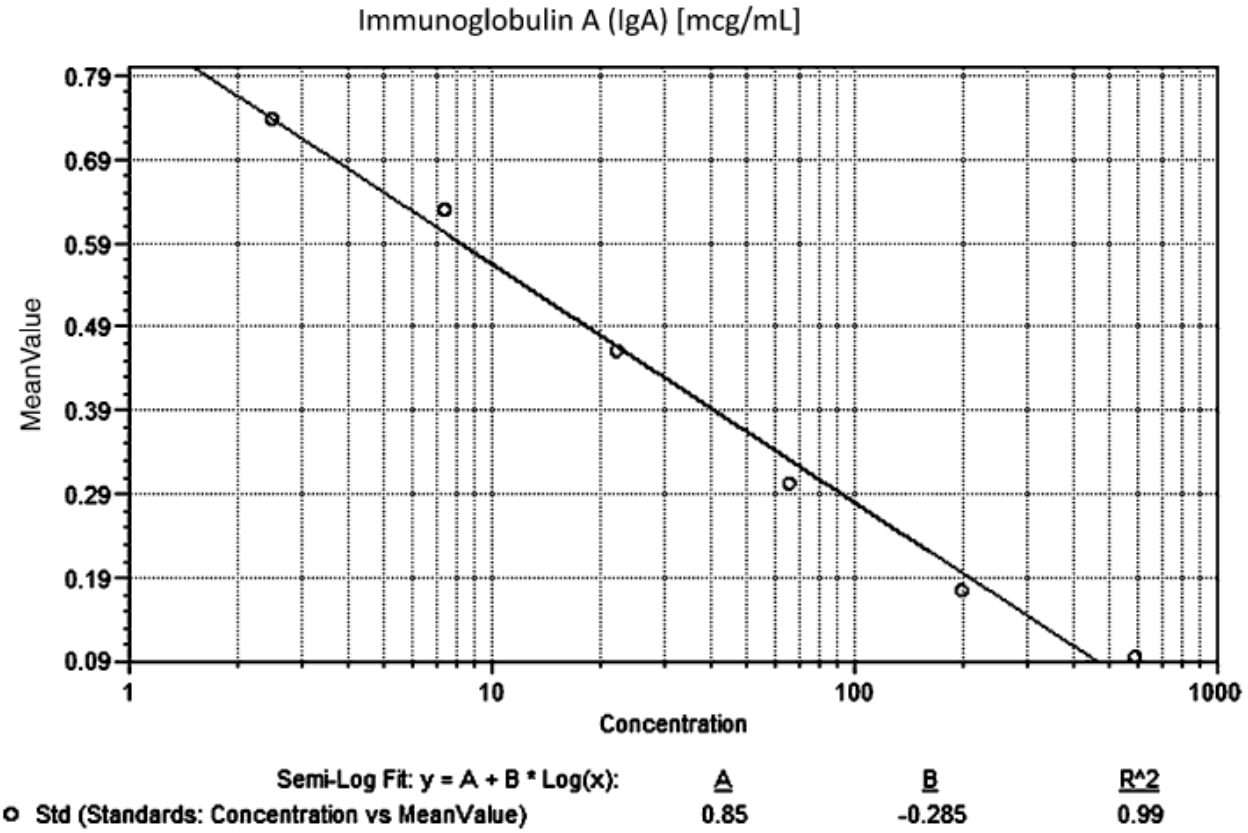


**Fig. 4.** Standard curve of sIgA, values in mcg/mL.




Moreover, animal models of disease are progressing towards providing excellent data for disease pathogenesis, leading several investigators utilizing this data to analyze human subpopulations. The same holds true for BPD, where investigators have brought animal data to clinical investigations in an effort to formulate disease targets for directed therapeutic purposes [30, 39].

Another promising direction may be the screening for specific stem cell biomarkers, which may reveal new mechanisms underlying BPD. Our previous work utilizing proteomic research analysis of MSC-CM identified two proteins of interest, mainly Opn and Csf1. These factors may possibly play a pivotal role in the protection against BPD and therefore, it is critical to conduct further research examiningthese two MSC signaling peptides within the TAF of premature infants at risk to develop BPD in order to define specific-targeted therapy. In this investigation, we demonstrated that stem cell peptides are secreted into the TAF of preterm infants and their concentrations can be easily measured during the first week of life using commercially available immunoassays. These can serve as potential biomarkers for predicting BPD in at risk preterm infants. Tracheal aspirates from infants less than 32 weeks' gestational age or birth weight under 1500 grams and less than 24 hours old were found to contain biomarkers including Opn levels ranging between 0.3–20 ng/mL, Csf1 levels ranging between 75–5000 pg/mL, and TGF-□ levels ranging between 30–2000 pg/mL. Opn was previously measured in various human clinical samples including spontaneous and provoked sputum [39]. Moreover, we showed that sIgA secreted in the TAF that could also be easily measured and used as a corrective factor for the biomarker levels given the lack of changes in sIgA levels with gestation and fluid volume.

Based on the successful measurement of stem cell biomarkers in the TAF of preterm infants, these data suggest that the aforementioned biomarkers will have valuable clinical significance. We also analyzed 4 full term infants who were intubated within first 48 hours of life for non-respiratory illness. The levels of these biomarkers were undetectable by same kits pointing to the fact that these are specific to the preterm infants only. Future studies aimed at determining the relationship between these biomarkers in the TAF of preterm infants and BPD is currently underway at our institution.

In conclusion, this pilot study's main aim was to detect biomarkers in the TAFs of preterm infants. Work done by Popova (2015) has also investigated proteins secreted by stem cells into the TAF of newborns

[30]. Interestingly, in our study, two biomarkers Opn and Csf1 represent potential targets for intervention in preterm infants at risk to develop BPD. Data from this study will form a basis to compare biomarker levels between lung-injured and healthy infants. Future studies are needed to determine a causal relationship between these biomarkers and BPD development and severity.

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