ASSOCIATION BETWEEN CYTOKINE PROFILE AND DISEASE SEVERITY IN CHILDREN INFECTED WITH RESPIRATORY SYNCYTIAL VIRUS CAUSING LOWER RESPIRATORY TRACT INFECTION

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine in the field of Clinical Microbiology and Infectious Diseases

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To my parents Matome and Tshamano Montlha; my brothers Moraba and Monkge Monhla for selfless sacrifices they endured in support of my studies and unconditional love they have given me; Katlego Moyaba for your patience and compassion towards me; to the loving memory of my sister, Sekgomane Sarah Monhla; and to all Lemba Clans.

PRESENTATIONS FROM THIS STUDY

- Poster presentation at *4th RSV Vaccines for the World*,
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  Poster Title: Association between cytokine profile and Respiratory Syncytial Virus causing lower respiratory tract infection

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  Abstract Title: Cytokine responses in South African children infected with Respiratory Syncytial Virus causing lower respiratory tract infection

ABSTRACT

Background: Respiratory Syncytial Virus (RSV) is a common cause of upper and lower respiratory tract infections (LRTI), primarily in children having severe disease manifestation. In South Africa, RSV is identified in approximately 25-30% of children hospitalized for LRTI. There is a spectrum of RSV-associated LRTI severity. Understanding associations between immune mediators and RSV-LRTI severity could assist clinicians in the triaging for level of care. Several cytokines have been implicated in RSV-LRTI severity.

Aim: Study the associations between cytokine levels from plasma and nasopharyngeal aspirate with RSV infection or RSV-associated LRTI severity in hospitalized infants ranging from 0-12 months of age. The correlation between plasma and nasopharyngeal aspirate cytokine concentrations was also evaluated.

Methods: Paired plasma and nasopharyngeal aspirate (NPA) samples were collected from polymerase chain reaction confirmed RSV-infected infants without coinfection with other pathogens that we investigated for. Paired samples were also collected from RSV negative-control infants (n=31) who did not have any respiratory symptoms. Control-infants were scheduled for elective surgery; samples were collected before administration of medication and surgical procedure. RSV associated LRTI episodes were grouped into mild (n=89) and severe RSV-LRTI (n=113) using the Respiratory Index of Severity in Children (RISC) Score. Interferon gamma (IFN-γ), interleukins (IL) IL-1α, IL-1β, IL-4, IL5, IL-6, IL-8, IL-9, IL-10, IL-12(p70)IL-13, macrophage inducing protein (MIP-1α), monocyte chemo attractant protein (MCP-1), tumour necrosis alpha (TNF-α), Regulated on activation, normal T cell expressed and secreted (RANTES) and were measured with multiplex immunosorbent assay using Luminex® technology. Cytokine profiles were evaluated for association of RSV-LRTI severity and between RSV LRTI cases and controls.

Results: Comparing hospitalized RSV-associated LRTI to control infants, RSV cases had elevated plasma TNF-α (0.7pg/ml vs. 0.5pg/ml; p=0.007), and IL-10 (1.0pg/ml vs. 0.6pg/ml; p=0.02) concentrations, and reduced plasma MIP-1α (12pg/ml vs. 28pg/ml; p=0.008) and IFN-γ (3pg/ml vs. 5pg/ml; p=0.02) levels. Nasopharyngeal aspirate TNF-α (8.0pg/ml vs. 1.0pg/ml; p=0.01), IL-8 (2682pg/ml vs. 184pg/ml; p=0.002), MCP-1 (287pg/ml vs. 66pg/ml; p<0.001), MIP-1α (27pg/ml vs. 6.7pg/ml; p=0.004) concentrations were elevated in RSV-LRTI cases compared to control infants. Infants hospitalized with severe RSV-associated LRTI (RISC score ≥2) were younger than mild cases (3.9 vs. 4.5 months; p=0.01). In RSV

cases, severe RSV-LRTI was associated with increased plasma IFN-γ levels (4pg/ml vs. 3pg/ml) and NPA MIP-1α concentrations (88pg/ml vs. 50pg/ml, mean; all other values medians) compared to mild RSV-LRTI. In a multivariate analysis, NPA MIP-1α levels remained associated with RSV-LRTI (p=0.05), but could not predict RSV-LRTI severity.

Conclusion: This study observed that during RSV-associated LRTI, immune response was directed at the respiratory tract. Reduced concentrations of plasma IFN-γ and elevated levels of cytokines in the NPA may suggest that the blood of RSV-LRTI cases had reduced number of IFN-γ producing cells. There was no evidence of distinct Th1 or Th2 type immune response and the cytokines associated with RSV-LRTI severity could not predict the outcome of severe RSV-associated LRTI.

Key words: Respiratory Syncytial Virus, Lower respiratory tract infections, Severity, Plasma, Nasopharyngeal aspirate, IFN-γ, MIP-1α, Luminex®

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CHBAH	Chris Hani Baragwaneth Hospital
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F- Protein	Fusion protein
G- Protein	Glycoprotein protein
HIV	Human immunodeficiency virus
HEU	HIV exposed uninfected
HREC	Human Research Ethics Committee
ICU	Intensive care unit
IFN-γ	Interferon gamma
γδ T cells	Gamma delta T cells
IL- 1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-9	Interleukin 9
IL-10	Interleukin 10
IL-12 (p70)	Interleukin 12 (p70)
IL-13	Interleukin 13
LRTI	Lower respiratory tract infection

MCP-1	Monocyte chemotactic protein 1
MFI	Mean fluorescence intensity
MIP-1 α	Macrophage Inflammatory Proteins 1 alpha
NOD	Nucleotide-binding oligomerization domain
NPA	Nasopharyngeal aspirate
NPS	Nasopharyngeal swab
PMT	Photo multiplier tubes
RANTES	Regulated on activation, normal T cell expressed and secreted
RMPRU	Respiratory and Meningeal Pathogens Research Unit
RSV	Respiratory syncytial virus
RPM	Revolutions per minute
SA-PE	Streptavidin-phytoerythrin
S	Standard
Th1	T helper type 1
Th2	T helper type 2
TNF	Tumour necrosis factors
URTI	Upper respiratory tract infections
UTM	Universal transport medium
WHO	World Health Organisation

Chapter 1: Literature Review

Respiratory Syncytial Virus (RSV) was initially identified in 1956 from a chimpanzee that had an upper respiratory tract infection; and named Chimpanzee Coryza Agent (1, 2). One year later, a similar virus now named RSV was isolated from infants with lower respiratory tract infection (LRTI) (3). The molecular characteristics of RSV were not elucidated for over two decades due to difficulty in culturing the virus (3), and finally published in 1981 (1). There is currently no licensed vaccine for RSV and medical interventions are largely supportive with no specific treatment against RSV (4). A prophylactic strategy is monoclonal antibody, palivizumab (Synagis®, MedImmune), which is recommended in infants with underlying risk of severe RSV disease and reportedly has 39-78% efficacy against RSV-associated hospitalization (5, 6).

1.1. Taxonomy of RSV

Human RSV belongs to the *Mononegavirales* order (7, 8) and is a member of the *Paramyxoviridae* family made of three genuses: *Morbilivirus* (example: measles virus), *Paramyxovirus* (examples: human parainfluenza virus types 1 to 4) and the *Pneumovirinae* genus (9, 10). RSV is classified into two subtypes RSV-A and RSV-B, based on genetic and antigenic variations of the surface G-protein (11-14). It is an enveloped virus with a single-stranded negative-sense ribonucleic acid (RNA) genome. The viral envelope includes two glycoproteins, the fusion protein (F-protein) and the glycoprotein (G-protein), which play key roles in disease pathogenesis (15). The G and F proteins are the most antigenic segments of the virus. These proteins are exposed on the surface of the RSV virions and induce neutralizing antibodies, therefore have been targeted in vaccine development (16).

To date 11 different RSV-A and 17 RSV-B genotypes have been identified globally (17). RSV-A includes SAA1 (South Africa, A1) (18), NA1, NA2, GA1 to GA7 (19) and ON1 genotypes (14, 17); and RSV-B includes GB1 to GB4, SAB1to SAB3 and BA1 to BA10 genotypes (17).

1.2. Structure of the Respiratory Syncytial Virus

The RSV virion is approximately 100-350 nm in diameter. The core of the virion consists of nucleocapsid proteins and an encapsulated genome with a lipid bilayer originated from the host cell membrane (1). The lipid bilayer from the host also contains viral encoded envelope glycoproteins of 11-20 nm in length (Figure 1.1) (20).

The RSV reference genome has 15 222 nucleotide (RSV A strain A2, GenBank accession number M74568) (1, 21). The genome is non-segmented, and is composed of an extended 3' leader region and 5' trailer regions (9), with 10 genes. The genome encode 11 proteins (15) categorised as: non-structural proteins, nucleocapsid-associated proteins, envelope glycoproteins, and regulatory proteins (Figure 1.1) (7).

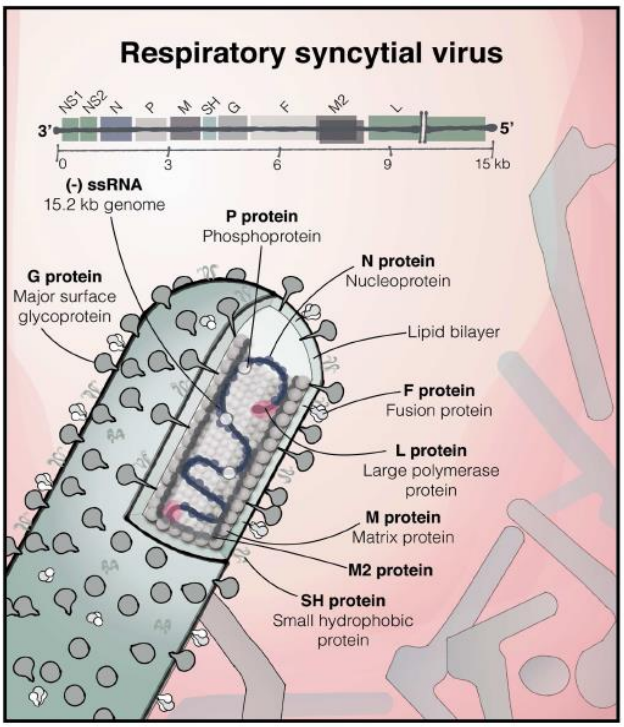


Figure 1.1. The structure of the RSV virion and encoded genome
From Lambert et.al (2013) *(11).*

1.3. RSV virulence factors

The RSV genome and proteins interact with host cell factors. Characterization of these interactions holds a key to the development of antiviral treatment and therapeutic intervention strategies.

1.3.1. Leader and trailer regions of RSV genome

According to Mink *et al.* (1991), the leader and trailer regions of the RSV genome include signals that are important in initiating viral RNA synthesis (9). The leader region is approximately 44 nucleotides long with two major functions: i) confers RNA replication signals to produce a full replicative antisense that directs the synthesis of the progeny genome, and ii) provides transcription signals that result in sub-genomic messenger RNA (mRNA) (22).

The trailer region of the RSV genome is approximately 155 nucleotides long. Studies by Hanley *et al.* (2010) have suggested that the trailer region enhanced the promoter signal activity involved in RSV replication. This allows the virus to replicate more rapidly and establish itself early in infection before the adaptive immune response has matured. As a result, RSV replicates rapidly in order to infect other hosts or host cells (22). Furthermore, the trailer region enables RSV to interact with and destabilize the host stress granule (SG) response. During environmental stress such as an infection, the SG response is an attempt by the host cells to inhibit viral RNA translation by forming stalling RNA-protein complexes in the cytoplasm known as stress granules. These complexes form a crucial link for host degradation machineries to recognise the viral RNA "tagged" for destruction (23, 24).

The ability of RSV to interact with the SG response can alter host cytokine response. During an infection, the interaction between the virus and host toll-like receptors (TLR) leads to the translation of large quantities of cytokines. The inhibition of SG response by the trailer region has the potential to augment the expression of pro-inflammatory cytokines, and this can lead to tissue damage by the immune response. It is therefore vital that the translation of cytokine mRNA is reduced to baseline once the healthy state has been achieved (24).

1.3.2. Non-structural proteins

The RSV non-structural (NS) proteins include NS1 and NS2 proteins. These proteins are encoded at the vicinity of the 3' leader region to ensure that they are highly expressed (7). Animal RSV challenge studies with RSV strains engineered to lack the function of non-structural proteins, demonstrated reduction in lung pathology (25). The NS1 and NS2 proteins antagonise the antiviral activity of interferon signals by up regulating the expression of suppressor of cytokines signalling (SOCS), a feedback loop which attenuates cytokine response (25).

1.3.3. Nucleocapsid-associated proteins

The RSV nucleocapsid is composed of nucleoprotein (N protein), polymerase (L protein), phosphoprotein (P protein) and matrix proteins (M2-1 and M2-2 proteins) that closely associate together (26). These proteins assemble to facilitate the transcription and replication of genomic RNA and mRNA (27, 28). The N protein associates with the negative sense genomic RNA and the antigenome (29). This interaction shields the genomic material from host intracellular recognition by the TLR and other innate immune system pro-inflammatory factors that would initiate antiviral responses (7), and also serves as a transcription start signal for the virus L protein. Furthermore, the N protein confers ribonuclease resistance to the genome and antigenome (26).

The L protein functions as the RNA dependent polymerase (28, 30). It replicates the negative sense genomic and anti-genomic RNA, and transcribes mRNA. It is also believed that the L protein has the ability to cap the 5' end of the mRNA and methylate the cap. Ribavirin, a ribonucleoside analogue, has been used to inhibit the functions of L protein, however has clinical limitations to treat RSV infection because of its toxicity (28).

The P protein provides stability to the nucleocapsid-associated proteins assembly complex during RNA synthesis (31). It also acts as L protein cofactor by directing the polymerase to the genomic RNA. The interaction of M proteins with the nucleocapsid-associated proteins is bridged by M2-1 protein (27). This protein mediates the assembly of new virions and their subsequent budding from the host plasma membrane (26). On the other hand, M2-1 protein has been reported to prevent early transcription termination (32). Recent *in vitro* and mice model studies by Bailly *et al.* (2016) inhibited the functions of M2-1 protein with the Smoothened Receptor (SMO) and demonstrated a reduction in viral transcription and virus

replication (32). SMO is a human protein that forms part of the signal transduction and belongs to G-Protein coupled receptors (33).

1.3.4. Envelope-associated proteins

The viral envelope consists of glycoproteins, small hydrophobic protein (SH), fusion protein (F-protein) and glycoprotein (G-protein), the latter two mediate infection of host cell (15). The SH is a transmembrane protein (34) and studies have shown that it can attenuate host cell apoptosis by inhibiting host cells from secreting tumour necrosis factor (TNF-α) and using alternative pathways, which still need to be explored (34, 35).

During RSV infection, the F-protein mediates the fusion of the RSV envelop with the host cell membrane (36). Furthermore, it facilitates membrane fusion of nearby RSV infected respiratory tissue cells to form a "giant cell" known as syncytia (37), and induces the expression of IL-1β by macrophages (38). F-protein interacts with immune cells such as neutrophils expressing toll-like receptor 4 (TLR4) (39). F-protein inhibits TLR4 signalling during cell entry. (38), This prevents neutrophils to form NETs (Neutrophil Extracellular Traps) in a process called NETosis (release of neutrophil extracellular traps, which has antimicrobial activity) (39).

Variations in the G-protein differentiate the RSV group A from B (13, 40). G-protein further facilitates the virus to escape the immune responses (13); rendering the host susceptible to reinfection by different RSV strains in a life-time (13). The secretion of early warning cytokines and other pro-inflammatory mediators is also antagonised by the G-protein (41).

Both F and G proteins work hand-in-hand to escape the host immune response. The heavily glycosylated G and F proteins are the most antigenic segments of the RSV and have been targeted in vaccine development as they induce neutralizing antibodies production (16).

1.3.5. Regulatory proteins

The viral M2-2 protein acts as the genomic negative sense RNA regulatory factor (42). *In vitro* studies demonstrated that the deletion of this protein was associated with reduced accumulation of RSV genomic and complementary genomic RNA which are important in RSV replication. The same study observed an increase in the accumulation of transcription products showing that M2-2 protein regulates RNA transcription and viral replication (43). It assures that sufficient infectious progeny viruses are produced by increasing replication.

1.4. Epidemiology of RSV disease

In 2015, pneumonia accounted for an estimated 15.5 % of under 5 years of age childhood mortality globally (Figure 1.2)(44). RSV is a common virus, causing upper and LRTI mainly in children below one year of age and the elderly (13, 45, 46). Approximately 80% of children worldwide are infected with RSV in their first year of life, of which an estimated one-third develop LRTI (11). According to Nair *et al.* (2010) globally RSV causes 30 million new LTRI episodes in children under the age of 5 years each year, resulting in 3.4 million hospitalizations associated with severe disease (47). It is estimated that 90% of the deaths due to RSV infection occur in low-middle income countries largely in Africa, South-East Asia, and Eastern Mediterranean regions (48). In line with these estimates, a study conducted in four South African provinces from 2009 to 2012 among children <5 years of age reported detection of RSV in 26% of LRTI hospitalized cases using real-time quantitative reverse transcription polymerase chain reaction (PCR) (49).

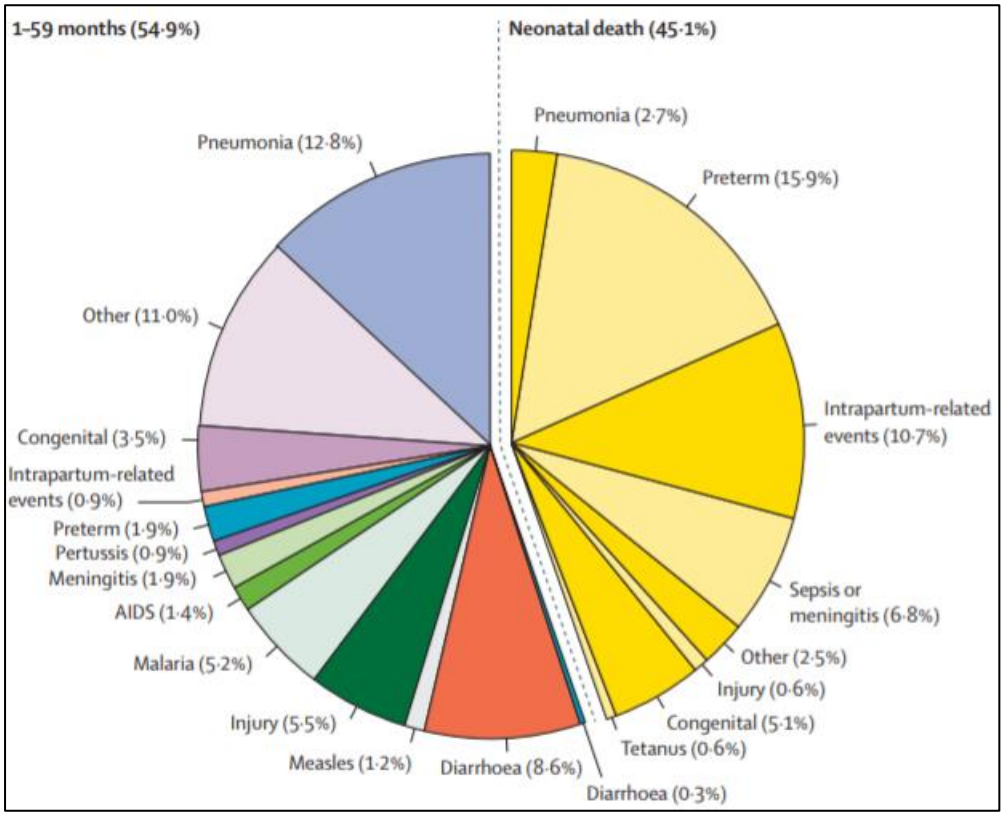


Figure 1.2. Global causes of under 5 years of age mortality

From Liu et al. (2016) (44).

1.5. Risk factors associated with RSV disease

Recognised risk factors for severe RSV-associated LRTI are low-socio economic conditions, prematurity, male gender, pre-existing chronic medical conditions, and young age (11, 50, 51). Nonetheless a substantial proportion of hospital admissions due to RSV are observed in previously healthy children with no other underlying risk factors other than young age (52, 53). In South Africa the incidence of RSV-associated LRTI is increased in Human Immunodeficiency Virus-1 (HIV-1) infected and HIV-exposed uninfected (HEU) infants (54). RSV-associated LRTI hospitalizations in Gauteng province are mostly limited to the pre-winter period, February-March (55). In KwaZulu-Natal RSV hospitalization is commonly between December-January and the Western Cape is limited to March (5).

1.6. RSV exposure and infection

RSV is highly contagious, and spreads through droplets containing the virus. The virus can also survive on surfaces such as hands and clothing, so it can easily spread through direct contact with contaminated surfaces and self-inoculation (56, 57). During a primary infection in the nose, the virus makes contact with nucleolin receptors on the apical surface of epithelial host cells (58) and enters the cells with aid of the F-protein (59, 60). In the nasopharyngeal epithelial cells, RSV incubates for two to eight days, replicates and may spread to the bronchioles (12, 56) after which more severe symptoms begin.

1.7. Clinical manifestation of RSV infection

RSV infection manifests with a variety of symptoms that are similar to other respiratory infections. One to three days following RSV infection, symptoms such as coughing, sneezing and wheezing may manifest, other respiratory symptoms may appear during the incubation period of the virus (61). Most upper respiratory tract infections (URTI) caused by RSV are characterised by inflammation of the mucosal membrane in the nose (13). Elderly individuals, premature infants, and children who are immune compromised or have chronic lung or congenital heart disease are at a greater risk of developing severe LRTI symptoms following exposure to the virus. RSV infecting the lower respiratory tract can result in pneumonia or bronchiolitis and approximately 25% to 40% of infants with primary RSV infection will show symptoms of pneumonia or bronchiolitis (62). RSV-associated pneumonia can be aggravated by superimposed bacterial infections, such as *Streptococcus pneumoniae* (63, 64). Those who are hospitalized may require oxygen supplementation or

mechanical ventilation (61) because RSV infection induces lung pathology by forming syncytia in the respiratory tissue (65). The preservation of the respiratory system's anatomical structure is essential for respiratory functions.

1.8. Immune response during RSV infection

RSV replicates, assembles new virions and spreads throughout the respiratory tissue at the expense of infected cells. Successful virus replication also relies on the virus taking over the host cell metabolic machinery; this can lead to host cellular starvation and cause tissue injury. Any microbe, including RSV which crosses the host physical barriers such as the skin and mucosal lining of the body is confronted by the innate immune response which is the first line of defence against microbes (Figure 1.3a) (16). Immune and epithelial cells detect viral infection using pattern recognition receptors (PRR) such as Toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD) like receptors (60, 66). PRR initiate host cellular responses in infected cells that inhibit virus replication, and secrete cytokines such as type I interferons which also inhibit RSV replication. As part of this intrinsic mechanism, RSV infected cells may undergo apoptosis, which halts the virus replication cycle and simultaneously initiates an inflammatory cascade in the respiratory tissue (67, 68). RSV infection may cause damage to the respiratory tissue either by viral replication or exacerbation of the host immune system, which itself may cause tissue damage. Immunity following first infection is incomplete and subsequent RSV infection can occur albeit usually milder. Hence RSV is characterized by recurrent infections throughout life, but which can also result in serious complication in older immune suppressed individuals (69).

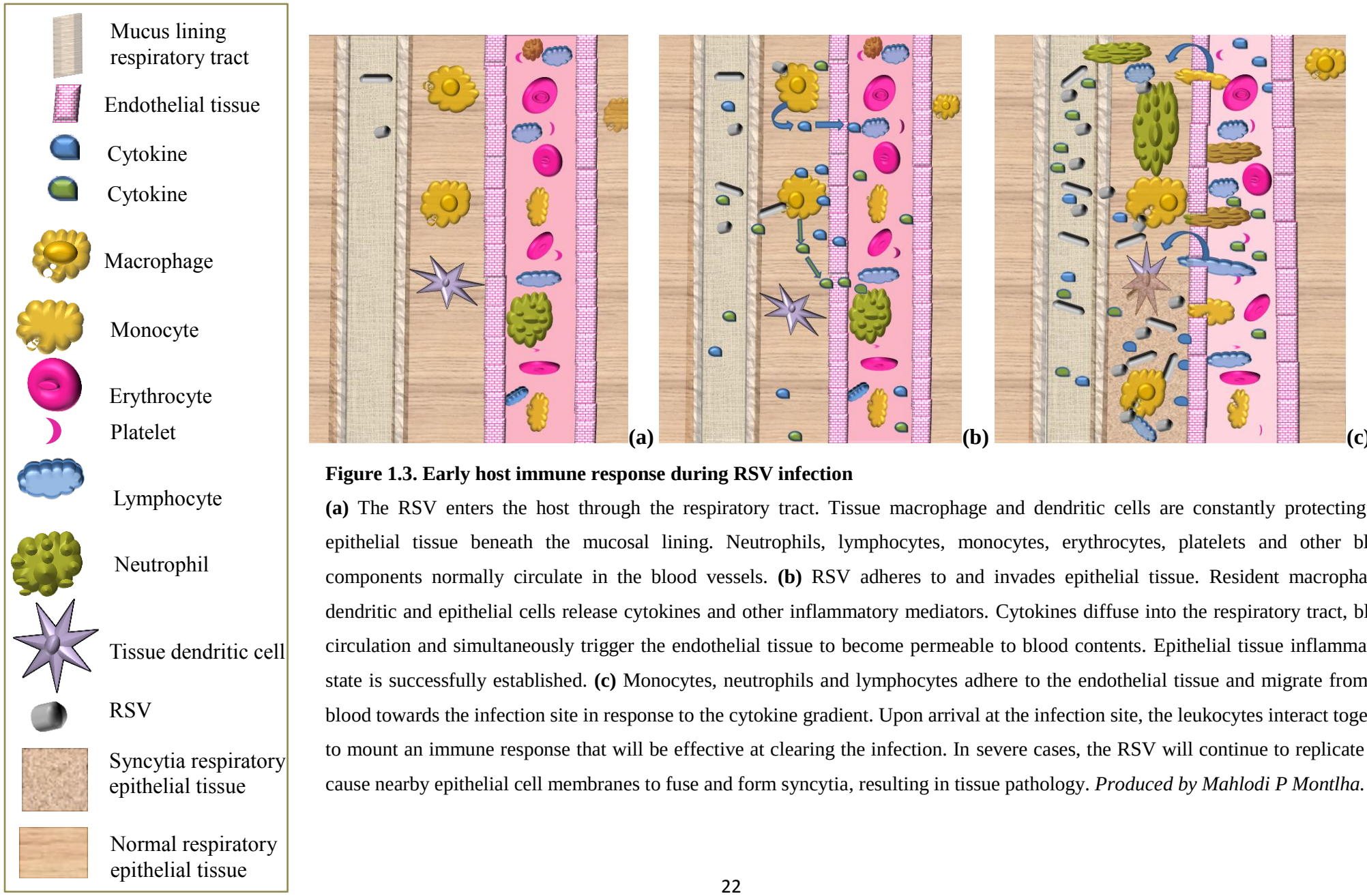


Figure 1.3. Early host immune response during RSV infection

(a) The RSV enters the host through the respiratory tract. Tissue macrophage and dendritic cells are constantly protecting the epithelial tissue beneath the mucosal lining. Neutrophils, lymphocytes, monocytes, erythrocytes, platelets and other blood components normally circulate in the blood vessels. **(b)** RSV adheres to and invades epithelial tissue. Resident macrophages, dendritic and epithelial cells release cytokines and other inflammatory mediators. Cytokines diffuse into the respiratory tract, blood circulation and simultaneously trigger the endothelial tissue to become permeable to blood contents. Epithelial tissue inflammatory state is successfully established. **(c)** Monocytes, neutrophils and lymphocytes adhere to the endothelial tissue and migrate from the blood towards the infection site in response to the cytokine gradient. Upon arrival at the infection site, the leukocytes interact together to mount an immune response that will be effective at clearing the infection. In severe cases, the RSV will continue to replicate and cause nearby epithelial cell membranes to fuse and form syncytia, resulting in tissue pathology. *Produced by Mahlodi P Montlha.*

1.9. Cytokines implicated in severe RSV infection

Cytokine is a general term used to describe a group of immune system mediator proteins (70). Interleukins are mediators between leukocytes (71) and monokines, are secreted by phagocytic cells such as macrophages and monocytes (72). Chemokines are secreted by leukocytes or other cells and cause migration of leukocytes towards the site of secretion (73) . Lymphokines are secreted by activated lymphocytes, especially helper Th1 cells-1 and Th2 cells (72). Cytokines have low molecular weight and a short half-life. They act as chemical messengers between the innate and adaptive immune systems (74, 75).

Cytokines form part of a large signalling cascade network which involves other proteins. The signals generated can protect host cells from infection and/or induce damage to host cells. Cytokines may have agonist, antagonist, or synergistic cellular effects (76). The most important characteristic of cytokines is that they are produced when they are needed; this is crucial to immune system regulation. Cytokines are produced by cells of both the innate and adaptive immune system including epithelial cells (77). The same cytokine molecule may have pleiotropic effects, and can be secreted by several cell types and also act on a variety of cell types, resulting in individual responses accordingly to the target cell. Cytokines of different origins and compositions may have overlapping functions, i.e. they may be partially redundant (76).

In broad terms cytokines can be largely classified into mediators of innate or adaptive immunity and stimulators of haematopoiesis (78). Within the mediators of innate and adaptive immunity groups there are pro-inflammatory and anti-inflammatory cytokines (Figure 1.4). Pro-inflammatory cytokines of the innate immunity include interleukin (IL), -1α, IL-1β, TNF-α, IL-12 and interferon gamma (IFN-γ) (79). Innate cells such as macrophages usually produce IL-10 as an anti-inflammatory cytokine (80), and chemokines such IL-8, macrophage inducing protein (MIP-1α), monocyte chemo attractant protein (MCP-1), and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) as attractants of different cell types from the blood circulation or lymph tissues (81). IFN-γ, TNF-α, and IL-10 are also produced by cells of the adaptive immunity.

The adaptive immunity pro-inflammatory cytokines are mostly produced by T-cells expressing the CD4+ or CD8+ markers, Th and cytotoxic T (Tc) cells (82). Helper T cells are subdivided into Th1, Th2, Th17 and T regulatory (Treg) cells on the basis of the cytokines they produce (83, 84). Th1-cytokines such as TNF-α, IFN-γ, and IL12 are crucial in initiating

cell mediated cytotoxicity immune responses largely facilitated by natural killer cells, natural killer T-cells, γδ T-cells and Tc cells (84). Th2-cytokines that are important in mediating pro-inflammatory associated with humoral immunity include IL-4, IL-13, IL-5 and IL-9 (84). IL-10 and transforming growth factor β (TGF-β) are classic anti-inflammatory cytokines of the adaptive immunity secreted mainly by Tregs (84, 85). Th17 cells produce IL-17 and are maintained by the presence of IL-23 which are strong pro-inflammatory cytokines associated with autoimmune diseases and maintain mucosal lining (86). Stimulators of haematopoiesis include granulocyte colony stimulating factor (GM-CSF) which stimulate the growth and differentiation of progenitors into monocytes and macrophages (87). These cytokines have been well studied during RSV infection and some have been associated with more severe outcomes. Table 1.1 shows a summary of the cytokines implicated in RSV-LRTI which the present study investigated.

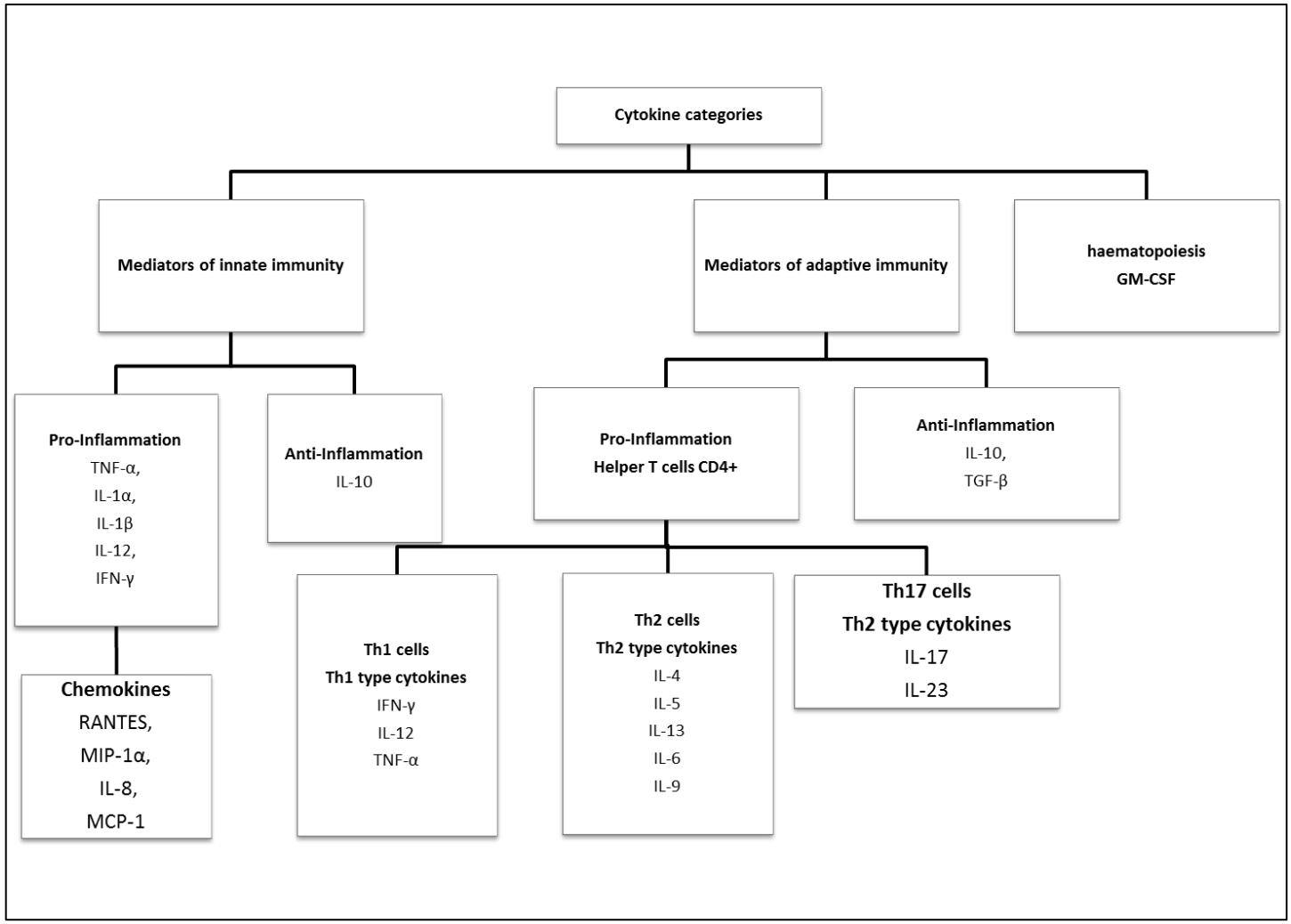


Figure 1.4. Cytokine categories

Cytokines can be grouped as mediators of innate or adaptive immunity and stimulators of haematopoiesis.

Table 1.1. Cytokines implicated in RSV disease

Cytokine	Function	Reference
IFN-γ	• Low serum levels measured in cases with mild disease • Levels increase with severity	(88)
	• High plasma levels of IFN-γ in infants with mild disease compared to severe and control groups. • High levels of IFN-γ and IL-12 reduce viral replication	(89)
IL-1α	• High concentration within nasopharyngeal aspirate was found to be related to severe disease • Recruit neutrophils to the infection site	(90-92)
IL-β	• Released by endothelial, monocytes and macrophages during RSV infection	(70)
IL-4	• Produced by peripheral T cells of infants with RSV	(88)
IL-5	• Detected in plasma of infants with severe disease • Recruits and prolongs the survival of eosinophils • Produced by CD4+ T cells that recognise RSV attachment G protein • Acts in concert with IL-4 and IL-10 to promote Th2 immune responses	(93, 94)
IL-6	• Enhances lymphocytes to differentiate into IL-17 producing cells during primary RSV infection • Increased levels found in nasopharyngeal aspirate of infected infants; enhances disease pathogenesis • Produced by alveolar macrophages and airway epithelial, and induces mucosal B cells	(95, 96)

IL-8	• Increased in nasal secretion during infection • Secretion by epithelial cells is readily induced by RSV • Attracts inflammatory immune cells that produce MCP-1 and MIP-1α • Recruit neutrophils, eosinophils, T cells to the infection site • Increased plasma level observed in severe disease	(91, 95, 97-99)
IL-9	• Stimulates mucus secretion and augment inflammatory response • Involved in pathogenesis of severe infection • Secreted by neutrophils	(100)
IL-10	• Anti-inflammatory cytokine • Up regulates anti-inflammatory mediators • Levels increase during infection • Up regulation correlated with IFN-γ	(70, 101)
IL-12	• Increased levels of IL-12 together with IFN-γ initiate defence mechanisms against RSV • Serum levels are increased in mild cases, suggesting a protective role • Production of IL-12 decreases during acute phase of infection as a result from viral inhibitory effects • Production also decreases in severe cases	(89, 102, 103)
IL-13	• Stimulate IgE synthesis • Activates and cause migration of eosinophils and basophils to infection site	(104, 105)

MCP-1	• Increased in nasal secretion during infection • Recruits monocytes during inflammation including neutrophils, basophils, monocytes, NK Cells and activated T cells • Important in pathogenesis of severe infection	(106, 107)
MIP-1α	• Increased quantities measured from severe cases • Initiates inflammatory response during infection • Induces mediator (histamine, cysteinyl leukotrienes) releases from basophils	(108-110)
RANTES	• Increased in nasal secretion during infection • Secretion by epithelial cells is readily induced by RSV	(111, 112)
TNF-α	• Produced by alveolar macrophages and airway epithelial cells • Increased levels observed in nasopharyngeal aspirates of severe infants compared to control • Induces the production of IL-6 • Recruit neutrophils and mononuclear cells to infection site • Associated with development of asthma	(96, 113)

1.9.1. Intruder alarms: TNF-α, IL-1 β and IL-1α

Tumour necrosis factor is a classic pro-inflammatory cytokine, is produced by activated antigen presenting cells such as macrophages, dendritic and B-cells in response to infection. Exposure of endothelial cells to TNF-α results in the expression of adhesion molecules like P-selectin, and causes the endothelial tissue to be permeable to the contents in the blood circulation (114). In most cases, TNF-α is the first cytokine to signal nearby cells of an infection and begins to co-ordinate the response of these cells. TNF-α is an important antiviral cytokine. The expression of TNF-α is normally followed by that of IL-1β which further augments local inflammation by inducing endothelial cells to express adhesion molecules that facilitate the migration of leukocytes from the pulmonary blood circulation to

the inflammation site (chemotaxis, Figure 1.3c) (79, 115). These immune cells will further contribute to the secretion of more pro-inflammatory or anti-inflammatory proteins at the infection site (92, 116). A recent study evaluating nasopharyngeal aspirates (NPA) from RSV infected infants reported IL-1β as a pro-inflammatory biomarker for disease severity (117).

Interleukin 1α is also a pro-inflammatory cytokine mainly produced by activated macrophages or by infected epithelial cells. In an elegant experiment, Chang *et al.* (2003) incubated endothelial cells with a cytokine-supernatant cocktail collected from an epithelial cell line inoculated with RSV. After treating the supernatant with UV radiation to attenuate the virus, the presence of IL-1α among other cytokines was investigated. This study observed that IL-1α induced the expression of adhesion molecules on endothelial cells. When IL-1α was blocked with a specific antibody, the endothelial cells failed to express adhesion molecules on their surface. This indicated that IL-1α facilitated a cascade that leads to migration of leukocytes from the pulmonary blood vessels into the airway spaces or lung tissue (92).

1.9.2. Anti-virus: TNF-α and IFN-γ

A study by Rutigliano and Graham (2004) demonstrated that TNF-α in addition to being an intruder alarm also played a role in clearing RSV infected cells in the early stages of infection (118). Elevated levels of interferon gamma (IFN-γ), a Th1 cytokine crucial in immune modulation during viral infection (119), have been reported to have anti-viral activity against RSV by stimulating the activity of Tc and natural killer cells. IFN-γ has also been implicated with RSV-associated wheezing (119). Studies evaluating IFN-γ association with severity have been contradictory; low levels of this cytokine have also been associated with the susceptibility to severe RSV disease (89). Kim *et al.* (2012) found that increased airway levels of IFN-γ during RSV bronchiolitis was associated with eosinophil inflammation and positively correlated with RANTES expression (119).

1.9.3. Mediators of attraction: RANTES, IL-8, MIP-1α and MCP- 1

Yamada *et al.* (2011) investigating the effect of Th1 and Th2 cytokines in an epithelial cell line found that the levels of these cytokines before RSV infection were important in the regulation of transcription of genes involved in the inflammatory response during RSV infection (120). In earlier studies, Domachowske *et al.* (2001) and Zhang *et al* (2001)

reported that RSV infection induced epithelial cells to secrete cytokines and chemokines involved in apoptosis responses, including IL-8, RANTES, MIP-1α, and MCP-1 (98, 121).

A study by Smyth *et al.* (2002) evaluated the nasopharyngeal secretions from infants infected with RSV, and identified increased concentrations of IL-8 and RANTES as risk factors for severe disease (122). According to that study during RSV bronchiolitis, increased levels of IL-8 resulted in accumulation of neutrophils into the alveolar space; and in most cases were directly correlated with increased white blood cell count and disease severity (122). RANTES also known as CCL5 has been detected in the nasopharyngeal secretions of infants with RSV infection and has been reported to recruit eosinophils cells to the infection site (123) resulting in airway mucosa inflammation (124). *In vitro* studies have also demonstrated that RSV induces alveolar macrophages to secrete RANTES (125).

During RSV-associated LRTI there is an increase in production of MIP-1α by the epithelial cells; this phenomenon has been observed in children with severe disease (126). It is believed that MIP-1α attracts monocytes, lymphocytes, mast cells, basophil and eosinophil cells, and possibly induces their degranulation to inhibit RSV replication (108, 127). An *in vitro* study has also shown that eosinophils can directly destroy extracellular virions (128).

MCP-1 also known as CCL2 is a monocyte chemokine that induces Th1 and Th2 inflammation. Numerous studies have found increased levels of MCP-1 both *in vitro* and in mice following RSV infection (125, 129). Clinical studies also found that levels of MCP-1 were elevated in nasopharyngeal aspirates during RSV infections, and were directly associated with disease severity (117). The expression of MCP-1 in cell lines that were previously primed with Th2 cytokines enhanced RSV replication, demonstrating that MCP-1 played a crucial role in RSV-host interaction pathogenesis (130).

1.9.4. Stimulators of cell growth and differentiation: IL-4, IL-13, IL-5 and IL-9

Interleukin 4 is a Th2 cytokine mainly produced by Th2 cells and macrophages. It mediates the development of new Th2 cells, and also stimulates the growth of differentiated Th2 cells that will activate B cells to produce antibodies (131). IL-4 promotes immunoglobulin isotype class switching, including to IgE.

IL-13 functions partially overlap with those of IL-4, including stimulating B-cell growth. Animal studies found that IL-13 was associated with reduced disease severity by preventing severe weight loss in RSV infected mice, and this was contradictory to humans (104).

Interleukin 5 is also produced by mast cells and Th2 cells (132); it stimulates the growth and differentiation of B cells, and activates differentiated eosinophils to release microbial toxic proteins (133, 134).

Interleukin 6 is a Th2 cytokine important in haematopoiesis. It facilitates the transition from innate immune response to a state of memory immune response by promoting the formation of plasma cells from B cells (135). Studies conducted by Becker *et al.* (1991) found that the interaction of RSV with alveolar macrophage led to the secretion of mediators which induced respiratory system epithelial cells to secrete IL-6 and IL-8 (136). In support to this, Jones *et al.* (2005) also suggested that alveolar macrophages in the presence of IL-6 and IL-8 inhibit RSV infection and support cellular and humoral immunity (135).

Interleukin 9 has pleiotropic effects; it has Th2-like functions and is important in stimulating activated Th2 cells to proliferate. Additionally, its secretion enhances the production of IgE from B cells and promotes the proliferation of haematopoiesis progenitors and mast cells. IL-9 has a synergic relationship with IL-1β and TNF-α. The latter cytokines act independently to stimulate the release of IL-9 by T-cells but this is not observed when both IL-β and TNF-α are present (115). IL-9 has been shown to be released by peripheral human blood eosinophil and modulates antiviral immunity during RSV infection by regulating T and B cells responses (137).

1.9.5. Immune regulator: IL-10

Interleukin 10 is an anti-inflammatory cytokine mainly produced by activated Th2 cells and macrophages. IL-10 can inhibit co-stimulatory signals required at the immunological synapse, the expression of human leukocytes antigen class II and cytokine production by activated macrophages. This total effect can regulate the strength of the immune response. IL-10 production may also inhibit the production of IFN-γ secreted by Th1 cells and thus favour Th2 cells to mount an immune response (138). When analysing nasopharyngeal aspirates of RSV infected infants, Murai *et al.* (2007) observed a synergistic role between IL-10 and RANTES. The levels of IL-10 and RANTES were elevated in RSV infected infants compared to the RSV uninfected group but association with severity was not reported (139).

1.10. Cytokines as potential biomarkers of RSV disease severity

During RSV pathogenesis, the levels of several host cytokines have been shown to be either increased or decreased; however whether cytokine levels have a protective role in disease progression remains controversial (16). There is a growing interest in evaluating cytokines as biomarkers of disease severity. A biomarker in this context is any measurable molecule (such as cytokines, complex carbohydrates, growth factors, lipids or hormones) that can be distinctively associated with a physiological change or pathological process (140). Biomarkers have been successfully used for prognosis and diagnosis of some autoimmune diseases, cancers and infectious diseases. These molecules can be detected and quantified from biological samples such as plasma, serum, nasopharyngeal aspirates or cell culture supernatant (141). Perturbations of cytokine levels are sometimes associated with a pathological response. Understanding the complex cytokine environment can serve as biomarker for disease severity (142, 143), and guide vaccine design and medical intervention strategies (144).

In South Africa cytokines associated with severe RSV infection have not been studied previously, and are most likely different from those documented from the Americas, Europe or Asia. Sub-Saharan Africa has a high burden of HIV and other comorbidities, as well as more prevalent malnutrition, all of which may play a role as risk factors of severe RSV infection. Cytokines can be quantified with immunoassays such as enzyme-linked immunosorbent assay (ELISA) (145) and Luminex assay (146). Luminex assays use microscale "ELISAs" on magnetic beads to detect and quantify molecules using a flow cytometer based detector (147). It allows evaluation of multiple cytokines simultaneously per experiment in contrast to single-plex ELISA (143, 146, 147).

1.11. Study rationale

Many infants with RSV-LRTI develop mild symptoms and do not need prolonged hospitalization or therapeutic support. Other infants after RSV infection they progress to severe illness necessitating prolonged hospitalizations and even intensive care admission. There are currently no biomarkers able to predict the disease progression and outcome of infants with RSV-LRTI. Host immune responses during the progression of RSV-LRTI could be important in determining the severity and outcome of the disease, and might serve as potential biomarkers. Several studies have in the past reported associations between cytokines and severe RSV-associated LRTI. The majority of the cytokines associated with RSV-LRTI

severity have been studied in non-African populations, to our knowledge this was the first study to evaluate these cytokines in African infants. Additionally we wanted to evaluate if the cytokines can predict severe RSV-associated LRTI in the South African population.

Recent studies by Nédélec *et al* (2016) and Quach *et al* (2016) have shown that the African population have a strong inflammatory response towards pathogens as compared to the European population (148, 149). This shows there is a need for the cytokines to be studied in the South African population, rather than incorporating into our intervention strategies the cytokine results obtained from populations of different ethnicity.

The aim of this study was to measure the levels of key cytokines previously implicated in RSV-LRTI and assess their potential as biomarkers for discriminating severe from mild RSV-associated LRTI in infants <12 months during the annual RSV epidemic season. Additionally we wanted to understand the immune response etiology involved in RSV-LRTI or mediating RSV-LRTI severity.

Study Objectives

a) Evaluate any association between RSV-associated LRTI severity and cytokine levels in the blood and nasopharyngeal aspirates.
b) Compare the cytokine profile in RSV associated LRTI cases to controls children without evidence of respiratory tract infection.
c) Evaluate the correlation between cytokine levels in the blood and in nasopharyngeal aspirate samples among RSV-LRTI cases.

Chapter 2: Materials and Methods

2.1. Ethics clearance and approval

Permission to conduct this study was obtained from the Human Research Ethics Committee (HREC) of the University of the Witwatersrand-Medical, Johannesburg, South Africa, with the clearance number: M160872. The clearance certificate is included as appendix A (Figure 1). This study was carried out according to the HREC guidelines. Signed informed consent was obtained from the parents or legal guardians of the participants of the study "Surveillance on pathogen-specific causes of pneumonia and diarrhoea hospitalization in children" (abbreviated as the "BoSS" study) before enrolment. The main study was an active surveillance study evaluating the burden of different respiratory pathogens such as RSV, *Bordetella* species, influenza and human metapneumovirus in hospitalized children. Clinical records and laboratory specimens from study participants were identified by a unique study identity number allocated during enrolment. Hard copy records were stored in a secured place, while electronic capturing of information was carried out with coded numbers only.

2.2. Study population and inclusion/ exclusion criteria

The study was conducted at Chris Hani Baragwanath Academic Hospital (CHBAH) located in the Soweto area in the city of Johannesburg, Gauteng, South Africa https://www.chrishanibaragwanathhospital.co.za/.

Participants were enrolled from 15 March 2016 to 29 August 2016. This study included infants between 0-12 months of age who were admitted at CHBAH. Potential case participants were identified as infants presenting with lower respiratory tract infection (LRTI). A convenience sample of control participants were infants scheduled for elective surgery with no respiratory symptoms or respiratory illness. Inclusion criteria for cases:

a. Child under 12 months of age admitted to the paediatric medical ward at CHBAH AND
b. In children under 3 months of age: Any child with diagnosis of suspected sepsis or physician diagnosed LRTI irrespective of signs and symptoms
c. In children above 3months of age: Admitting diagnosis of acute pneumonia or other physician diagnosed LRTI

   AND

   Symptom onset less than 7 days prior to hospitalisation

Soweto has a high prevalence of HIV. As a consequence HIV infected and exposed infants were included in this study. Table 2.1 below summarises the HIV status of the control infants and RSV cases.

Infants were excluded from the study if they were receiving corticosteroid, had congenital heart disease or had an underlying medical condition at the time of enrolment. Enrolled infants that met case definition but tested positive for other respiratory pathogens (*Bordetella* species, influenza and human metapneumovirus) other than RSV were also excluded from the study. For both case and control infants who had no paired plasma and NPA samples were excluded. Figure 2.1 shows the study design.

After the consenting progress Respiratory and Meningeal Pathogens Research Unit (RMPRU) study staff, administered the study questionnaire and collected the study related samples. Following collection, samples were immediately transported on ice to the RMPRU laboratory.

Table 2.1 HIV status of the control infants and RSV cases

HIV Status (N)	**Control n=31**	**Mild n=89**	**Severe n=113**
HIV Uninfected			
Unexposed	22	73	82
Exposed	9	9	10
Unknown exposure	-	2	9
HIV Infected	0	5	12

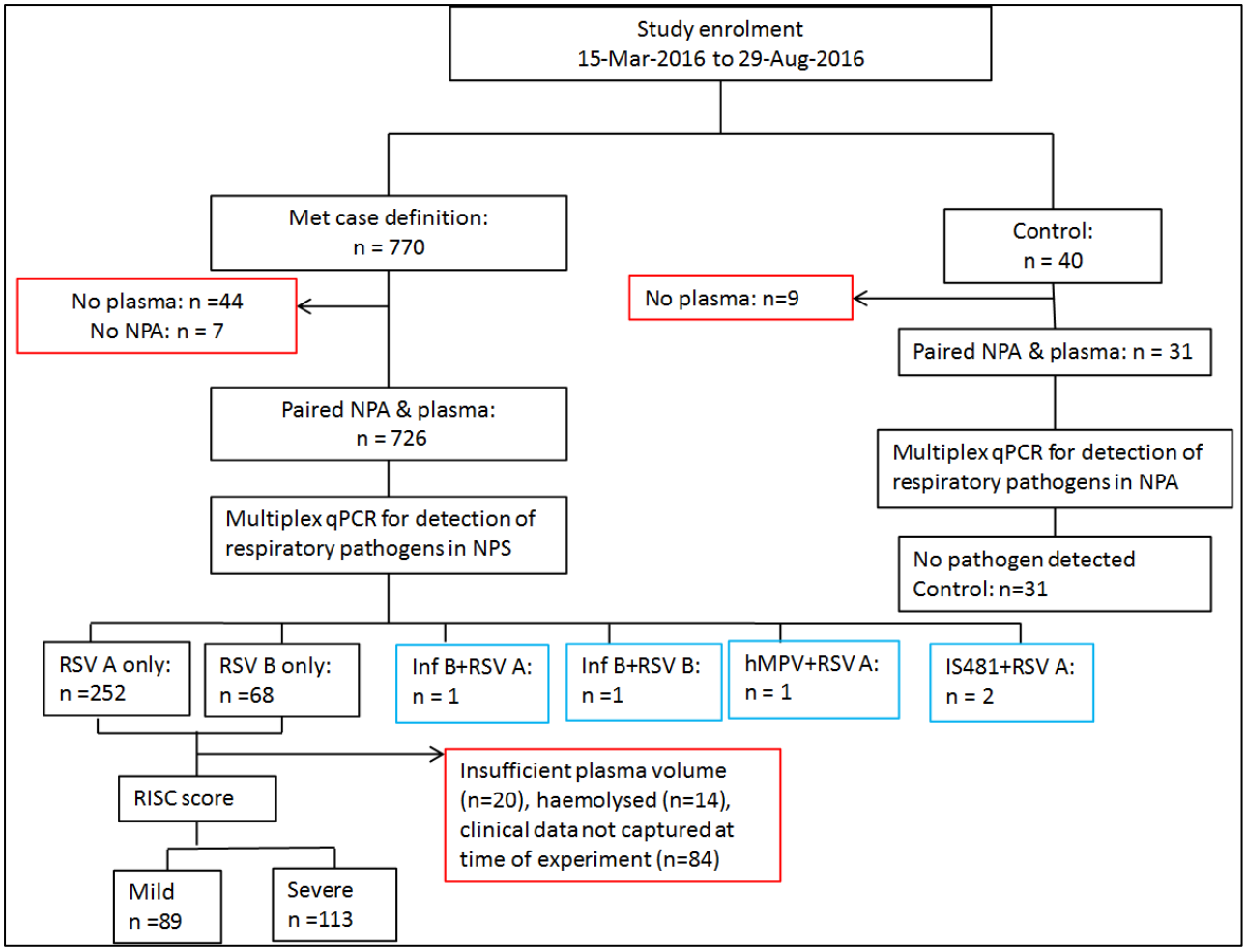


Figure 2.1. Study design

Participants that were enrolled and subsequently excluded from the current analysis are shown in red. The samples were excluded as a result of unpaired sample, insufficient plasma volume, uncaptured clinical data before cytokines were measured. The blue rectangles show the number of participants who had RSV coinfections and were also excluded.

2.3. Scoring RSV-LRTI severity

Infants with RSV infection were divided into two groups: i) mild and ii) severe disease, based on the previously described Respiratory Index of Severity in Children (RISC) Score (150). The first group included hospitalized infants with mild LRTI who had a RISC score from -2 to 3 inclusive. The second group included infants with severe LRTI with a RISC score above 3. HIV-unexposed-uninfected and HIV-exposed-uninfected infants were scored using RISC criteria for HIV-uninfected patients. HIV-infected infants were scored using the RISC score criteria for HIV-infected. The clinical RISC score algorithm is included as appendix C. Although there are other clinical scores that have been used to score RSV disease severity (151, 152), the RISC score was developed in the context of South African infants hospitalized for LRTI, incorporating HIV-infection and nutritional status.

2.4. Sample collection

Nasopharyngeal swabs (NPS) and nasopharyngeal aspirates (NPA) were collected into universal transport medium (UTM; Copan, Brescia, Italy) during hospitalization – of the participants. NPS or NPA were used for testing of respiratory pathogens including RSV A and B using an in-house 1-step multiplex real time polymerase chain reaction (PCR) assay. Only NPA were collected from control participants. Total nucleic acids were extracted from NPS or NPA using an automated nucleic acid extraction machine, NucliSENS® easyMAG® (BioMerieux, France). The in-house PCR assay was setup to test for RSV A and B, influenza A and B, human metapneumovirus and *Bordetella* species. Positive controls for pathogens as well as RSV A and B were included in each multiplex PCR reaction, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a human internal control. Children who tested positive for RSV A or B in the PCR (i.e. quantitation cycle value $\leq$35) were classified as cases. RSV cases had no PCR confirmed coinfection with other pathogen that we investigated for; i.e. as we tested limited number of respiratory pathogens, we cannot be conclusive that we excluded all coinfections. Surgical participants who tested positive for any of the respiratory pathogens tested in the PCR assay were excluded from the study. Furthermore, during RSV season (when approximately 70% of LRTI are due to RSV), children enrolled into the main BoSS study also consented to have a blood draw (collection of 2-3 ml). All control children had a blood draw at enrolment. Paired RSV (A or B) positive NPA and whole blood samples were used to evaluate the cytokine profile of the hospitalized infants.

2.4.1. Plasma sample preparation

Approximately 2-3 ml of whole blood was collected into ethylene-diamine-tetra-acetic-acid (EDTA) containing tubes following venepuncture. EDTA is an anticoagulant that maintains whole blood in liquid form and protects easy to denature blood components such as cytokines (153). Following blood collection, the tube was inverted several times to mix whole blood and EDTA. Samples were transported to the RMPRU laboratory in a cooler bag for processing within 1 hour.

Upon arriving at the laboratory, samples were verified not to have haemolysed; haemolysed samples were not used for the experiment. Whole blood samples were centrifuged at 3320 revolutions per minute (rpm) for 15 minutes at 4˚C using a bench top centrifuge (Eppendorf, United kingdom) to separate the whole blood sample into plasma, buffy coat, and erythrocytes layers. The plasma, the first layer, was transferred into a sterile polypropylene tube, and centrifuged again at 3320 rpm for 10 minutes at 4˚C to completely remove remaining precipitates and platelets. Approximately 250 µl of plasma was aliquoted into each of the three cryovials for single-use to prevent freeze thaw cycles. Plasma samples were stored at -70˚C until further testing.

2.4.2. Nasopharyngeal aspirate sample preparation

Nasopharyngeal aspirates were collected by introducing a suctioning tube into the nasopharynx and approximately 1.5ml of sterile saline was instilled. The suctioning tube was connect to mucous trap and fitted to a syringe. The aspirate was suctioned and collected into a sterile tube containing 3ml of Universal transport medium (UTM) (see below for details). The samples were immediately transported to RMPRU laboratory for processing within 3 hours. NPA were centrifuged at 3320 rpm for 15 minutes at 4˚C to pellet the cells using a bench top centrifuge (97). The supernatant was aliquoted into three cryovials to avoid unneeded thaw cycles of samples as this may damage the cytokines. The supernatant was stored at -70˚C until testing.

Universal transport medium is an isotonic solution, non-toxic to human cells and maintains the viability of viruses; it is formulated with Hank’s balanced salt solution, supplemented with bovine serum albumin, sucrose, cysteine and glutamic acid. Furthermore UTM contains the antibiotics vancomycin, amphotericin and colistin which minimise the growth of

competing yeast, fungi and bacteria. UTM is buffered with HEPES buffer at pH 7.3 ± 0.2 at room temperature and contains phenol red to indicate any pH deviation (154).

2.5. Quantitative measurement of cytokines using commercially available Luminex assay

A commercial custom made multiplex bead immunoassay "Human Cytokine 15-Plex" Kit Catalogue Number: LHB001M and LCP000SA (Invitrogen, Life Technologies Corporation, California USA) was used to measure the cytokine levels in the study samples. The kit was designed to detect and measure the following human cytokine panel: IFN-γ; IL-1α; IL-1β; IL-4; IL5; IL-6; IL-8; IL-9; IL-10; IL-12(p70); IL-13; MCP-1; MIP-1α; RANTES; TNF-α These cytokines were selected based on the literature (Table 1.1). This kit included: validated cytokine-specific antibody bead concentrate, wash solution, biotinylated antibody and biotin diluent, 15-Plex cytokine standards (concentrations shown in Table 2, appendix D), streptavidin-PE concentrate and diluent, assay diluent, incubation buffer, and a 96-well flat bottom plate. All reagents were stored at 4˚C and brought up to room temperature before use. Cytokines levels were measured and quantified on a Luminex xMAP instrument (Bio-Rad Laboratories Inc., USA). Step-wise details of reagent preparations are reported in appendix D and for Luminex® instrument setup are reported in appendix F. Kit manufacturer instructions were closely followed during the study and amendments were noted and reported. All assays were conducted at RMPRU.

2.5.1. Principles of Multiplex Bead Immunoassays with Luminex

Multiplex bead immunoassays allow simultaneous detection of multiple cytokines from one sample using fluorescent dyed microspheres. The principles of this technique are based on sandwich ELISA. The microspheres are magnetic beads each containing a unique spectral property that permits discrimination of one bead from another. Magnetic microspheres were covalently cross-linked to a monoclonal capture antibody; the antibodies are specific for each cytokine of interest.

A flat bottom 96-well plate, designed for use with a magnetic handheld washer, was coated with 25µl of 1X magnetic microspheres conjugated with primary antibodies against 15 cytokine panel. The coated plate was washed twice with 200µl of 1X wash solution using the handheld magnetic washer (Bio-Rad Laboratories Inc., USA). Incubation buffer (50µl) was added to all wells. This was followed by the addition of test samples (50µl of plasma or 70µl

of NPA diluted with 50µl of assay diluent) or cytokine standards of known concentrations in assay diluent. The first 2 wells of the plate served as blank samples and no test sample or cytokine standard was added. Cytokine standard, test and blank samples were setup in duplicates. This multiplex assay suspension was incubated at room temperature in the dark on an orbital shaker (Daigger Scientific, USA) (500 rpm) for 2 hours. This allowed the cytokines of interest in the samples to react and bind with the specific primary antibodies coupled to the magnetic microspheres. The incubation period was followed by a series of washes (always using the handheld magnetic washer appendix E) to remove unbound cytokines.

1X biotinylated secondary detection antibody (100µl) was added to the plate and, incubated at room temperature in the dark on an orbital shaker for 1 hour to react with the cytokine monoclonal capture antibody. This resulted in a sandwich complex. The biotinylated secondary antibody serves as an amplification step that intensifies the detection signal and allows the visualization of the antibody binding to the cytokine of interest.

This was followed by washing off unbound biotinylated detection antibody and adding 100µl of 1X streptavidin conjugated to phycoerythrin fluorescent protein (SA-PE conjugate) to form a detection complex which was incubated at room temperature in the dark on an orbital shaker for 30 minutes. At the end of this incubation period the liquid was decanted and the assay plate was washed three times. 1X wash solution (150µl) was added to each assay well, covered and incubated in the dark on an orbital shaker at 500 rpm for 3 minutes. The plate was uncovered and loaded onto the Luminex 200 platform and the assay was acquired to quantify the specific cytokines.

The biotin SA-PE conjugate is the preferred detection method for molecules such as cytokines as they are occasionally expressed at low levels that cannot be detected using solely labelled antibody. The signal amplification takes advantage of the strong affinity that streptavidin protein has for the tetrameric biotin. Four SA-PE conjugates bind to a single biotinylated antibody and the phycoerythrin fluorophore serves as the reporter signal. Cytokine standard proteins provided with the kit generated a range of concentrations during the assay to allow extrapolation of cytokine levels within the test samples.

During data acquisition the multiplex assay suspension was drawn from the plate into the Luminex instrument. A red laser of 635nm interrogated the spectral property of each microsphere to identify the cytokine, while the green laser of 532nm simultaneously excites PE linked to streptavidin to generate a reporter signal that is detected by the photomultiplier

tube (PMT). Bio-Plex Luminex instrument employs modified flow-cytometry-based laser technology and is fitted with a high speed digital processor to generate a comprehensive data output. The Bio-Plex Manager Software version 5 (Bio-Rad Laboratories Inc., USA) reports the data as mean fluorescence intensity (MFI) and automatically makes the conversion to concentration (pg/ml). The concentration of each cytokine is proportional to the MFI of the PE (147).

2.5.2. Standard curve dilution matrix for NPA assays

Tabarani *et al* (2013) studying cytokines in NPA from children infected with RSV causing severe illness also used a commercial multiplex bead immunoassay similar to that used in our study (117). The kit used in this study was validated and optimised for cytokine detection from serum, plasma, and tissue supernatant; but it was not optimised for cytokine detection in NPA. Certain aspects of the kit had to be adjusted for detection of cytokines from NPA collected into UTM. During NPA sample assay, the standard vials were reconstituted with 50% assay diluent (provided by manufacturer) and 50% UTM. This facilitated the diluted standards to match the NPA samples dilution, since the NPA was collected into UTM. Both the standards and the NPA samples were therefore diluted in the identical buffer preparation.

2.5.3. Estimation of dilution factor introduced during NPA collection

Collection of NPA by direct aspiration without using any fluid does not provide enough samples to perform clinical assays. Also fluid such as saline solution is used during aspiration to reduce discomfort associated with aspiration of nasal secretions. While this favoured accumulation of adequate assay volume; there is an unknown dilution factor which is introduced during NPA collection. It is important to correct for this dilution factor when reporting cytokine concentrations to prevent the distortion of results with clinical significance (155). During this study, the NPA was collected into 3ml UTM using 1.5ml 0.9% saline. The dilution factor that was introduced during NPA collection was estimated to be 1.25-fold using the formula below:

$$Introduced\ NPA\ dilution\ factor = \frac{Total\ volume\ of\ fluid}{total\ volume - 1\ ml}$$

$$Estimated\ total\ volume = 1.5\ ml\ saline + 0.5\ ml\ NPA + 3\ ml\ UTM = 5\ ml$$

$$Introduced\ NPA\ dilution\ factor = \frac{5}{5-1}$$

$$Introduced\ NPA\ dilution\ factor = 1.25\ fold$$

This formula was obtained from Heikkenen et al. (1999) (155).

During assay preparation 70µl of NPA was diluted with 50µl of assay diluent (provided with the kit) resulting in 120µl total volume of NPA loaded into assay wells. The dilution factor during sample loading into assay wells was estimated as 1.71-fold as shown by the equation below.

$$Dilution\ factor\ during\ sample\ loading = \frac{Volume\ of\ \ NPA\ in\ assay\ diluent}{NPA\ volume}$$

$$Dilution\ factor\ during\ sample\ loading = \frac{120\ \mu l}{70\ \mu l}$$

$$Dilution\ factor\ during\ sample\ loading = 1.71\ fold$$

The total dilution factor of each NPA sample was approximated as 2.14-fold using the formula below.

$$Final\ NPA\ dilution\ factor = (Introduced\ dilution\ factor)(Dilution\ factor\ during\ sample\ loading)$$

$$Final\ NPA\ dilution\ factor = 1.25\ X\ 1.71$$

$$Final\ NPA\ dilution\ factor = 2.14$$

The original cytokine concentration within the nasal cavity was obtained by multiplying the measured cytokine concentration by the *Final NPA dilution factor*. Similarly, during an assay preparation 50µl of plasma was diluted with 50µl of assay diluent (provided with the kit) resulting in 100µl total volume of plasma loaded into assay wells. The dilution factor during

sample loading into assay wells was estimated as 2-fold. The original plasma concentration was obtained by multiplying the measured concentration with 2-fold.

2.6. Assay reproducibility

Inter assay variation was used determine the assay reproducibility. Inter assay variation for each plasma or NPA assay was determined by testing independent duplicate of prepared positive control samples. For each cytokine studied, a Levey Jennings quality control chart was generated to assess reproducibility of the results as per day to day variation.

2.6.1. Preparation of positive control samples for plasma and NPA assays

Whole blood used to prepare positive control samples for the plasma assay was donated by a single non-respiratory illness healthy adult. Ten vials with 100µl of plasma were prepared according to section 2.4.1. One plasma vial was thawed and included as plate control during each plasma assay. During the preliminary analysis of the results from plasma plate 1 (which was the first experimental plate), it was noted that some cytokines in the positive control sample were expressed at undetectable or reduced levels. As a consequence of these results, the remaining nine vials were retrieved from the freezer (handled on dry ice) and spiked with 60µl of reconstituted cytokine standard of known concentration provided with the kit and returned for storage at -70˚C.

NPA assay control samples were obtained from a single control participant. Ten vials of 100µl NPA sample (prepared according to section 2.4.2) were spiked with 50µl of reconstituted cytokine standard of known concentration provided with the kit before being stored at -70˚C. One NPA vial was thawed and included during each NPA assay as plate control.

2.6.2. Generation of Levey Jennings quality control charts

Control samples for plasma and NPA spiked with known concentrations of cytokine standards were used to develop Levey Jennings quality control charts for each cytokine of interest as a measure of inter assay variability. The Levey Jennings rule assumes that the cytokines within the different vial aliquots of either plasma or NPA are stable and have minimum cytokine concentrations variation. Measuring cytokines from these single use aliquot samples during each assay over the period of the study has the ability to characterise technical errors or assay imprecisions (156). A sample of a given concentration would

naturally give different mean fluorescence intensity (MFI) values for different plates. Sometimes this difference is substantial, and it is corrected for by running a standard curve on every plate. MFI values for samples tested on different plates are expected to vary. It is therefore appropriate to generate the Levey Jennings on the output concentrations, which we expect to be similar for the same sample and not on the MFI. A quality control chart for each of the 15 cytokine was prepared for both plasma and NPA. The chart features the cytokine concentration on the *y*-axis and the assay plate number on the *x*-axis. A horizontal line was drawn at the cytokine mean, lower and upper limits. The limits included -2 and +2 standard deviation ($\bar{x}$ ± 2SD), and $\bar{x}$ ±3SD which were calculated using the measures generated from all the plasma or NPA control samples. Finally the individual cytokine concentration from each assay plate was plotted on the chart. A stable and precise assay will produce cytokine concentration within the $\bar{x}$ ± 2SD lower and upper limit. An assay was rejected when cytokine concentration from the control sample exceeded the $\bar{x}$ ± 3SD control limits. Cytokine concentration from the control that exceeded $\bar{x}$ ± 2SD limits during an assay warranted further investigation before the assay results could be accepted or rejected.

2.6.3. Levey Jennings assessment criteria for cytokine concentration exceeding ± 2SD

A control sample with cytokine concentration between the $\bar{x}$ ±2SD and $\bar{x}$ ±3SD control limits was considered "suspicious" (156). Suspicious cytokine concentration obtained from control samples were analysed using the Levey Jennings assessment criteria. The criteria were applied to determine the need to reject and repeat the assay or to accept and report the assay results. An assay that was suspicious was tested for analytical systemic error with assessment criteria (explained below) and also tested for random error with assessment criteria. An assay violating any of these rules was rejected. According to Westgard (1981), the laboratory should decide which rules are significant for the assay and laboratory conditions (156). The assessment criteria that were applied during this study were the following:

a) Systematic error test: 2_{2SD}

The assay was rejected provided that 2 control samples from consecutive assays exceeded the $\bar{x}$ ±2SD control limit. This rule was applicable even though a new aliquot of control sample (from same batch as previous assay run) was used in the consecutive assay.

b) Systematic error test: 4_{1S},

This rule rendered the assay to be rejected when four consecutive assay runs exceeded the $\bar{x}$ ±1SD. This was acceptable since control samples of the same batch were used and there was no change in control sample batch during this study. In total there were 7 control samples for all plasma assays and 7 control samples for all NPA assays; therefore the rule could only be applied starting from the 5th plasma or NPA assays.

c) Random error test: 1_{3S}

This is the optimal rejection criteria, $\bar{x}$ ±3SD control limit. A control observation that exceeds this limit was rejected and it was often due to undeterminable random analytical errors. The occurrence of this error should be an alert signal for assays to follow.

In summary, these assessment criteria were used to assist resolve ambiguity when cytokine concentration in the control sample exceeded the $\bar{x}$ ±2SD control limit.

2.6.4. Inter-assay precision

The cytokine concentrations observed from the control samples were within the Levey Jennings control limits (appendix H) for most cytokines with exception for RANTES NPA plate 4. Figure 2.2 below shows inter assay variation for RANTES concentration over 7 NPA assays. This Figure shows that 6 out of 7 RANTES control observations were within the $\bar{x}$ ±1SD control limit. The control observation on plate 4 exceeded however the $\bar{x}$-2SD control limit. This warranted further interpretation before RANTES assay results from plate 4 were accepted or rejected.

According to rule $2_{2SD,}$ the assay should be rejected provided that 2 concentration readings of the RANTES from 2 previous consecutive assays exceed the $\bar{x}$±2SD control limit. The RANTES concentration on plate 2 and 3 observations were both within the $\bar{x}$±2SD control limit, therefore this rule was not violated. The second test 4_{1SD} was also applied. 4_{1SD} rule renders the assay to be rejected when 4 previous consecutive assays runs exceed the $\bar{x}$±1SD. This rule was also not violated because the concentration from plate 1, 2 and 3 were within the control limits. Additionally, test 1_{3SD} was also not violated because plate 4 concentrations did not exceed the optimal rejection criteria, $\bar{x}$±3SD control limit. Taking all this into consideration the RANTES results on plate 4 were accepted as none of the Levey Jennings decision criteria tests were violated.

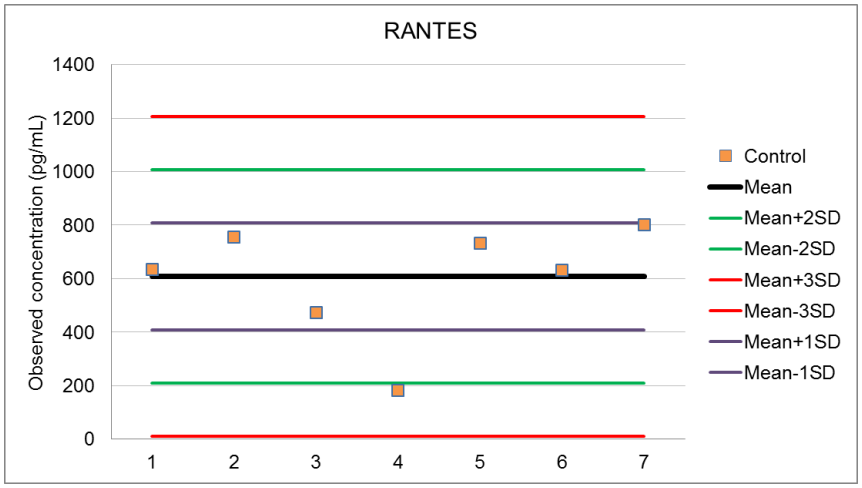


Figure 2.2. MFI Inter assay variability for RANTES over 7 NPA assays

2.7. Sensitivity of the Luminex assay and curve fitting algorithm

The limit of detection (LOD) for each cytokine from either the plasma or NPA assay was calculated as the average of recorded blank sample MFI plus 2 standard deviations. The blank sample included all assay reagents but no plasma, NPA or standard (S) was added to it. MFI measured from the blank sample could have been a result of fluorophores not bound to specific target or from auto fluorescence of reagents used during the assay preparation.

LOD was defined as the lowest cytokine concentration that could be reliably distinguished from the blank sample MFI. The upper limit of detection was not calculated; any sample with cytokine concentrations above S1 would had to be diluted and re-tested so that the concentration could be estimated within the reliable standard curve and later corrected for the dilution factor. During this study all samples' cytokines were estimated within the standard curves.

Table 2.2 depicts the LOD for cytokines. The average standard curve equation estimated by the Bio-Plex software manager was used to calculate the LOD concentration. The average MFI for the blank sample and standard deviation was calculated for the plasma and NPA assays. For each cytokine 2 standard deviations (+2SD) were added to the calculated average MFI of the blank sample. The LOD for each of the 15 cytokines was determined as the concentration at the "average MFI +2SD", and it was obtained by solving the 5 parameter logistic regression (PL) curve equation for "X" following substitution of average "MFI (+2SD)" into the equation. Bio-Plex software manager generated 5PL curve for each cytokine standard curve;

$$y = d + \frac{a - d}{[1 + \left(\frac{X}{C}\right)^{b}]^{g}}$$

Where: a = estimated response at zero concentration

b = slope factor

c = mid-range concentration (C_{50})

d = estimated response at infinite concentration

g = asymmetry factor

y = average MFI +2SD

A 5 parameter logistic regression equation is a commonly used curve fitting method for estimating concentrations from a standard curve, i.e. plot of concentration versus fluorescence intensity response in immunoassays. The equation generates a well fitted curve model for standard curves that display symmetrical or asymmetrical MFI responses; Figure 2.3 shows an example of a typical curve generated with the Bio-Plex Manager software.

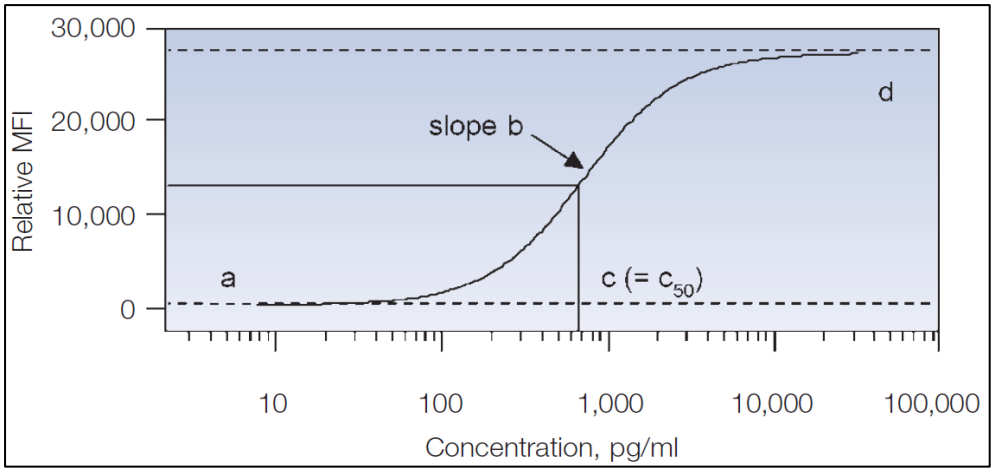


Figure 2.3. Example of Bio-Plex cytokine standard curve.

From "Bio-Plex™ suspension array system" technical note (157).

Participants with cytokine concentrations that were below the LOD were assigned an arbitrary value that was ($\frac{1}{2}\ LOD$) of the specific cytokine to facilitate data analysis. The calculations of LOD for each cytokine in both the plasma and NPA assays are detailed in appendix G.

Table 2.2 Limit of detection for cytokines in plasma and NPA samples

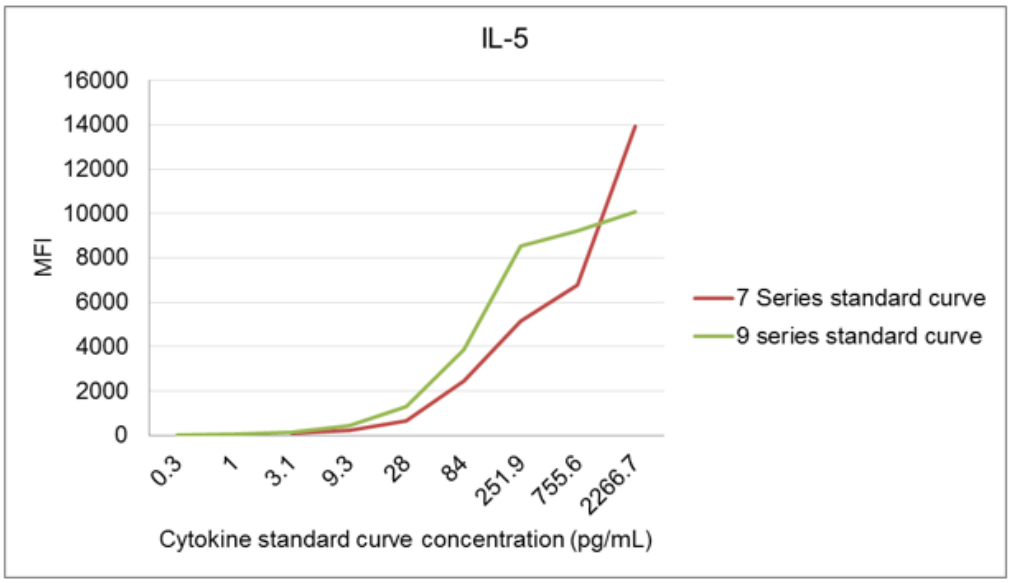
Cytokine	Limit of detection (pg/ml)	
	Plasma	**NPA**
IFN-g	1.0	1.1
IL-1α	8.9	7.8
IL-1β	31.7	17.3
IL-4	3.2	4.6
IL-5	0.2	0.6
IL-6	0.6	0.7
IL-8	3.1	2.6
IL-9	2.0	1.2
IL-10	0.6	0.8
IL-12p70	10.0	15.7
IL-13	1.6	2.9
MCP-1	31.8	35.1
MIP-1α	6.3	6.7
RANTES	6.6	5.3
TNF-α	0.5	1.0

2.8. Plasma and NPA assay standard curves

The protocol included with the detection kit suggested that the standard curves should be prepared as 7 series (S1 to S7) in three-fold dilution. This was followed for the first 4 plasma assay plates. Analysis of the results from the first four plasma assay plates showed that the 7 series standard curve did not reach a plateau (estimated MFI response at infinite concentration) and had an exponential shape. The remaining 3 plasma assay plates and all 7 NPA assay plates were assayed with a 9 series standard curve, which generated a sigmoidal shaped curve. Figure 2.4 shows an example of 7 and 9 series standard curve projection for IL-5 from plasma assay; similar projections were observed for other cytokines. The bottom window of Figure 2.4 is a magnified lower end of 7 and 9 series standard curves respectively. The 7 series standard curve has a high MFI at S7 compared to the 9 series standard curve. The lowest IL-5 concentration that could be interpolated from the plasma samples with a 7 and 9 series standard curve were 3.1pg/ml and 0.35pg/ml respectively.

Analysis of immunoassays using the Bio-Plex manager software can result normally in 3 types of standard curve response; low-response, high response or sigmoidal response curve as shown in Figure 2.5 (157). The 7 series curves produced a high response curve (Figure 2.5b) which facilitated quantitation of high cytokine concentration from the samples. Cytokines that were expressed at low levels were reported by the Bio-Plex Manager software as under range. These could have been incorrectly assigned as below the LOD, had the standard curve not been diluted to S9.

Sometimes a high response standard curve is not physiologically useful since most cytokines even when expressed at low concentrations have the ability to trigger a physiological response during disease (158). Analysis of the generated standard curves showed that this protocol adjustment yielded cytokine standard curves that more properly covered the physiological range of cytokine concentrations that were measured in this study (i.e. sigmoidal curve). Extending the lower end of the standard curve from S7 to S9 presumably permitted cytokines concentrations with MFI values extending below the S7 of 7 series curve to be more accurately quantified from the samples that would have been reported as undetectable.



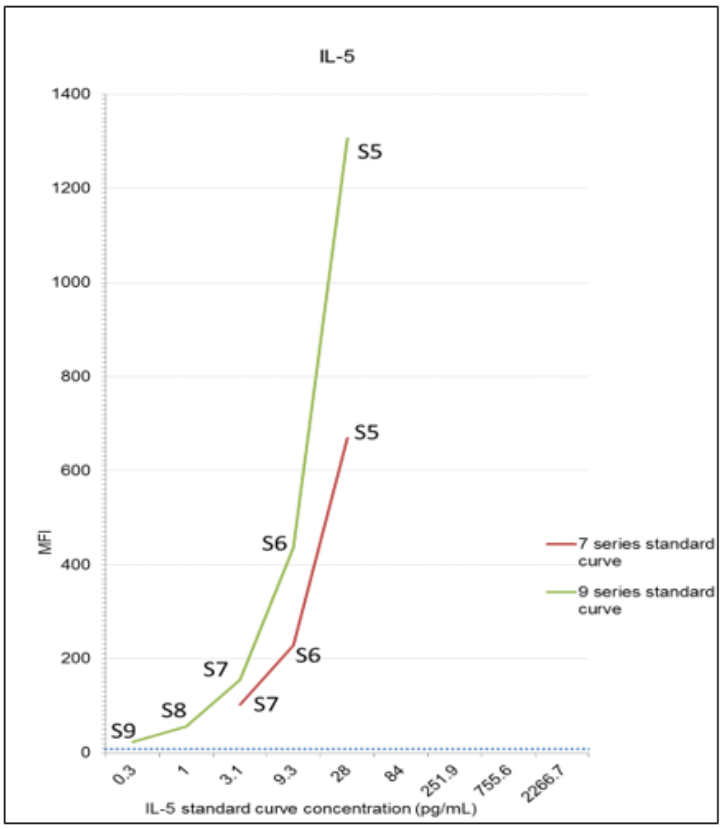


Figure 2.4. IL-5 standard curves

Red curve shows a typical 7 series suggested by the protocol and green curve shows a 9 series standard curve of three-fold dilutions. The bottom window shows a magnification of the upper window and it compares the low end of S9 and S7 series standard curves. The blue dotted line shows the background MFI for IL-5.

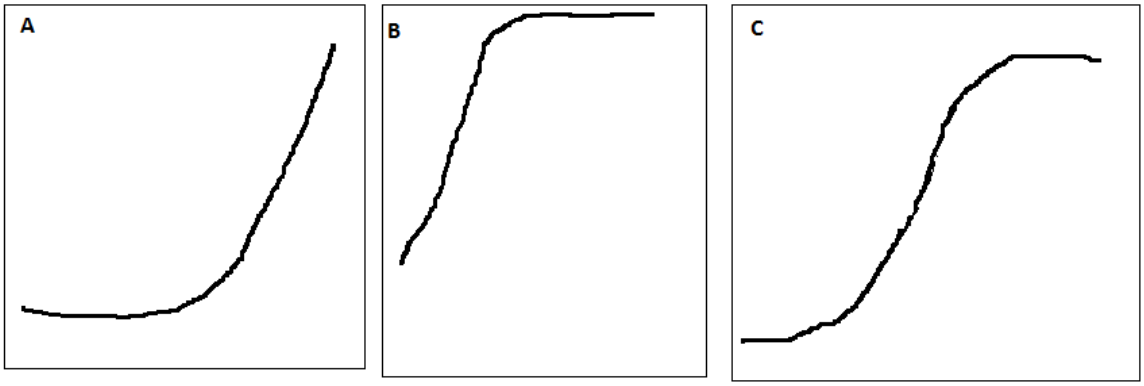


Figure 2.5. Possible standard curve response

(a) Low response. (b) High response. (c) Sigmoidal response. *Adapted from Bio-Plex ™ suspension array system (157).*

2.9. High dose hook effect for plasma IL-13, MIP-1α and RANTES standard curves

The standard curves of IL-13, MIP-1α, and RANTES from plate 6 plasma assay had a significantly reduced MFI at S1 and S2 when compared with the standard curves generated on the remaining 6 plates. The lower MFI was not corresponding with the expected cytokine concentrations at S1 and S2 which should be the highest in the 9 series standard curve. Although the cause of this was not investigated, the projection of these three cytokines could have demonstrated a phenomenon known as "high dose hook effect", which is a form of antibody interference. The inferred problem is that the washing steps did not remove all the cytokines remaining in solution, i.e. that were not bound to the capture antibody/beads. These soluble cytokines would then compete for binding of the secondary antibody to the beads and reduce the MFI signal. The capture antibody is conjugated to the magnetic bead; all assay components not sandwiched to it are washed off, such as any soluble secondary antibody-cytokine complexes (159-161).

The hook's effect for IL-13 and MIP-1α standard curves in plasma assay plate 6 occurred at MFI above the range of samples reading that were measured. The participants' concentration for both IL-13 and MIP-1α could be estimated in the region below the hook effect (Figure 2.6 a-b). The IL-13 and MIP-1α observations from these participants were included in the data analysis. On the other hand, the hook's effect encountered with the RANTES standard curve was in the same MFI and concentration range as the participants' samples' concentration (Figure 2.6c). The concentration of RANTES within those samples could not be estimated with certainty using the standard curve generated in plate 6 assay. The RANTES standard curve that was chosen for extrapolating participants' concentration was picked based on the

lower range of the substituting curve having similar MFI to the lower range of plate 6 RANTES. Detailed standard curves of NPA assays and for the remaining plasma assays are shown in appendix H.

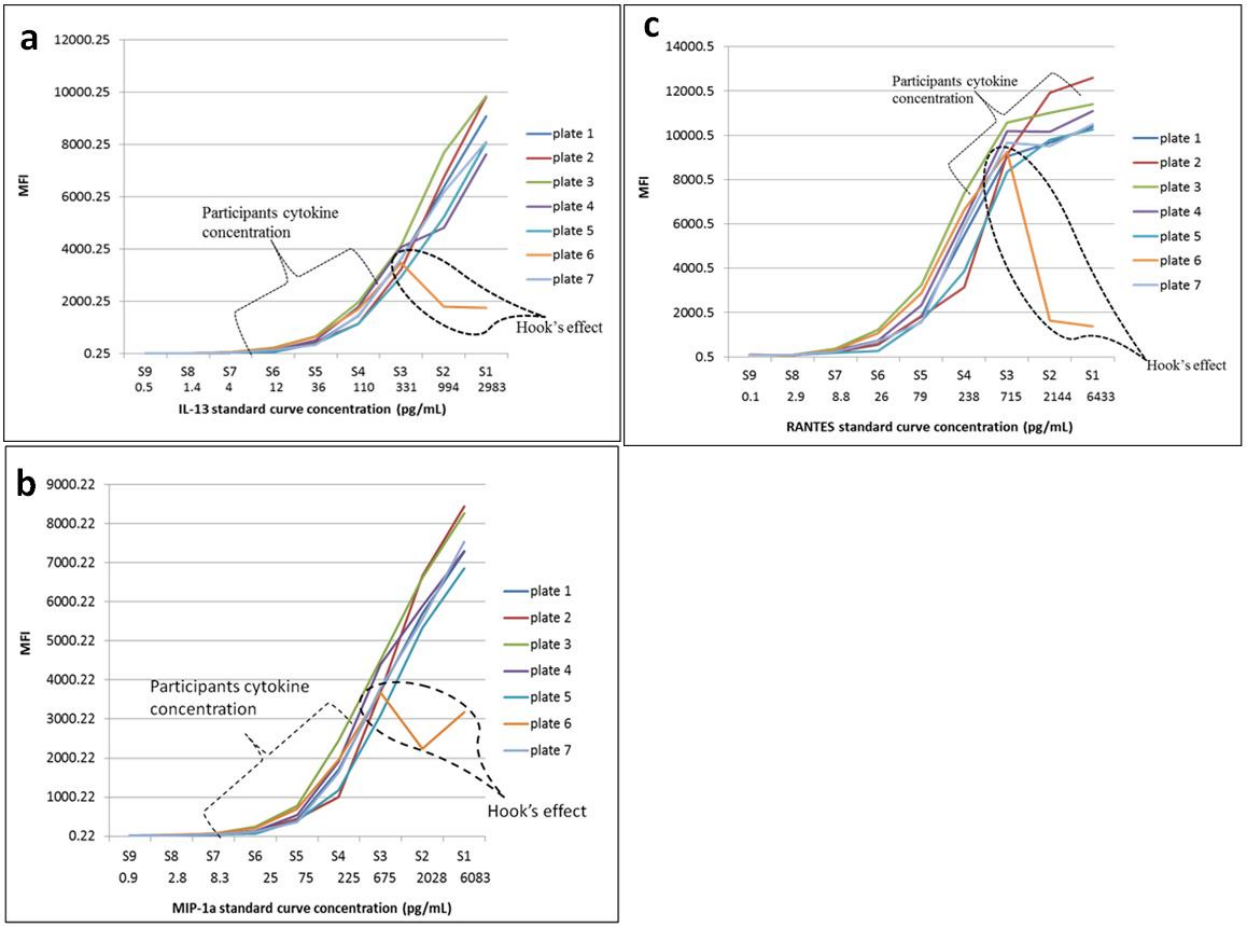


Figure 2.6. Standard curves and hook effect for IL-13, MIP-1α and RANTES

(a-b) The participants MFI for IL-13 and MIP-1α were below the hook effect region; therefore the concentration of these samples could be reliably predicted from the same standard curve. (c) The participants MFI for RANTES was in the same range as the hook effect region; therefore the concentration of these samples could not be reliably estimated from the same standard curve and the standard curve from plate 5 was used.

2.10. Statistics for data analysis

The statistical analyses of this study were performed using STATA version 13, (Stata Corp LP, USA) statistical software. Graphpad Prism 5 (GraphPad, San Diego) was used to draw box and whiskers diagrams. The Shapiro Wilk and Kurtosis test for normality were used to assess the normality distribution of all continuous variables.

Demographic characteristics of the study participants were described using frequency Tables with percentage for categorical variables. Continuous variables were described using mean and standard deviations or median and interquartile range depending on the normality distribution of continuous variables. Median and interquartile range (25th and 75th percentiles) were used to describe the cytokine levels as they were not normally distributed. The 25^{th} and 75^{th} percentile is less affected by skewed data and it has been reported as a good measure of central tendency compared to 5^{th} and 95^{th} percentile (162).

The Kruskal-Wallis test was initially used to evaluate demographic and cytokine variables for any existing difference between all three groups. For cytokine analysis, Bonferroni correction was made to the Kruskal-Wallis test and the critical p value (α=0.05) was divided by 15 to account for the number of cytokine comparisons. Following the Bonferroni adjustment, a $p \leq 0.003$ was considered to be the threshold for statistical significance. The Bonferroni correction was applied to decrease the chances of obtaining false-positive results. Demographic and cytokines variable that were not significant with the Kruskal-Wallis test were excluded from further analysis.

If group differences were significant for the demographic variables, then a Mann-Whitney test was used for one to one group comparison for continuous variables that were not normally distributed; alternatively a two-way student t-test was used for normally distributed demographic variable. Fisher's Exact test was used for categorical variables. Demographic variables that were significantly different in one to one group comparison were considered as covariates.

A covariate adjusted logistic regression was used to evaluate the relationship between each cytokine and the two levels of outcome i.e. control versus all RSV-LRTI or mild versus severe RSV-LRTI. The strength of association for all cytokines significant in severity was further quantified in a covariate adjusted multivariate logistic regression model. Variables with $p<0.2$ were included in a multivariate logistic model. Coefficients of variations were

reported as a measure of association between the RSV LRTI severity and the covariates. Hosmer Lemeshow test was used to investigate goodness of fit on the final model.

MEDCALC software (MedCalc, USA) was used to generate receiver operation characteristic (ROC) curves, sensitivity and specificity report on the ability of significant demographic characteristic or cytokines to discriminate between a mild or severe RSV-LRTI.

Correlation analyses using Spearman's Correlation Coefficients were performed to compare the cytokines concentration between NPA and plasma samples.

Chapter 3: Results

3.1. The epidemiology of study participants

Paired peripheral whole blood and NPA samples were collected from infants with PCR confirmed RSV-LRTI (n=202). The samples were also collected from control infants (n=31) without respiratory illness symptoms or pathogens detected (that we tested for) and were admitted to the hospital for elective surgery. RSV-LRTI cases were grouped into mild (n=89) and severe (n=113) based on their Respiratory Index of Severity in Children (RISC) score. Tables 3.1 and 3.2 depict the demographic characteristics and clinical symptoms at hospital admission of the participants included in the present study.

3.1.1. Growth standards and demographics

The control infants participants were younger than RSV-LRTI cases (median age 1.5 months, $p<0.001$). The majority of the participants were of black African origin. In the control group 94% of the infants were males and this was significantly higher than in the severe (53%) and mild (49%) group ($p<0.001$) for both comparisons.

Infants with severe RSV-associated LRTI were younger (median age 3.9 months) than those with mild RSV-LRTI (median age 4.5 months, $p\leq0.01$). No difference was observed between the mild and severe RSV-LRTI cases in the proportions of full term vs. pre-term birth participants ($p=0.1$).

Household characteristics were similar between the control group and, mild and severe RSV-LRTI cases. Equal proportion of infants from the control group and RSV-LRTI cases were exposed to smoke *in utero;* 16% of infants with mild disease attended day care compared to the 6% severe group ($p>0.05$).

The feeding habits were evaluated for all enrolled infants. A large portion of RSV-LRTI cases (mild=79% and severe=74 %) were never breastfed compared to the control group (39%, $p<0.001$); this analysis was adjusted for age at hospital admission because the control infants were younger than RSV-LRTI cases. Equal proportions of mild and severe RSV-LRTI were never breastfed (79% vs. 74%).

Table 3.1 Demographic characteristics of RSV-LRTI cases and control infants

Characteristics	Control n = 31	Mild n = 89	Severe n =113	Control vs. All RSV	Mild vs. severe
Growth standards					
Age, month, median (IQR)[b]	1.5 (0.43 - 2.8)	4.5 (2.4 - 8.6)	3.9 (1.7 - 6.4)	**<0.001**	**0.01**
Admission weight, kg, median[b] (IQR)	5 (4 - 6)	6.7 (5.5 - 8.5)	5.6 (4.2 - 7)	**<0.001**	**<0.001**
Gender					
Female	2 (6)	45 (51)	60 (53)	**<0.001**	0.86
Male	29 (94)	44 (49)	53 (47)		
Gestation age					
Full term	31 (100)	61 (69)	68 (60)		
Pre term	-	13 (14)	26 (23)	**<0.001**	0.1
Unknown	-	15 (17)	19 (17)		
Ethnicity					
Black	28 (90)	86 (97)	107 (95)	NS	NS
Coloured	3 (10)	3 (3)	6 (5)		
Underlying medical condition					
No	31 (100)	75 (84)	105 (93)	NS	NS
Yes	-	1 (1)	1 (1)		
Unknown	-	13 (15)	7 (6)		
Household background					
Smoke exposure during pregnancy					
No	29 (94)	83 (93)	108 (96)	NS	NS
Yes	2 (6)	6 (7)	6 (4)		
Alcohol exposure during pregnancy					
No	31 (100)	89 (100)	108 (96)	NS	NS
Yes		-	5 (4)		

Smoke exposure from caregiver No Yes	31 (100)	87 (98) 2 (2)	111 (98) 2 (2)	NS	NS
Day care attendance No Yes	31 (100)	75 (84) 14 (16)	106 (94) 7 (6)	NS	NS
Feeding practices during the first 4 months of life					
Ever breastfed Never breastfed	19 (61) 12 (39)	19 (21) 70 (79)	29 (26) 84 (74)	**<0.001**[*]	0.5[*]

Abbreviations: IQR, Interquartile range; kg, kilogram; Non-significance, NS. Data are presented as number (%) unless otherwise specified. A Kruskal-Wallis test was performed to evaluate differences within the three groups. A $p \leq 0.05$ was considered to be statistically significant. Not significant (NS) was assigned to all group comparisons for measures with Kruskal-Wallis $p \geq 0.05$; these data were not tested any further. If group differences were significant, then Fisher's exact test was used for one to one group comparisons to test differences in the frequency of categorical variable. [b]Mann-Whitney test was used for one to one group comparisons to test for equality of the characteristic's median. [*]Age at hospital admission adjusted logistic regression analysis.

3.2. Clinical symptoms presented by RSV-LRTI cases at hospital admission

Infants with RISC score (150) <2 were categorised as mild RSV-LRTI cases and those with a score ≥2 as severe RSV-LRTI cases. Figure 3.1 depicts the RISC score distribution of RSV LRTI cases. Table 3.2 includes the clinical data used to score the disease severity among the cases. Pulse oximetry oxygen saturation measures were available for 108 severe RSV-LRTI cases and 72 mild RSV-LRTI cases, of who 31% and 1% had oxygen saturation ≤90%, respectively. For the participants with no oxygen saturation information available, points to calculate the RISC score were allocated based on whether they had received oxygen supplementation or not; 58% of infants with severe RSV-LRTI required oxygen supplementation compared to 7% with mild RSV-LRTI. 92% of the RSV-LRTI infants with a severe RSV-LRTI presented with hypoxia at hospital admission. The RISC criteria for HIV-uninfected were applied to HIV-uninfected infants including those who were HIV-exposed but remained uninfected; similarly the HIV-infected RISC criteria were used to score the

RSV-LRTI severity of HIV-infected infants. The proportions of infants who were HIV-infected or exposed were not different between all three study groups.

The respiratory symptoms presented by the RSV-LRTI infants at hospital admission are also included in Table 3.2. RSV-LRTI infants more often (>93%) had a history of cough or presented with cough symptoms at examination. The symptoms included runny nose or coryza, nasal flaring and chest crepitation. Expiratory grunting was observed in 5 RSV-infected infants and apnoea in 4; stridor was observed in 3 infants in the severe group. The vital signs recorded at hospital admission included heart rate, respiratory rate and temperature. The median heart rate presented by mild (n=71) and severe (n=102) RSV-LRTI cases was 164 versus 157 beats per minute respectively. The median respiratory rate observed for mild RSV-LRTI (n=80) was 57 breaths per minute and 60 breaths per minute for severe RSV-LRTI cases (n=101). Both the mild (n=81) and severe (n=97) RSV-LRTI cases had a median body temperature of 36.8°C at hospital admission.

Only 1 RSV-LRTI case was admitted to intensive care unit. Infants with severe RSV-LRTI had extended length of hospital stay compared to mild RSV-LRTI (3 days vs. 2 days, $p<0.001$). None of the participants died during hospitalisation.

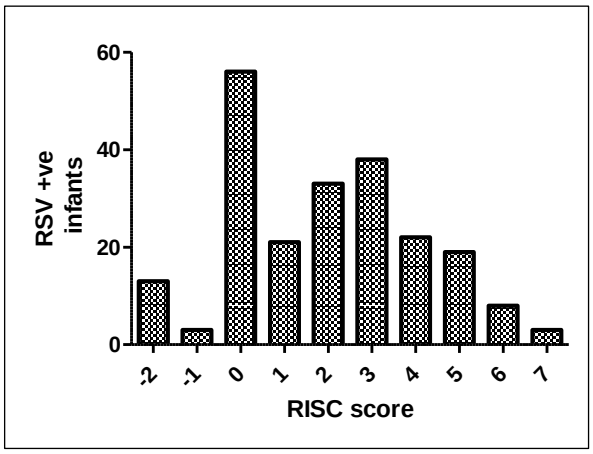


Figure 3.1.Distribution of RISC score in RSV-LRTI infants

The RISC score for HIV uninfected (exposed and unexposed) ranged from -2 to 6. The maximum RISC score for HIV infected infants was 7.

Table 3.2 Clinical symptoms presented by RSV-LRTI cases at hospital admission

Clinical symptoms at hospital admission N (%)	**Mild n = 89**	**Severe n = 113**
RISC Score		
Oxygen saturation %, median (IQR)	96 (94 - 98)	97(90- 100)
Observed frequency	n = 72	n = 108
Oxygen saturation ≤ 90 %	1 (1)	33 (31)
Oxygen supplementation No	83 (93)	48 (42)
Yes	6 (7)	65 (58)
Chest wall retraction No	43 (48)	9 (8)
Yes	46 (52)	104 (92)
Wheezing No	24 (27)	76 (67)
Yes	65 (73)	37 (33)
Feeding intolerance No	78 (88)	86 (76)
Yes	11 (12)	27 (24)
WHO weight for age z (WAZ) score, mean ± SE	- 0.2 ± 0.1	- 1.1 ± 0.2
HIV Uninfected	84 (94)	101 (89)
Unexposed	73 (82)	82 (73)
Exposed	9 (10)	14 (12)
Unknown exposure	2 (2)	5 (4)
HIV Infected	5 (6)	12 (11)
Respiratory system symptoms		
Cough history or at examination No	5 (6)	2 (1)
Yes	83 (93)	108 (96)
Unknown	1 (1)	3 (3)
Post-tussive vomiting No	1 (1)	1 (1)
Yes	11 (12)	18 (16)
Unknown	77 (87)	94 (83)
Runny nose/ coryza No	58 (65)	76 (67)
Yes	30 (34)	36 (32)
Unknown	1 (1)	1 (1)

Nasal flaring	No	67 (75)	77 (68)
	Yes	21 (24)	36 (32)
	Unknown	1 (1)	-
Apnoea	No	87 (98)	111 (98)
	Yes	1 (1)	2 (2)
	Unknown	1 (1)	-
Crepitation	No	49 (55)	40 (35)
	Yes	40 (45)	73 (65)
Stridor	No	0 (100)	110 (97)
	Yes	-	3 (3)
Expiratory grunting	No	88 (99)	109 (96)
	Yes	1 (1)	4 (4)
Vital signs at admission			
Heart rate (beats per minute), median (IQR)		164 (148 -174)	157 (147-172)
Observed frequency		n = 71	n = 102
Respiratory rate (breaths per minute) median (IQR)		57 (46 - 63)	60 (52 -68)
Observed frequency		n = 80	n = 101
Temperature (°C), median (IQR)		36.8 (36.5 - 37.3)	36.8 (36.5 - 37.5)
Observed frequency		n = 81	n = 97

Abbreviations: IQR, Interquartile range. Data are presented as number (%) unless otherwise specified.

3.3. RSV Testing

The nasopharyngeal swabs from RSV-LRTI cases and nasopharyngeal aspirate from controls were tested for RSV infection by PCR. The median cycle of quantification (Cq) was similar among the mild and severe RSV-LRTI (27.2 vs. 28.3, p=0.2) data not shown. Since Cq is a proxy for viral load this suggests that viral load did not influence the outcome of severity in this study.

3.4. Plasma cytokines

Plasma concentrations for cytokines IL-1α, IL-1β, IL-5, IL-9, and IL-12p70 were below the limit of detection (LOD) across all study groups included (Table 3.3a). Plasma IL-8 was observed at levels above LOD, but these levels were not different between control infants and RSV-LRTI cases (Table 3.3a). Cytokine levels from Table 3.3a that were different between two or all three groups (p<0.003) were further studied to evaluate their association with RSV-LRTI and RSV-LRTI severity (Table 3.3b).

3.4.1. Plasma cytokines: Control versus RSV-LRTI cases

Table 3.3b includes the cytokine levels in the plasma for control versus RSV-LRTI. IFN-γ (3 pg/ml vs. 5 pg/ml, p=0.02) and MIP-1α (12 pg/ml vs. 28 pg/ml, p=0.008) were reduced in RSV-LRTI cases compared to control infants. RSV-LRTI was associated with increased levels of plasma TNF-α when compared to control infants (0.7 pg/ml vs. <0.5 pg/ml, p=0.007). Anti-inflammatory cytokine IL-10 was elevated in the plasma of RSV-LRTI cases compared to control infants (1.0 pg/ml vs. <0.6 pg/ml, p=0.02). Plasma levels for IL-4, IL-6, and RANTES were not different between RSV-LRTI cases and controls.

3.4.2. Plasma cytokines: Mild versus severe RSV-LRTI

Median level of plasma IFN-γ was higher in severe RSV-LRTI (4 pg/ml) than mild RSV-LRTI (3 pg/ml, p=0.055). Additionally, Table 3.3b shows that the plasma TNF-α, Th2 type, pyrogenic, chemokines and anti-inflammatory cytokines were not significantly different between mild and severe group.

In general, the IFN-γ and MIP-1α plasma levels declined as a result of RSV-LRTI. Higher levels of plasma IFN-γ were associated with the outcome of severe RSV-LRTI. TNF-α was also elevated as a consequence of RSV-LRTI. RSV-LRTI induced a systemic immune regulation as indicated by elevated levels of plasma IL-10.

Table 3.3a Plasma cytokine concentrations (pg/ml) in control infants, mild and severe RSV-LRTI cases

Cytokines	LOD	Control n = 31	Mild n= 89	Severe n = 113	All groups
Th1 type					
IFN-γ	1	5 (3-5)	3 (2-4)	4 (3-5)	**<0.001**
IL-1α	8.9	0.2*	0.2*	0.2*	NS
IL1-β	31.7	31.7*	31.7*	31.7*	NS
IL-2p70	10	10.0*	10.0*	10.0*	NS
TNF-α	0.5	0.5*	0.7 (0.5*-1)	0.7 (0.5*-1)	**<0.001**
Th2 type					
IL-4	3.2	3.2*	5 (3.2*-6)	3.2* (3.2*-5)	**<0.001**
IL-5	0.2	31.8*	31.8* (31.8*-0.3)	31.8*	NS
IL-9	2	2.0*	2.0*	2.0*	NS
IL-13	1.6	1.6*	1.6* (1.6*-2)	1.6*	**NS**
Pyrogenic					
IL-6	0.6	0.6*	0.6 (0.6*-2)	1.0 (0.6*-3)	**<0.001**
Chemokines					
IL-8	3.1	10.3 (3.1*-20.0)	4.9 (3.1*-11.2)	6.3 (3.1*-14)	NS
MCP-1	31.8	102 (69-230)	63 (41-139)	64 (3-126)	**NS**
MIP-1α	6.3	28 (26-37)	14 (10-22)	11.3 (7-20)	**<0.001**
RANTES	6.6	552 (509-591)	498 (328-940)	431 (348-536)	**0.002**
Anti-inflammatory					
IL-10	0.6	0.6*	1.2 (0.7-3)	1.1 (0.6*-2)	**<0.001**

Abbreviations: not-significant, NS. Data are presented as median (25th - 75th percentile). Kruskal-Wallis test was performed to evaluate median differences between study groups and a Bonferroni correction was applied; critical *p* value (α=0.05) was divided by the number of cytokines (i.e. 15) being compared in plasma samples. Following the Bonferroni adjustment, a $p \leq 0.003$ was considered to be the threshold for statistical significance. If the Kruskal-Wallis was not significant, the cytokine was excluded in further analysis. If group differences were significant, then a covariate adjusted logistic regression was used to evaluate association between cytokine and RSV-LRTI or RSV-LRTI severity. * Cytokine level below limit of detection (pg/ml)

Table 3.3b Plasma concentrations (pg/ml) of the cytokines that were different in control, mild and severe RSV-LRTI infants

Cytokines	LOD	Control n = 31	RSV LRTI n=202	Mild n = 89	Severe n = 113	Control vs. RSV LRTI[a]	Mild vs. severe[b]
Th1 type							
IFN-γ	1.0	5 (3-5)	3 (2-4)	3 (2-4)	4 (3-5)	**0.02**	**0.055**
TNF-α	0.5	0.5*	0.7 (0.5*-1)	0.7 (0.5*-1)	0.6 (0.5*-1)	**0.007**	0.6
Th2 type							
IL-4	3.2	3.2*	3.7 (3.2*-5)	5 (3.2*-6)	3.2* (3.2*-5)	0.4	0.9
Pyrogenic							
IL-6	0.6	0.6*	0.8 (0.6*-2)	0.6 (0.6*-2)	1.0 (0.6*-3)	0.1	0.8
Chemokines							
MIP-1α	6.3	28 (26-37)	12 (8-21)	14 (10-22)	11.3 (7-20)	**0.008**	0.9
RANTES	6.6	552 (509-591)	448 (338-583)	498 (328-940)	431 (348-536)	0.08	0.1
Anti-inflammatory							
IL-10	0.6	0.6*	1.0 (0.6-2)	1.2 (0.7-3)	1.1 (0.6*-2)	**0.02**	0.9

Notes: Data are presented as median (25th - 75th percentile). $p \leq 0.05$ was statistically significant. *Cytokines levels below the limit of detection. [a]Covariate adjusted (age at hospital admission, gender, gestation age, breast milk feeding) logistic regression for plasma cytokines associated with RSV-LRTI. [b]Covariate adjusted (age at hospital admission) logistic regression for plasma cytokines associated with RSV-LRTI severity.

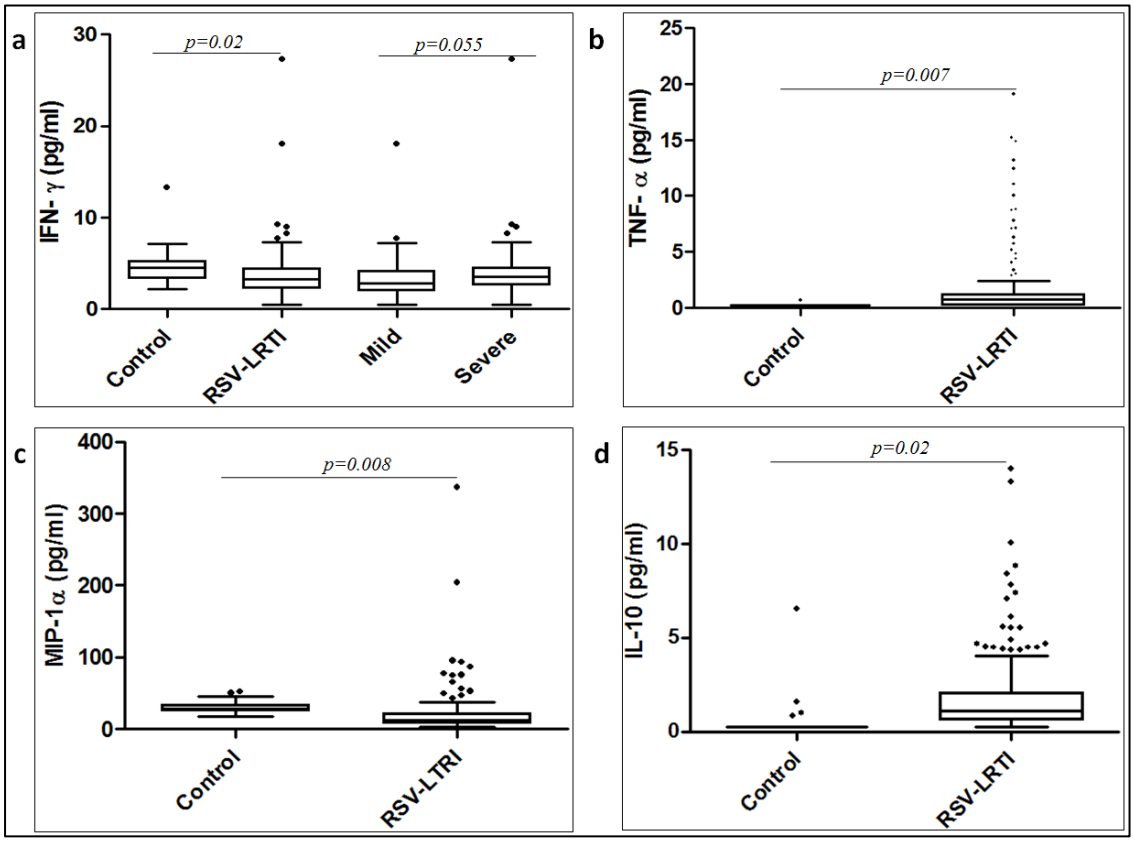


Figure 3.2. Plasma cytokines that were different in control vs. RSV-LRTI or mild and severe RSV-LRTI infants.

(a)The IFN-γ and (c) MIP-1α plasma levels declined as a result of RSV-LRTI. (a) Higher levels of plasma IFN-γ were associated with the outcome of severe RSV-LRTI. (b) TNF-α levels were higher in RSV-LRTI compared to control. (d) RSV-LRTI induced systemic immune regulation as shown by higher levels of IL-10 in RSV-LRTI cases compared to control.

3.5. Nasopharyngeal aspirate cytokine

Nasopharyngeal aspirate IL-1β, IL-9, and RANTES concentration were below LOD (Table 3.4a). IFN-γ, IL-1α, IL-12p70, and IL-4 were detected at levels above the LOD in RSV-LRTI cases but not in control infants. The levels of IL-13 were equal in both RSV-LRTI cases and control infants. Cytokines levels from Table 3.4a that were different between two or all three groups (p<0.003) were further studied to evaluate their association with RSV-LRTI and RSV-LRTI severity (Table 3.4b).

3.5.1. Nasopharyngeal aspirate: Control versus RSV-LRTI cases

Th1 type cytokine TNF-α levels were higher in the NPA of RSV-LRTI cases than in control infants (8pg/ml vs. 1.4pg/ml, p=0.01). Pyrogenic cytokine IL-6 median levels were also higher in the NPA of RSV-LRTI cases than in control infants (43pg/ml vs. 3pg/ml, p=0.07). IL-8 (2682pg/ml vs. 184pg/ml, p=0.002), MCP-1 (287pg/ml vs. 66pg/ml, p<0.001) and MIP-1α (27pg/ml vs. <6.7pg/ml, p=0.004) were elevated in RSV-LRTI cases compared to control infants. IL-8, MCP-1 and MIP-1α are all important chemokines that attract immune cells to the infection site. Anti-inflammatory IL-10 median levels were also increased in RSV-LRTI cases than in control (3pg/ml vs. <0.8pg/ml, *p=0.4*).

3.5.2. Nasopharyngeal aspirate: Mild versus severe RSV-LRTI

Although the median levels were slightly higher in mild compared to severe RSV-LRTI (29pg/ml versus 27pg/ml, p=0.05), the spread of the interquartile range showed that in general severe RSV-LRTI cases (12pg/ml, 67pg/ml) had higher MIP-1α levels compared to mild RSV-LRTI (10pg/ml, 103pg/ml) respectively. In a multivariate logistic regression (Table 3.8), coefficient of variation for NPA MIP-1α was positive indicating that increased cytokine levels were associated with increased severity. This was also true for a univariate logistic regression (data not shown). Additionally, Figure 3.3d below shows that severe cases appear to have increased levels of MIP-1α than the mild cases. For this reason, the mean concentrations (88pg/ml vs. 50pg/ml) of MIP-1α for severe and mild RSV-LRTI are shown in the abstract and not medians.

In general, the median levels of pro-inflammatory cytokines TNF-α, IL-6, IL-8, MCP-1, MIP-1α, were increased in RSV-LRTI cases compared to the control group. Increased levels of MIP-1α were associated with the outcome of severe RSV-LRTI. Taken together, this indicated that the immune response was targeted towards the respiratory tract. Median level of anti-inflammatory cytokine, IL-10 was also increased in RSV-LRTI cases. This strongly suggests that the pro-inflammatory immune response was regulated.

Table 3.4a Nasopharyngeal aspirate cytokine concentrations (pg/ml) in control infants, mild and severe RSV-LRTI cases

Cytokines	LOD	Control n =31	Mild n = 89	Severe n = 113	All groups
Th1 type					
IFN-γ	1.1	1.1*	1.1* (1.1*-2)	1.1* (1.1*-2)	NS
IL-1α	7.8	7.8*	7.8* (7.8* - 9)	7.8*	NS
IL1-β	17.3	17.3*	17.3*	17.3*	NS
IL-12p70	15.7	15.7*	15.7*	15.7* (15.7*-16)	NS
TNF-α	1	1.4 (1.0*-6)	7.4 (3-22)	8 (2-34)	**<0.001**
Th2 type					
IL-4	4.6	4.6* (4.6*-8)	5.2 (4.6*-11)	4.6* (4.6*-9)	NS
IL-5	0.6	0.6* (0.6*-1)	0.6*	0.6*	NS
IL-9	1.2	1.2*	1.2*	1.2*	NS
IL-13	2.9	2.9* (2.9*-4)	2.9* (2.9*-4)	2.9* (2.9*-4)	NS
Pyrogenic					
IL-6	0.7	3.1 (0.9-10.1)	50 (18-100)	40 (1-102)	**<0.001**
Chemokines					
IL-8	2.6	184 (91.0-778)	3437 (1551-7690)	2598 (1133-6072)	**<0.001**
MCP-1	35.1	66 (37-195)	307 (205-363)	274 (170-372)	<**0.001**
MIP-1α	6.7	6.7* (6.7*-11)	29 (12-67)	27 (10-103)	<**0.001**
RANTES	5.3	5.3*	5.3*	5.3*	NS
Anti-inflammatory					
IL-10	0.8	0.8 (0.8-3)	3.2 (1-8)	3 (1-7)	**0.003**

Abbreviations: not-significant, NS. Data are presented as median (25th - 75th percentile). Kruskal-Wallis test was performed to evaluate median differences between study groups and a Bonferroni correction was applied; critical *p* value (α=0.05) was divided by the number of cytokines (i.e. 15) being compared in plasma samples. Following the Bonferroni adjustment, a $p \leq 0.003$ was considered to be the threshold for statistical significance. If the Kruskal-Wallis was not significant, the cytokine was excluded in further analysis. If group differences were significant, then a covariate adjusted logistic regression was used to evaluate association between cytokine and RSV-LRTI or RSV-LRTI severity. * Cytokine level below limit of detection (pg/ml).

Table 3.4b Nasopharyngeal aspirate concentrations (pg/ml) of the cytokine that were different in control, mild and severe RSV-LRTI infants

Cytokines	LOD	Control n = 31	RSV LRTI n = 202	Mild n = 89	Severe n = 113	Control vs. RSV LRTI[a]	Mild vs. severe[b]
Th1 type							
TNF-α	1.0	1.4 (1.0*-6)	8 (2-31)	7 (3-22)	8 (2-34)	**0.01**	0.3
Pyrogenic							
IL-6	0.7	3 (0.9-10)	43 (14-99)	50 (18-100)	40 (1-102)	0.07	0.9
Chemokines							
IL-8	2.6	184 (91-778)	2682 (1165-6072)	3437 (1551-7690)	2598 (1133-6072)	**0.002**	0.1
MCP-1	35	66 (37-195)	287 (186-372)	307 (205-363)	274 (170-372)	**<0.001**	0.8
MIP-1α	6.7	6.7* (6.7*-11)	27 (10-82)	29 (12-67)	27 (10-103)	**0.004**	**0.05**
Anti-inflammatory							
IL-10	0.8*	0.8* (0.8*-3)	3 (1-7)	2.5 (1-8)	3 (1-8)	0.4	0.6

Notes: Data are presented as median (25th - 75th percentile). $p<0.05$ was statistically significant. *Cytokines levels below the limit of detection. [a]Covariate adjusted (age at hospital admission, gender, breast milk feeding) logistic regression for NPA cytokines associated with RSV-LRTI. [b]Covariate adjusted (age at hospital admission) logistic regression for NPA cytokines associated with RSV-LRTI severity. * Cytokine level below limit of detection (pg/ml).

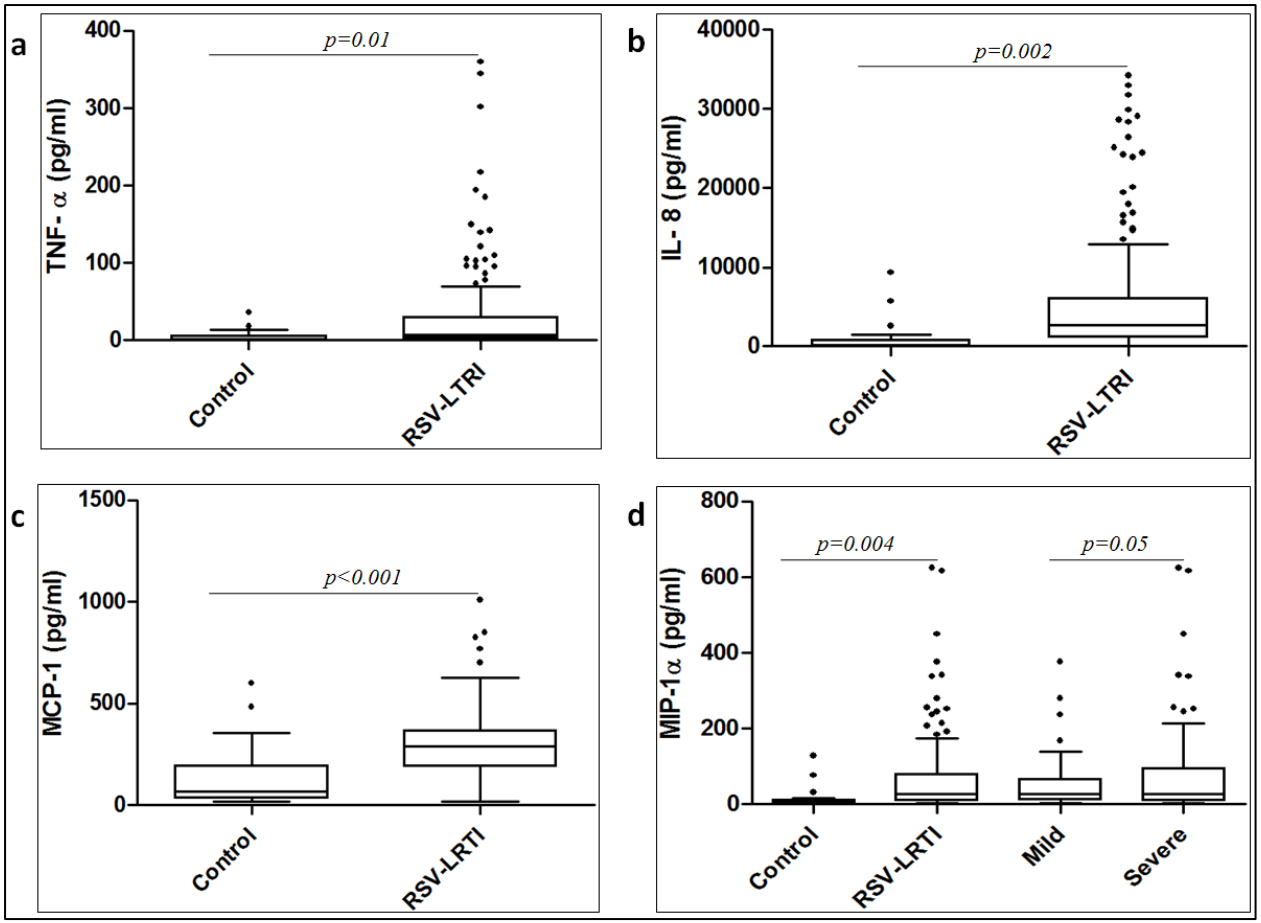


Figure 3.3.Nasopharyngeal aspirate cytokines that were different in control vs. RSV-LRTI or mild and severe RSV-LRTI infants.

(a)-(c) TNF-α, IL-8, and MCP-1 were elevated due to RSV-LTRI. (d) Severe RSV-LRTI cases had higher MIP-1α levels compared to mild RSV-LRTI.

3.6. Correlations of plasma and nasopharyngeal aspirate cytokine levels

Spearman correlation analyses were used to investigate the relationship between cytokines concentration in plasma and nasopharyngeal aspirate (NPA) of mild and severe RSV-LRTI infants (Table 3.5). During RSV-LRTI episodes, increases in plasma IL-10 was associated with increased NPA IL-10 (r=0.25, $p<0.001$), MCP-1 (r=0.16, $p=0.02$) and IL-8 (r=0.15, $p=0.03$). Additionally, plasma IL-6 had a positive correlation with NPA TNF-α (r=0.19, $p=0.01$). There was an inverse relationship between plasma IFN-γ and NPA IL-8 (r=-0.17, $p=0.01$). Figure 3.4 shows significant correlation between plasma and NPA cytokines.

The reason for observing a low strength of correlation with significant *p* value is that, this study had a large sample size to show small effect or association between cytokines. For some RSV-LRTI cases, the relationship between certain plasma and NPA cytokines obeyed a positive or negative correlation. This resulted in a significant *p* value. Since other RSV-LRTI cases were clustered around the fitted linear model for positive or negative correlation, this resulted in a weak strength of correlation. In this study, the results with ($p<0.05$) are meaningful; as they show that plasma and NPA cytokines are related to each other, and not that one cytokine in the plasma or NPA directly or indirectly caused the other and vice versa (163, 164).

Table 3.5. Correlations between plasma and nasopharyngeal aspirate cytokine concentrations of mild and severe RSV-LRTI infants

Cytokines (n=202)		**Nasopharyngeal aspirate**					
		IL-10	**IL-6**	**MIP-1α**	**MCP-1**	**TNF-α**	**IL-8**
Plasma	**IL-10**	**0.25**	0.10	0.08	**0.16**	0.07	**0.15**
	IL-6	0.06	0.04	0.04	0.08	**0.19**	-0.03
	RANTES	0.04	0.11	0.02	0.06	0.07	-0.14
	MIP-1α	-0.04	-0.01	-0.01	-0.04	0.01	-0.08
	IFN-γ	0.05	0.11	0.08	0.06	0.00	**-0.17**
	TNF-α	-0.03	0.00	-0.04	-0.02	-0.12	-0.35
	IL-4	-0.54	-0.02	-0.05	-0.11	0.05	-0.07

Notes: Correlation coefficients between cytokines in plasma and nasopharyngeal aspirates for RSV-LRTI cases. Correlation coefficients shown in bold are significant ($p<0.05$).

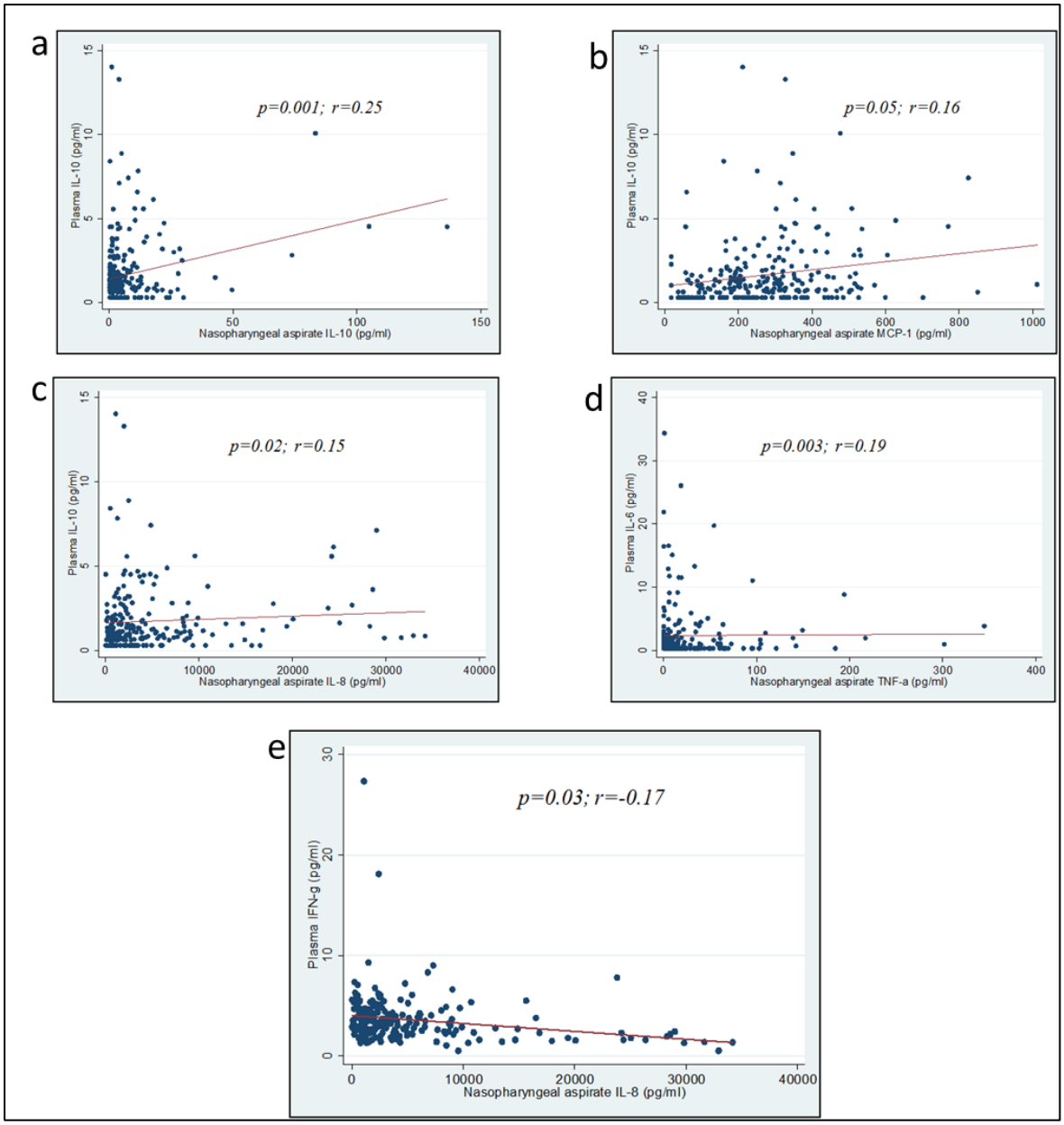


Figure 3.4. Cytokines that are significantly correlated between plasma and nasopharyngeal aspirates. Correlations between: (a) plasma IL-10 and NPA IL-10, (b) plasma IL-10 and NPA MCP-1, (c) plasma IL-10 and NPA IL-8, (d) plasma IL-6 and NPA TNF-α, (e) plasma IFN-γ and NPA IL-8.

3.7. Discriminating severe from mild RSV disease

The strength of association between cytokine concentrations and RSV-LRTI severity observed in Tables 3.3b and 3.4b was quantified with a multivariate logistic regression analysis (Table 3.6) adjusted for age at hospital admission. Cytokines with $p<0.2$ for comparison of mild versus severe RSV-LRTI were included. In a multivariate model, plasma IFN-γ ($p=0.2$) was associated with increased RSV-LRTI severity in concert with other cytokines. Nasopharyngeal aspirate MIP-1α was strongly associated with RSV-LRTI severity ($p=0.05$) with a coefficient of variation positive indicating that a higher MIP-1α level at hospital admission was associated with increased RSV-LRTI severity. Increased levels for plasma RANTES (*p=0.06)* and nasopharyngeal aspirate IL-8 ($p=0.06$) were associated with reduced RSV-LRTI severity.

Table 3.6. Criteria associated with RSV-LRTI severity

Logistic regression analysis (n = 202)			
RSV-LRTI severity	**Coefficient of variation**	**95 % Confidence Interval**	***p* value**
Age at admission	-0.05	-0.15-0.05	0.4
Plasma IFN-γ	0.11	-0.07-0.28	0.2
Plasma RANTES	-0.0007	-0.001-0.00003	0.06
NPA IL-8	-0.00003	-0.0001-8.08e^{-07}	0.06
NPA MIP-1α	0.004	0.00003-0.008	0.05
Goodness-of-fit		0.4	
Hosmer-Lemeshow		0.2	

Plasma IFN-γ and RANTES, nasopharyngeal aspirate IL-8 and MIP-1α were analysed further with the receiver operating characteristics curves in order to determine the cut-off values for discriminating the two levels of RSV-LRTI severity (Table 3.7). Severe RSV-LRTI cases were characterised by the following cytokine concentrations; plasma IFN-γ >2.7pg/ml and RANTES ≤550pg/ml, nasopharyngeal aspirate IL-8 ≤3593pg/ml and MIP-1α >104pg/ml. The cytokines could not solely discriminate RSV-LRTI cases at a risk of developing severe RSV-LRTI from those who are most like to have a mild RSV-LRTI. It is most likely that the combination of all the cytokines in the multivariate model contribute to severity rather than an individual cytokine.

Combination of markers had high sensitivity but the specificity remarkably declined. This suggests a relatively low reliability for using even the combined model to identify children at risk of severe LRTI upon hospital admission. Therefore, the results of this model should be interpreted with other clinical data when predicting RSV-LRTI severity at hospital admission.

Table 3.7. Ability of markers to predict disease severity

Marker	Cut-off value (pg/ml)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC
Plasma IFN-γ	>2.7	74	50	34	85	0.62
Plasma RANTES	≤550	78	40	60	64	0.55
NPA IL-8	≤3593	63	48	59	73	0.58
NPA MIP-1 α	>104	25	90	55	10	0.51
Combination of Markers						
Plasma IFN-γ & RANTES		86	37	63	67	0.68
Plasma IFN-γ & NPA IL-8		88	31	62	65	0.66
Plasma IFN-γ & NPA MIP-1α		76	47	65	61	0.65
Plasma RANTES & NPA IL-8		85	35	62	65	0.62
Plasma RANTES & NPA MIP-1α		90	24	60	66	0.59
NPA IL-8 & NPA MIP-1α		90	18	58	59	0.64
Performance of all combined markers						
Plasma IFN-γ		76	47	65	61	0.69
Plasma RANTES						
NPA IL-8						
NPA MIP-1 α						

Abbreviations: PPV (%), positive predictive value; NPV (%), negative predictive value; AUC, area under the curve.

Chapter 4: Discussion

In this study we measured the levels of the most relevant cytokines described in the literature to be implicated in RSV-LRTI severity as potential markers for severe or mild RSV-LRTI in children less than 12 months of age. In this study we found that RSV infection was associated with increased expression of MIP-1α, MCP, IL-8, and TNF- α in nasopharyngeal aspirate (NPA) and, IL-10 and TNF- α in plasma. IFN-γ levels were lower in the plasma of mild RSV-LRTI than in severe RSV-LRTI cases. Furthermore severe RSV-LRTI was associated with increased levels of NPA MIP-1α. In line with RSV literature, we also found that infants with severe RSV-LRTI were in general younger at hospital admission and experienced breathing problems.

4.1. Severe disease is associated with breathing problems

We observed that severe disease was associated with breathing difficulty and the need for oxygen supplementation during hospitalisation. This was expected since the RISC model uses these variables as part of its severity categorisation, in accordance with Tabarani *et al* (2013) and Diaz *et al* (2015). In addition, these studies found that IL-1β was also elevated in infants requiring oxygen supplementation. We were unable to detect IL-1β in any of the studied groups. This could have been a consequence of our assay having a high LOD for IL-1β or simply because the cytokine was not secreted. Also, it has been suggested that increased levels of MIP-1α, MCP-1, and TNF-α in respiratory secretions during RSV infection may be associated with the need for oxygen therapy (117, 165). Although in our study, these cytokines were elevated in NPA of RSV infected infants they were not associated with severity except for MIP-1α.

RSV-infected infants (52% of mild and 92% severe) presented with chest retraction. The respiratory rate of infants with severe disease was also elevated at hospital admission compared to infants with mild disease. Taken together these signified that RSV infection caused breathing difficulty and supported the use for oxygen supplementation. It was noted that a high proportion of RSV infected infants presented with wheezing and this was consistent with many other studies (166-169). In our study wheezing was associated with reduction of severity and it was largely observed among infants with a mild disease, suggesting that wheezing may be associated with physiological protection during RSV infections.

Most RSV infected infants had a history of cough or were coughing during admission and also had chest crepitation. Cold-like symptoms such as runny nose and nasal flaring were also frequent in RSV infected infants; what could mislead clinicians to dismiss RSV cases as common cold illness. Infants with severe RSV-LTRI had an extended length of hospital stay; which according to McMillan *et al* (1988) might be associated with hypoxia (169).

4.2. Breast milk feeding protects against RSV LRTI-associated hospitalization

There is overwhelming evidence from epidemiological studies demonstrating that breast milk feeding can be beneficial against respiratory pathogens (170, 171) and RSV-LRTI (172). In line with previous studies (173), we also observed that lack of breast milk feeding was associated with RSV hospitalization. Breast milk is a complex nutrition source that contains antimicrobial substances, immune regulators with capabilities to provide infants with passive protection and influences the development of mucosal immunity (174). Many studies have measured various cytokines in human breast milk (175-178) that were otherwise found to be reduced in infants (179-181). The study by Wong and Orgra (1986) demonstrated that RSV replication was reduced in RSV challenged neonatal cotton rats that were foster nurtured with breast-milk containing RSV acquired antibodies (182). Both these observations suggest that breast milk compensate for the late development of infants immune system. Breast milk feeding may however remain a challenge for HIV-infected mothers.

4.3. PCR quantitation cycle as proxy of viral load in nasopharyngeal aspirate was not associated to RSV-LRTI severity

Polymerase chain reaction Quantitation cycle (Cq) value can be used as a proxy for viral load. A high Cq value means that the RSV RNA was not detected or viral load levels were low, and a low Cq value signifies that the RSV RNA was abundant, indicating high viral load. In accordance with Brand *et al* (2013), our study supports the findings that Cq value cannot differentiate infants with severe LRTI from those who have mild LRTI (183). This was in contradiction with the findings of a Kenyan study which suggested that Cq values could be a useful tool to diagnose severe LRTI (184). We speculate that these authors found an association between Cq value and severity because their study was community based. During RSV infection children begin to shed the virus between days 6-8 following infection (185). At this stage the viral load is very high and may vary according to levels of RSV severity. Most frequently the child would still be at home, thus collecting samples at this stage may have biased the Kenyan study to detect an association between RSV-LRTI severity

and Cq value. In contrast, infants in hospital-based studies such as ours may have been hospitalized at variable times along the course of infection. If time during the course of infection independently affects viral load, the association between viral load and severity may be lost in hospital admission studies. The viral load begins to decline at day 9 post-infection as it is contained by the immune system (186). At this time the clinical symptoms may start and only then, the parent of the child is alarmed and concerned enough to consult the hospital. In our study, this was the time samples were collected and the viral load that could predict RSV-LRTI severity may have already decreased. Similarly Wright *et al. (*2002) observed that the viral load in intubated children was not higher than in children who had mild RSV-LRTI (185). Thus, at the time we collected NPA samples, the severity may have already been defined by the viral load that was present before hospital admission.

4.4. Cytokines associations with RSV-LRTI and severity

The type of immune respond that is induced during an infection depends on the cytokines that are produced by host cells such as epithelial cells, Th1 and Th2 T-cells or tissue residing macrophages. In this study we observed no evidence for distinct Th1 and Th2 cytokines in mild and severe RSV-LRTI cases.

4.4.1. Plasma cytokines and associations with RSV-LRTI

The plasma of RSV-LRTI cases had reduced levels of IFN-γ compared to the control infants. This was in line with Aoyagi *et al.* (2002) who observed reduced production of IFN-γ by gamma delta (γδ) T-cells from RSV-LRTI cases (187). These authors also showed that low levels of IFN-γ were accompanied by increased proportion of IL-4 producing cells. In our study IL-4 was measured above the LOD in RSV-LRTI cases but this was not associated with RSV-LRTI. It is possible that the IL-4 detected in our RSV-LRTI cases was associated with young age and not solely of RSV-LRTI. Studies from Bendelja *et al.* (2000) and Bont *et al.* (1999) showed that IFN-γ levels were reduced in the plasma of RSV-LRTI cases that had oxygen saturation below 95 % measured on oximeter or required ventilation (42, 99). Our study findings were in line with those studies, as most of our RSV-LRTI cases had breathing difficulty, required oxygen supplementation in addition to reduced IFN-γ. It is also possible that IFN-γ levels may have declined due to the IL-10 which was produced as a consequence of RSV-LRTI.

RSV-LRTI cases, had median plasma TNF-α concentrations of 1.0pg/ml slightly above the LOD (0.5pg/ml) while TNF-α was below LOD in control infants. According to Mella *et al.* (2013), TNF-α plasma levels below 1000pg/ml during RSV-LRTI indicated the need for hospitalization (188). This was in line with our study as all the RSV-LRTI cases were hospitalized. Bendelja *et al*, (2010) suggested that the low levels of plasma TNF-α produced during RSV-LRTI was a consequence of reduced response by monocytes expressing TLR-8 (189).

In our study, infants with RSV-LRTI had low levels of plasma MIP-1α compared to control infants. This result was in line with Moreno-Solis *et al* (2014) where the concentration of plasma MIP-1α decreased during RSV-LRTI (190). MIP-1α is mainly produced by monocytic phagocytes (128, 191). The declining plasma levels observed during RSV-LRTI may be a consequence of MIP-1α producing cells migrating from the peripheral blood towards the respiratory tract (190), possibly also contributing to airway obstruction. If indeed MIP-1α producing cells migrated away from the peripheral blood during RSV-LRTI, then this would explains the inadequate production of TNF-α observed in the plasma.

RSV-LRTI cases had elevated plasma levels of IL-10 compared to controls. These findings were consistent with the study by Brand *et al.* (2013) conducted in the Netherlands which measured IL-10 in plasma of RSV-LRTI cases (183), and observed that the median concentration IL-10 was not associated with RSV-LRTI severity. IL-10 is an important immunoregulator during viral infections which alleviates excessive production of IFN-γ and TNF-α (192), thus preventing the progression from mild to severe RSV-LRTI by reducing immunopathology mediated by IFN-γ (138). Monocytes have been reported to be the producer of plasma IL-10 during RSV infection (193).

4.4.2. Plasma IFN-γ is associated with RSV-LRTI severity

The plasma of infants with mild RSV-LRTI was characterised by low levels of IFN-γ compared to severe RSV-LRTI. In a whole blood culture and cytokine induction study, Bendelja *et al.* (2002) demonstrated that, the peripheral blood mononuclear cells (PBMC) from mild RSV-LRTI cases had increased expression of IL-4 and reduced IFN-γ (94). Similarly Chen *et al.* (2002) observed that the production of IL-4 was suppressed and that IFN-γ was upregulated in infants with severe RSV-LRTI (89). The results of these studies and ours are however in contradiction with the findings of Brandenburg *et al.* (2000). In a similar whole blood culture study, Brandenburg *et al.* observed that RSV-LRTI cases had

reduced levels of IL-4 and high levels of IFN-γ irrespective of disease severity, and concluded that Th1-type immune response was present in RSV-LRTI infants (194).

We hypothesize that mild RSV-LRTI cases may have a more competent immune response to RSV compared to those with severe RSV-LRTI. Majority of the leukocytes from mild RSV-LRTI cases could migrate to the respiratory tract and limit the spread and replication of RSV. As a consequence of this immunological "calling" the peripheral blood of mild RSV-LRTI cases may have reduced number of IFN-γ producing cells.

4.4.3. Nasopharyngeal aspirates cytokines and associations with RSV-LRTI

In our study the concentration of many cytokines and chemokines involved in innate immunity were elevated in NPAs from RSV-LRTI cases suggesting that the immune response was being targeted to the respiratory tract (123). The control infants had low levels of nasopharyngeal aspirate chemokines. The immune cells responding to the nasopharyngeal aspirate chemokines in these infants were possibly required to fight low level of infections introduced by breathing. The respiratory tract of RSV-LRTI cases was characterised by increased levels of IL-6, IL-8, IL-10, MCP-1, MIP-1α, and TNF-α compared to control infants. Both macrophages and epithelial cells secrete IL-8, MCP-1, and MIP-1α in response to RSV (128, 195).

Tsutsumi *et al.* (1996) using nasal washes also detected that IL-6 and TNF-α were secreted during RSV infection (196). Our results were also in agreement with Diaz *et al.* (2013) who observed that TNF-α nasopharyngeal aspirate levels increased during RSV-LRTI (197). Production of these cytokines is attributed as an early warning to infection and initiates an inflammatory response. IL-6 together with TNF-α stimulates the hypothalamus and pituitary gland inducing fever (198). IL-6 is also known to activate lymphocytes and to promote the development of adaptive immune response (135). TNF-α induces endothelial cells lining the blood vessels to become permeable and allows leukocytes to migrate from the blood (199). In the presence of increased levels of NPA MIP-1α, MCP-1 and IL-8, the activated granulocytes migrate from the blood to the respiratory tract. Activated lymphocytes also respond to MIP-1α and MCP-1and could migrate to the respiratory tract (126, 200).

The study of McNamara *et al.* (2005) showed that in nonbronchoscopic bronchoalveolar lavage IL-8 was secreted in large quantity compared to other chemokines what is in agreement with our study where we observed that NPA IL-8 was present in higher

concentration compared to other chemokines. Neutrophils are attracted to the infection site by following the IL-8 gradient concentration (201).

As expected a large portion of the pro-inflammatory response occurred within the respiratory tract, and there was evidence of immune regulation. IL-10 triggered the immune regulation needed to reduce antigen presentation and secretion of other pro inflammatory mediators in order to reduce pathology caused by the immune system (138, 202).

IFN-γ which was found to be associated with RSV-LRTI severity in the plasma and nasopharyngeal levels between control infants and RSV-LRTI cases were comparable. In the respiratory tract were viral particles are abundant; the RSV non-structural proteins (N1 and N2) may have been successful at antagonising the interferon signals by upregulating the expression of suppressor of cytokines signalling (SOCS). This could have consequently prevented IFN-γ associated antiviral activities to take place at the site of infection (25, 203).

4.4.4. Nasopharyngeal aspirates MIP-1α is associated with RSV-LRTI severity

The elevated NPA cytokines observed in our study is in line with the results of Bonville *et al.* (1999) who observed that MIP-1α was secreted into nasal washes during on-going RSV-LRTI. High levels of MIP-1α were found to be a signature of RSV-LRTI (compared with adenovirus, herpes simplex virus-1, cytomegalovirus and rhinovirus infections) (123).

In our study, MIP-1α was also associated with RSV-LRTI severity. MIP-1α is produced by tissue residing macrophages, lymphocytes and dendritic cells (204). MIP-1α attracts T lymphocytes, basophils and neutrophils to the infection site, and activates mast cells to degranulate (126). In the presence of MCP-1 as observed in this study, basophils will be activated to release histamine (126). Higher levels of MIP-1α were measured from the nasopharyngeal aspirate of severe RSV-LRTI cases than mild RSV-LRTI cases. In this study, the hallmark of MIP-1α in RSV-LRTI severity is that it acts in concert (with other chemokines) as a potent chemo attractant for different type of granulocytes and induces their effector functions. When an antibody receptors (Fc receptors) on the surface of cells (205), such as neutrophils, eosinophils, and natural killer cells make contact with viral particles opsonised by immunoglobulin (Ig) G or IgA (206), reactive oxygen species and anti-microbial proteins (207) are released. These may limit RSV from replicating and spreading to other areas (208). This may mediate immunopathology and exacerbate respiratory tissue damage causing severe RSV-LRTI.

4.5. Correlation between plasma and nasopharyngeal aspirates cytokine levels

We detected positive correlations for plasma IL-10 and NPA IL-10, IL-8, and MCP-1. The strength of correlation observed between the two compartments for these cytokines was modest but significant. Additionally, direct correlation was also observed for plasma IL-6 with NPA TNF-α. Both plasma and NPA TNF-α were directly correlated to each other. Moreno-Solís *et al.* (2015) studied the correlation between cytokine levels in plasma and nasal washes (209). In contrast to our study, these authors observed a positive correlation between plasma and nasal wash MIP-1α. In the same study, plasma IL-8 increased with nasal wash levels.

An inverse correlation was noted between plasma IFN-γ and NPA IL-8. This strengthens the hypothesis that during RSV infection the cells that produce plasma IFN-γ migrate out of the blood in response to the increased chemokine concentration within the respiratory tract. As a consequence, the plasma of RSV infected children had lower levels of IFN-γ compared to the controls. These results may suggest that the plasma and respiratory tract constitute independent compartments with regard to immune response and the two compartments are interconnected by chemokines. The respiratory chemokines might be the key mediators that influence the migration of cells from the blood towards the RSV infected compartment (190).

4.6. Cytokines below limit of detection compared with previous studies

We were unable to detect plasma IL-1α, IL-1β, IL-9, IL-12p70, and NPA IL-1β, IL-9, and RANTES yet previous studies were able to measure them. Some cytokines were observed in this study at high or low magnitude compared to previous studies investigating RSV severity. This section discusses and compares to previous studies, the cytokines that were not detected in this study and yet have been described to be key to RSV immune response.

4.6.1. Plasma cytokines not detected

We did not observe plasma IL-1β. These results were consistent with the study by Brand *et al.* (2016) who studied cytokine responses in plasma and NPA of infant with RSV infection from the Netherlands and observed no IL-1β in plasma (Table 4.1) (183). These conflicted with Bermejo-Martin *et al* (2007) and colleagues who studied cytokine responses to RSV infection Spanish infants and detected IL-1β (median, 17.5pg/ml) in the plasma of RSV infants (210). If IL-1β was secreted into the plasma of our study participants at similar levels to those observed in Spanish infants, we were unlikely to have detected this range of

concentration since this value is below our LOD (31.7pg/ml). The study by Brand *et al* (2016), which had IL-1β LOD of 11.2pg/ml, should have been able to measure IL-1β at the levels reported in the Spanish study but it was not reported in Brand *et al.* (2016) study.

IL-12p70 was not detected in our study nor a Dutch study (10.0pg/ml vs 0.6pg/ml LOD respectively) (183). Bermejo-Martin *et al.* (2007) study reported much higher levels of IL-12p70, median concentration of 28.9pg/ml. Brand *et al.* (2016) and our study was capable of measuring similar levels to the ones observed in the Bermejo-Martin *et al.* (2007) study if secreted at all.

Our plasma results agree better with those of Brand *et al.* (2016) than with Bermejo-Martin *et al.* (2007); the latter which also used a Luminex based assay to measure cytokines similarly to our study. Other likely explanations for the observed difference could be that, during RSV infection cytokines are secreted differently in infants based on population variation. We also cannot rule out that unstable cytokines could have been too labile once they are collected into tubes and samples are handled. Nonetheless, a review of the literature did not uncover unequivocal evidence that such degradation could explain our inability to detect IL-1β or IL-12p70.

Table 4.1. Plasma cytokine below LOD compared to previous studies

Cytokines	Soweto study	Previous studies				
	LOD (pg/ml)	**Observed median concentration in RSV cases (pg/ml)**	**Technique used**	**Measure of Severity**	**Study country**	**Reference**
IL-1β	31.7	17.5	Luminex	RSV versus control	Spain, Bermejo-Martin *et al.* (2007)	(210)
		Undetected; LOD =11.2	Flow cytometer (Functionally similar method to Luminex)	No supportive intervention=mild Oxygen need =moderate Ventilation = severe	Netherlands Brand *et al.* (2013)	(183)
IL-12p70	1	28.9	Luminex	RSV versus control	Spain, Bermejo-Martin *et al.* (2007)	(210)
		Undetected; LOD =0.6	Flow cytometer (Functionally similar method to Luminex)	No supportive intervention= mild Oxygen need = moderate Ventilation = severe	Netherlands Brand *et al.* (2013)	(183)

4.6.2. Nasopharyngeal aspirate cytokines not detected

IL-1β, IL-9, and RANTES were also below the limit of detection (LOD) in nasopharyngeal aspirates from RSV-LRTI infants. We specifically analysed IFN-γ because it has been highlighted as important for RSV immune responses (119). Kim *et al.* (2012) from South Korea, detected high levels of IFN-γ (median 4.4pg/ml) in nasal washes of RSV infected infants compared to control (below LOD) and associated this with eosinophilia inflammation (119). The opposite was observed in our study as the median IFN-γ level was equivalent to the LOD (1.1pg/ml). Additionally our results were similar to Brand *et al.* (2016) who also did not detect IFN-γ (LOD =1.2pg/ml) within the nasal wash of RSV infants.

The median NPA levels of IL-1β observed from Bermejo-Martin *et al.* (2007), Brand *et al.* (2016) and 2 USA studies, Christiaansen *et al.* (2016) and Tabarani *et al.* (2013), all varied in magnitude as shown in Table 4.2. Both Bermejo-Martin *et al.* (2007) and Christiaansen *et al.* (2016) measured cytokines from RSV and control infants using Luminex technique. The median IL-1β levels reported in RSV cases varied widely: 15.6pg/ml to 468pg/ml. Brand *et al.* (2016) and Tabarani *et al.* (2013) studies used different techniques to measure cytokines; nonetheless both these studies showed that the levels of IL-1β increased with severity. Intriguingly, the most severe group from these two studies were infants admitted to ICU. Even though the severe groups were similar, IL-1β in infants from Brand *et al.* (2016) study was almost 4 times higher (1093pg/ml) than in infants (300pg/ml) from Tabarani *et al.* (2013) study. The different techniques used to measure cytokines, does not appear to be decisive in influencing the cytokine levels observed. If techniques had any influence, surely the levels observed from Bermejo-Martin *et al.* (2007) study (468pg/ml) which used Luminex would have differed greatly with the mild group from the Brand *et al.* (2016) study (552pg/ml) which used flow cytometer. An alternative explanation is that the affinity of the antibodies used in the detection kits for IL-1β could have varied from one study to the next.

These previous studies have shown that IL-1β was secreted at various levels among different population and that the magnitude was not consistent with various measures of severity. Three studies reported concentrations in RSV cases above our calculated LOD. If IL-1β was secreted into the nasopharyngeal aspirate of our study infants, we would have had the ability to detect it since the LOD (17.3pg/ml) was lower compared to the measured medians levels of the 3 previous studies. The same would have been true for RANTES which was also not

detected in the nasopharyngeal aspirates from this study regardless of low LOD (5.3pg/ml) compared to median levels observed by 3 previous studies.

Additionally, IL-9 was not detected in the nasopharyngeal aspirates from our study. IL-9 was differently expressed in two studies from UK (McNamara *et al*. (2014) and Semple *et al.* (2007)) both using ELISA. The McNamara *et al*. (2014) study showed that IL-9 levels declined with increasing severity, while the study by Semple *et al.* (2007) implicated high levels of IL-9 with severity. McNamara *et al.* (2014) however included pre-term infants as severe group and this could have influenced the different IL-9 levels observed among the 2 studies, since prematurity is a well-known risk factor for severe RSV. Furthermore, our study included infants who had not been ventilated similarly to the non-severe group of McNamara *et al.* (2014) study. If IL-9 was secreted into the nasopharyngeal aspirate, we could have measured this cytokine because the LOD (1.2pg/ml) from our study was lower than the median level (1.9pg/ml) detected in the non-severe non-ventilated full term infants from McNamara *et al*. (2014) study.

The studies published to date reported a very wide range of median concentrations for IL-1β, IL-9, and RANTES in NPA. Although it may be possible that cytokines are secreted differently among various ethnicities during RSV infection, some of the variation reported was within one country (IL-9 concentrations differing by about 50-100 fold in the two UK studies). The median concentrations reported were above our LOD in 3 of 4 studies for IL-1β, 3 of 3 studies for RANTES and 1 of 2 studies for IL-9, suggesting that cytokines' degradation in the sample during processing could be possible, particularly for RANTES, although there is no direct evidence to support this. It also remains possible that ethnic difference could play a role in the differences observed when comparing our South African data to the European/USA data in the literature summarised here. According to Hoffmann *et al.* (2002), high and low levels of cytokine production are greatly influenced by polymorphisms within the regulatory region of the cytokine genes (211, 212), and IL-1 gene cluster is a well know cytokine whose secretion is greatly influenced by ethnicity (212). Secretion of different cytokines levels have been measured in two different ethnic groups (from same geographical area) infected with Dengue virus (213). It is likely that the same is true for RSV infection. Polymorphisms of various cytokines have also been implicated in cytokine production levels including RSV severity (214-216).

Table 4.2. Nasopharyngeal aspirate cytokines not detected compared to previous studies

Cytokine	Soweto study	Previous studies				
	LOD (pg/ml)	Observed median concentration in RSV cases (pg/ml)	Measure of Severity	Technique used	Study country	Reference
IL-1β	17.3	468	RSV versus control	Luminex	Spain, Bermejo-Martin *et al.* (2007)	(210)
		15.6	RSV versus control	Luminex	USA, Christiaansen *et al.* (2016)	(217)
		552	No supportive intervention= mild	Flow cytometer (Functionally similar method to Luminex)	Netherlands, Brand *et al.* (2013)	(183)
		1670	Oxygen need = moderate			
		1093	Ventilation = severe			
		126	Non-Hospitalised	Luminex	USA, Tabarani *et al*. (2013)	(117)

		149	Hospitalised not in ICU			
		300	Admission to ICU			
RANTES	5.3	78	RSV versus control	ELISA	USA, Sheeran *et al.* (1999)	(218)
		23.51	RSV versus control	Luminex	USA, Christiaansen *et al.* (2016)	(217)
		116	No supportive intervention= mild	Flow cytometer (Functionally similar method to Luminex)	Netherland, Brand *et a.l* (2013)	(183)
		74	Oxygen need = moderate			
		48	Ventilation = severe			
IL-9	1.2	1.9	Non-severe = Full term non-ventilated	ELISA	UK,McNamara *et al*. (2004)	(219)
		0.4	Severe = Preterm ventilated			

		112	No oxygen =mild	ELISA	UK, Semple *et al.* (2007)	(220)
		210	Oxygen = moderate			
		113	Ventilation = severe			
IFN-γ	1.1	4.4	RSV vs. control	Luminex	South Korea, Kim et al. (2012)	(119)
		LOD =1.2	No supportive intervention= mild	Flow cytometer (Functionally similar method to Luminex)	Brand *et al.* (2013)	(183)
			Oxygen need = moderate			
			Ventilation = severe			

4.7. Soweto study: what did we learn?

There are many studies around the world which have investigated association of cytokines with disease severity during RSV infection. Here we have glanced at few important studies from various countries; all these studies are in agreement that the levels of NPA cytokines and chemokines involved in the innate immunity increased during RSV infection regardless of the cytokine detection kit used. Additionally, the immune response observed in plasma varied greatly. The similarities observed in the expression of IL-6, IL-8, IL-10, MCP-1, MIP-1α, and TNF-α in the respiratory tract may signify that there is a common pathway in which RSV or infected epithelial cells induce an immunological response. The most striking conclusion of this exercise comparing different studies is how different the studies' results are from each other, even when comparing ethnically similar populations. This raises the possibility that technical differences may play a role; including affinities of antibodies used in detection kits, veracity of cytokine standard preparations and other factors associated with the details of how each set of experiment may have been performed.

4.8. Conclusion and future perspectives

Severity of RSV-LRTI was associated with elevated plasma IFN-γ and nasopharyngeal aspirate (NPA) MIP-1α concentrations. There was an indirect correlation observed between plasma IFN-γ and nasopharyngeal aspirate IL-8. Supported by the observed correlation, the blood of mild RSV-LRTI cases may have had reduced number of IFN-γ producing cells. This suggested that in these cases, the immune response was directed towards the respiratory tract as a consequence of elevated NPA cytokine levels. This could have reduced progression to severe disease. This response may be impaired in more severe cases of RSV-LRTI; high levels of plasma IFN-γ may suggest that the cells remained in the blood. Since IFN-γ was not detected in the nasopharyngeal aspirate; future studies should not only study cytokine responses in plasma or nasopharyngeal aspirates but also study the immune cells in order to understand the complete immune response during RSV-LRTI. Additionally, the stability of nasopharyngeal aspirate cytokines collected into universal transport media should be investigated as we only adjusted for the dilution factor introduced during NPA collection. We observed no evidence of canonical Th1 or Th2 type cytokine responses. The concentrations of plasma IFN-γ and nasopharyngeal aspirate MIP-1α, which were associated with RSV-LRTI severity, could not independently predict RSV-LRTI cases at risk of severe RSV-

associated LRTI. We suggest that the results of this study should be interpreted together with clinical data when clinicians triage for level of care during RSV-LRTI.

Chapter 5: References

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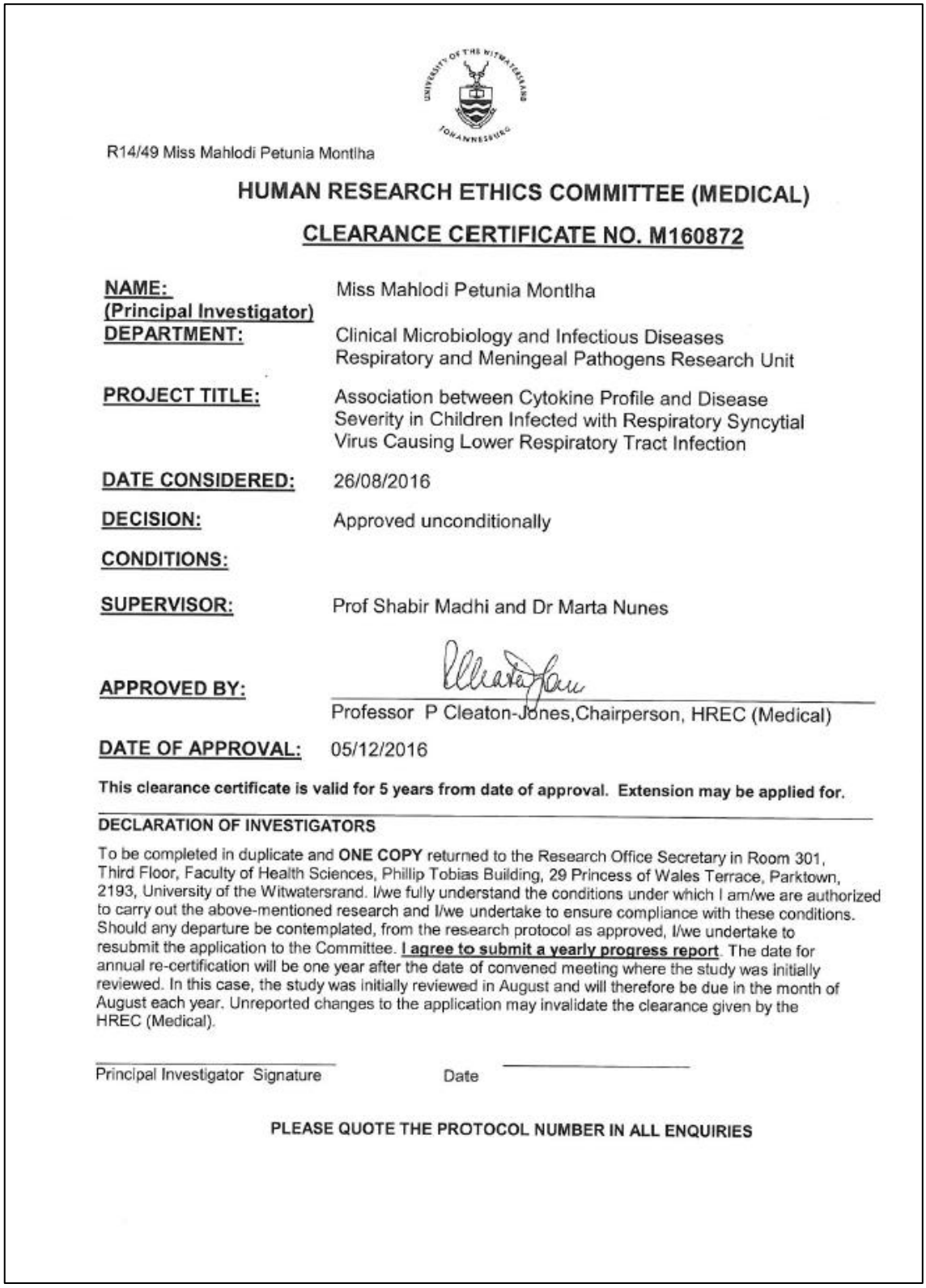
Appendix A: Ethics certificate



R14/49 Miss Mahlodi Petunia Montlha

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160872

NAME: (Principal Investigator)	Miss Mahlodi Petunia Montlha
DEPARTMENT:	Clinical Microbiology and Infectious Diseases Respiratory and Meningeal Pathogens Research Unit
PROJECT TITLE:	Association between Cytokine Profile and Disease Severity in Children Infected with Respiratory Syncytial Virus Causing Lower Respiratory Tract Infection
DATE CONSIDERED:	26/08/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Shabir Madhi and Dr Marta Nunes
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	05/12/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in August and will therefore be due in the month of August each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Figure 1.Copy of the ethics clearance certificate received from the HREC.

Appendix B: Turnitin Report

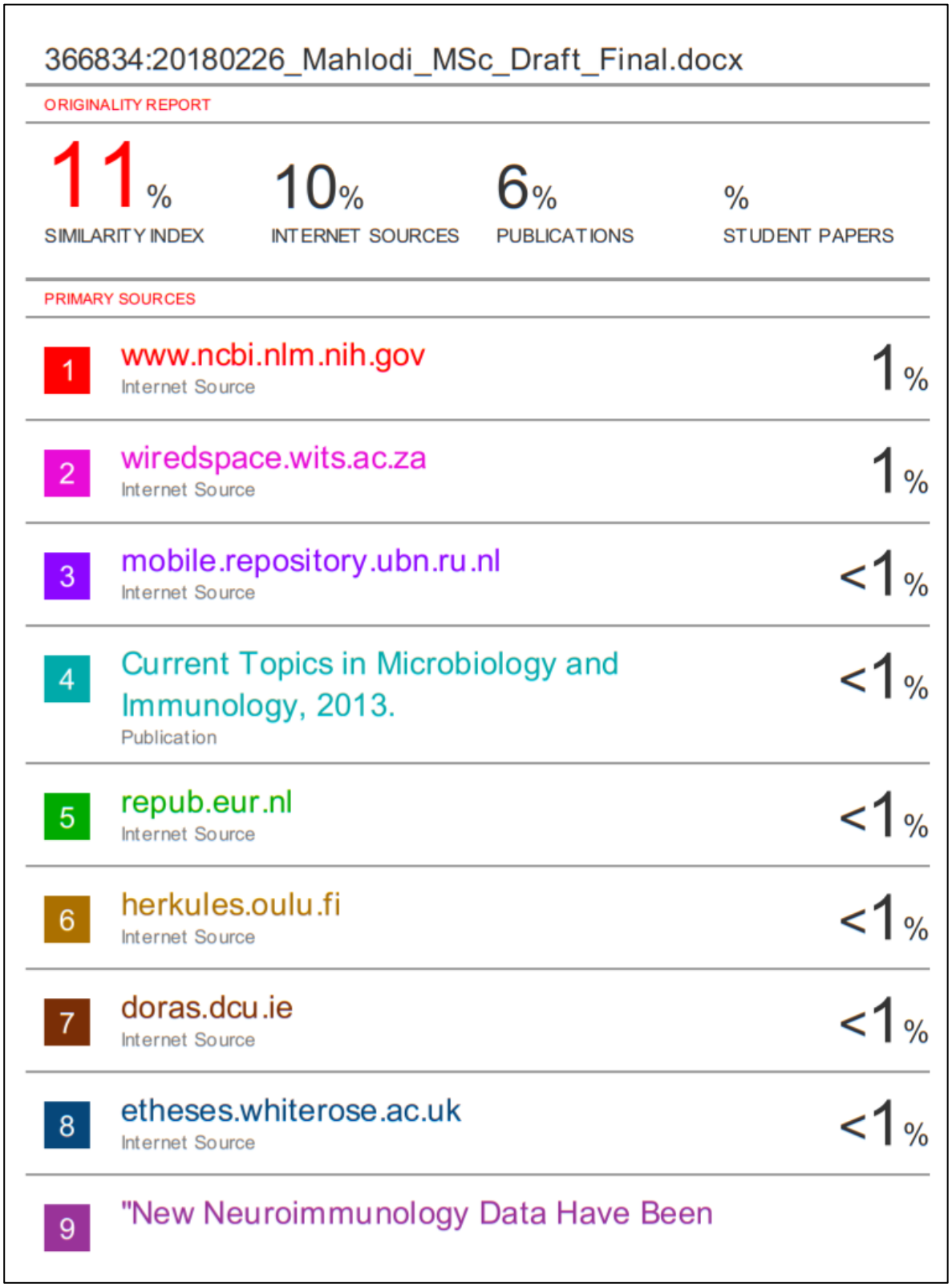


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Figure 2. The Turnitin report showing originality of the dissertation and study contents.

Appendix C: Respiratory Index of Severity in Children (RISC) Score

The RISC score was used to group RSV infected infants into mild or severe disease. HIV positive and negative infants were scored accordingly as shown below. RISC score was obtained from Reed *et al.* (2012) (150).

HIV non-infected children

Severity of respiratory signs on physical exam:

1. Oxygen supplementation?	Yes/No:	3 points
2. Does the child have chest indrawing?	Yes/No Indrawing:	2 points
3. Does the child have wheezing?	Yes/No Wheezing:	-2 points
4. Has the child been refusing feedings?	Yes/No Refusal to feed:	1 point

Growth standards:

5. Weight for age ≤ 3?	Yes/No	2 points
6. Age ≤ 90 days?	Yes/ No	1 point

Total points: _____

(Maximum: 6)

HIV infected children

Severity of respiratory signs on physical exam:

1. Oxygen supplementation?	Yes/No:	2 points
2. Does the child have chest indrawing?	Yes/No indrawing:	1 point
3. Does the child have wheezing?	Yes/No Wheezing:	-1 points
4. Has the child been refusing feedings?	Yes/No Refusal to feed:	1 point

Growth standards:

5. Age	0–2 months:	2 points
	3–12 months:	1 points
	12 months:	0 points
6. HIV Clinical Classification	C:	2 points
	A/B:	1 point
	None:	0 points

Total points: _____

(Maximum: 7)

Appendix D: Reagent and sample preparation for assays

Wash solution preparation

A wash solution 10X concentrate that had formed a precipitate was dissolved by floating the wash solution 10X concentrate bottle in a 37 °C water bath until the precipitate had dissolved. 10X wash solution concentrate (15ml) were added to 285ml of distilled, autoclaved, filtered water.

Preparing 1 X antibody beads

The 10 X antibody bead concentrate was vortexed for approximately 1 minute prior to use. This action deviated from the protocol which was suggesting 30 seconds vortex plus 30 seconds of sonication. The frequency of sonication was not provided by the manufacturer and we optioned to vortex longer. 2.5µl of the 10 X antibody bead concentrate was diluted in 25µl of working wash solution to obtain a 1:11 dilution factor. This provided excess volume to make up loss of volume due to pipetting errors that may have accrued during bead transfer to the plate. Each assay well required 25µl of the diluted 1 X antibody bead. During the assay Table 1 below was used to prepare the scaled volume of 1X antibody bead solution from the 10 X antibody bead. Both the stock and working solution were always protected from light. The beads were cross linked to fluorophore which lose fluorescence intensity when exposed to light; loosing fluorescence intensity could result in low levels of cytokines being falsely reported.

Table 1.Preparing volumes of 1X antibody beads working solution

Number of wells to use on the plate	Volume of 10 X antibody bead concentrate (µl)	Volume of working wash solution (ml)
24	60	0.6
32	80	0.8
40	100	1.0
48	120	1.2
56	140	1.4
64	160	1.6
72	180	1.8
80	200	2.0
88	220	2.2
96	240	2.4

The Table was adapted from Human cytokine 10-plex panel user manual page 10 (221).

Standard curve preparation

The lyophilised cytokine standards (S) were reconstituted within 1 hour of performing the assay. To reconstitute the cytokine standards, 0.334ml of assay diluent was added to each of the three cytokine standard vials when preparing standards for plasma samples assay.

When preparing standards for NPA samples assay, 0.334ml constituting 50% assay diluent 50% UTM mixture was added to each standard vial. This facilitated the standard matrix to match the NPA cytokines matrix collected into UTM and will be subjected to equal background fluorescence scatter. Plasma and NPA standards were prepared and assayed on separate plates with appropriately diluted standards.

The vial stopper was replaced and allowed to stand for 10 minutes undisturbed. Thereafter the vials were gently swirled and gently inverted 3 times to facilitate complete reconstitution. The standard vials were further allowed to stand at room temperature for another 5 minutes. The cytokine concentration of reconstituted standards from the kit insert is shown below in Table 2. At the end of incubation period, 0.333ml of standard was pipetted from each of the three standard vials into 1 sterile tube and created a multiplex cytokine standard with a total volume of approximately 1ml. This was labelled as standard (S) 1 and had a starting 3-fold dilution factor. This multiplex S1 cytokine standard cocktail included 15 cytokines this study

was investigating. S1 was the highest concentrated standard of the 3-fold 9 serial dilutions that was performed. The starting cytokine concentration of S1 following mixing of the three vials are also shown below in Table 2. Figure 2b below show the preparation of the 7 and 9 series standard curve.

Table 2.Cytokine concentration of reconstituted standards

Vial number & Lot Number	Cytokine name	Reconstituted standard concentration (pg/ml)	S1 concentration (pg/ml)
1- Lot number: A18815	IL-1α	6000	2000
2- Lot number:1470602	MCP-1	27600	9200
	MIP-1α	18250	6083.33
	RANTES	19300	6433.33
3- Lot number: 1654899	IFN-γ	3850	1283.33
	IL-1β	7800	2600
	IL-4	67000	22333.33
	IL-5	6800	2266.67
	IL-6	6850	2283.33
	IL-8	1950	650
	IL-9	2850	950
	IL-10	28650	9550
	IL-12(p70)	8650	2883.33
	IL-13	8950	2983.33
	TNF-α	11250	3750

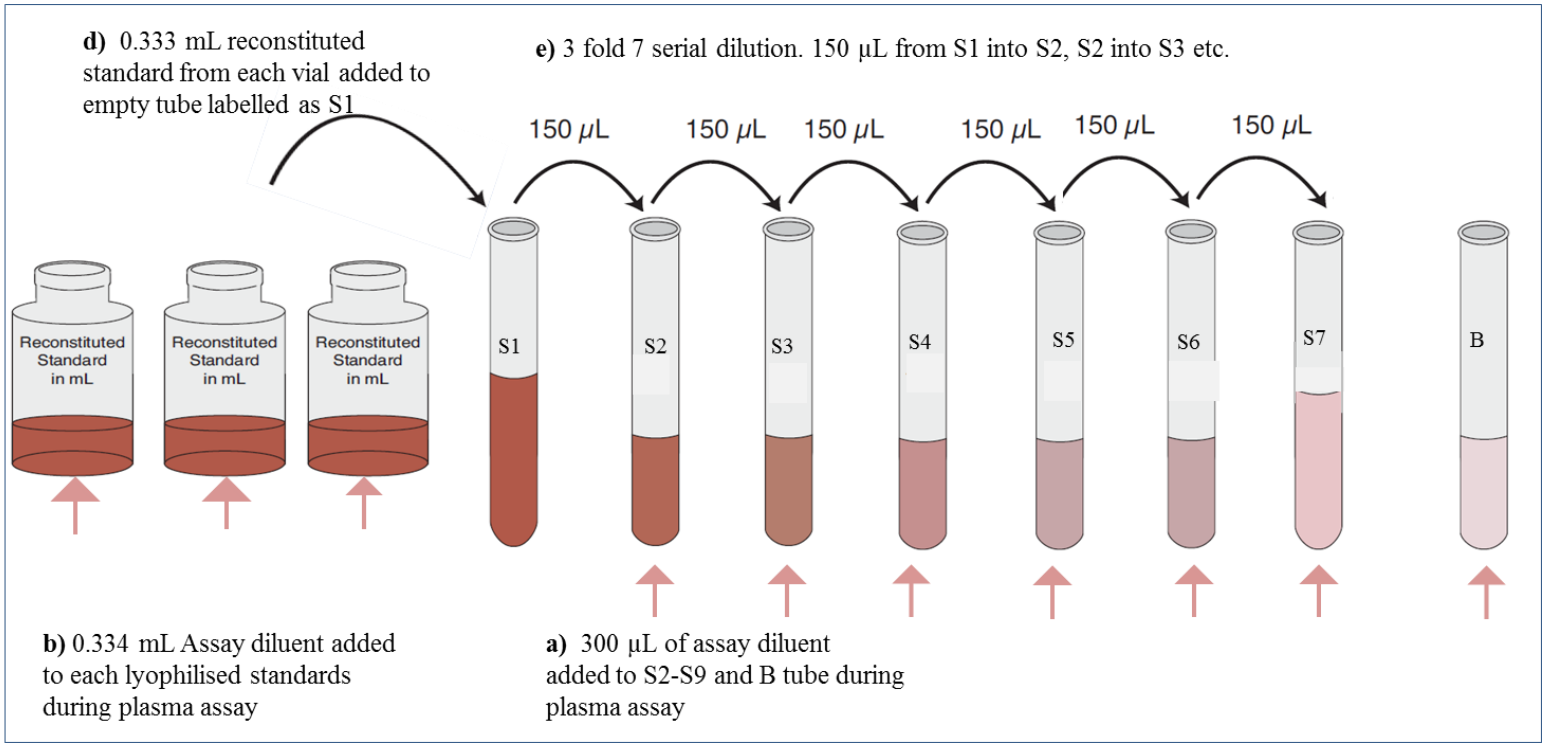


Figure 2a.Preparation of cytokine standard curve for the first four plates.

This Figure was adapted from the protocol insert provided with the kit. The Figure is also present in the Human cytokine 10-plex panel user manual page 9 (221).

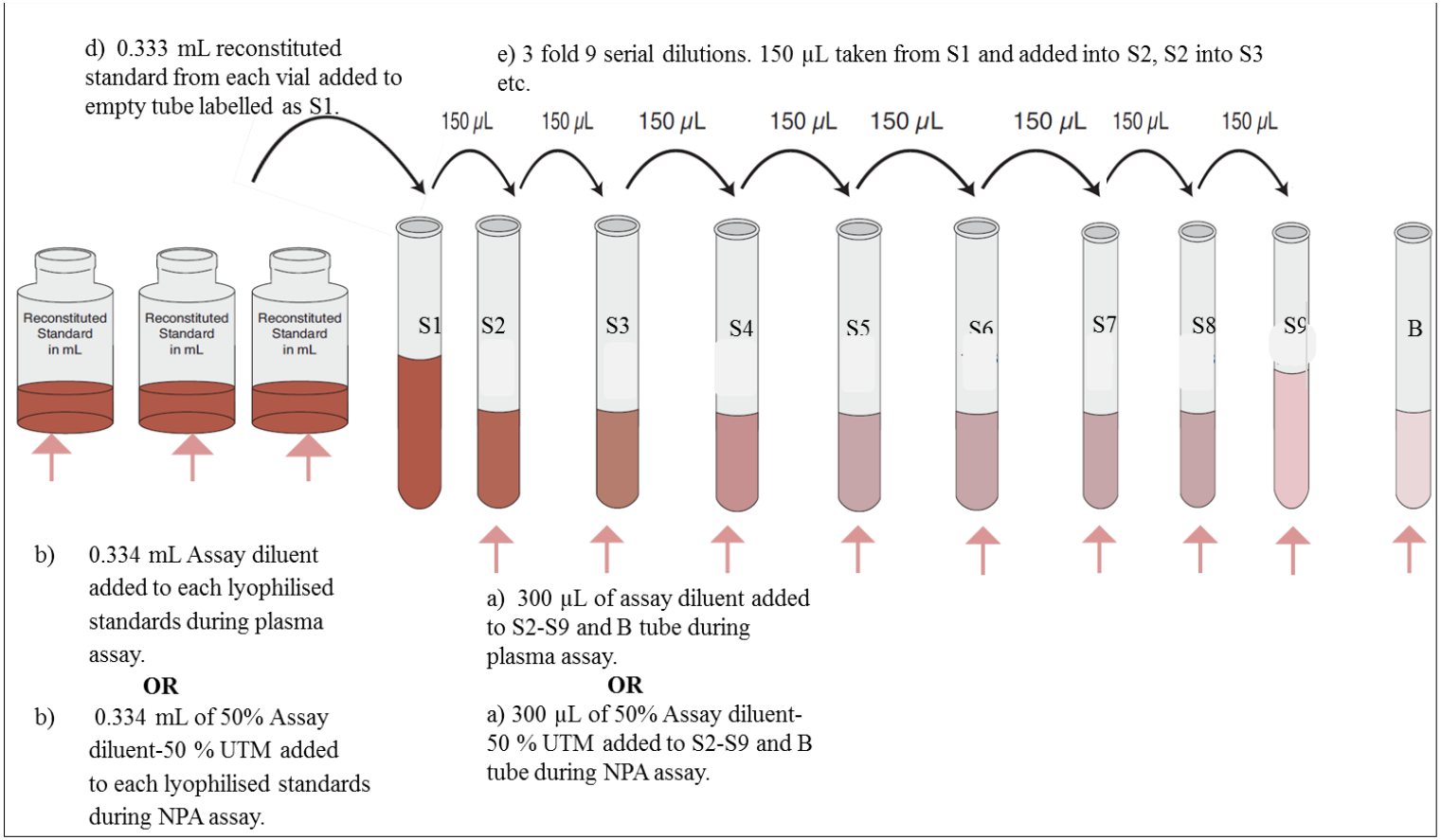


Figure 2b.Cytokine standard preparations used from the fourth plasma plate assay and the entire NPA plate assays.

This Figure was adapted from the Figure provided with the protocol. The Figure is also present in the Human cytokine 10-plex panel user manual page 9 (221).

Sample preparation for immunoassay

Previously prepared plasma or NPA supernatant required for one assay analysis were removed from the -80 ˚C ultra-low freezers and allowed to thaw on ice. The samples were gently mixed by hand to avoid foaming. The plasma samples were diluted 1:1 (50µl of plasma and 50µl of assay diluent provided with the kit) relative to the standards, and the NPA samples were diluted 1,4ml:1ml (70µl of NPA and 50µl of assay diluent). All samples were kept at room temperature until loaded onto the plate. No test sample or cytokine standards were added to the wells designated for the blank samples.

Plate layout

The diagram below shows the plate layout that was used during the assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	S6	S2	X3	X7	X11	X15	X19	X23	X27	X31	X35
B	B	S6	S2	X3	X7	X11	X15	X19	X23	X27	X31	X35
C	S9	S5	S1	X4	X8	X12	X16	X20	X24	X28	X32	X36
D	S9	S5	S1	X4	X8	X12	X16	X20	X24	X28	X32	X36
E	S8	S4	X1	X5	X9	X13	X17	X21	X25	X29	X33	X37
F	S8	S4	X1	X5	X9	X13	X17	X21	X25	X29	X33	X37
G	S7	S3	X2	X6	X10	X14	X18	X22	X26	X30	X34	X38
H	S7	S3	X2	X6	X10	X14	X18	X22	X26	X30	X34	X38

Figure 3. Plate layout showing the position of blank (B), standards (S), and samples (X). Position X38 was allocated to spiked control sample.

Preparing 1 X Biotinylated Antibody

The biotinylated antibody was provided with the kit as a 10 X concentrate. 10 X biotinylated antibodies (1ml) was diluted in 10ml of biotin diluent to obtain a 1 X working solution with 1:11 dilution factor which covered for extra pipetting error. Table 3 and equation 2 below were used to prepare the required volume of 1X biotinylated antibody according to the number of assay wells to be used during the assay; each well required 100µl of the 1X biotinylated antibodies.

Table 3. Preparing 1 X biotinylated antibody working solutions.

Number of wells to be used on the plate	Volume of 10X biotinylated antibody (µl)	Volume of working wash solution (ml)
24	240	2.4
32	320	3.2
40	400	4.0
48	480	4.8
56	560	5.6
64	640	6.4
72	720	7.2
80	800	8.0
88	880	8.8
96	960	9.6

The Table was adapted from Human cytokine 10-plex panel user manual page 13 (221).

Streptavidin-PE preparation

The streptavidin-PE was supplied as a 10 X concentrate and had to be diluted into a working solution before use. This reagent was prepared 15 minutes before the end of cytokine detection incubation period and was continuously protected from light. 10 X streptavidin-PE (10µl) was diluted in 100µl of streptavidin-PE diluent to obtain a 1X working solution. The dilution factor was 1:11 to allow for extra pipetting volumes. Each well required 100µl of the 1 X streptavidin-PE, Table 4 below was used to prepare the 1 X streptavidin-PE according to the number of wells to be used during the assay.

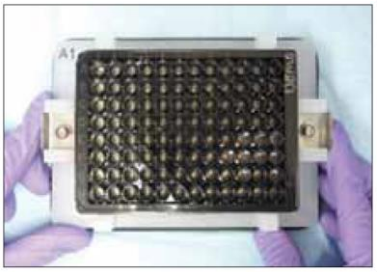
Table 4.Preparing streptavidin-PE working solution

Number of wells to be used on the plate	Volume of 10X streptavidin-PE (µl)	Volume of working wash solution (ml)
24	240	2.4
32	320	3.2
40	400	4.0
48	480	4.8
56	560	5.6
64	640	6.4
72	720	7.2
80	800	8.0
88	880	8.8
96	960	9.6

This Table was adapted from Human cytokine 10-plex panel user manual page 15 (221).

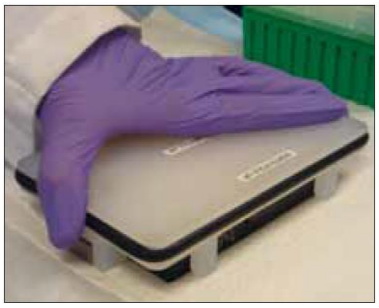
Appendix E: Using handheld magnetic washer

The 96-well flat bottom plate containing assay beads was placed onto the handheld magnetic washer, and safely secured with clips (step 1). The plate was undisturbed for 1 minute to allow the beads settle to the bottom of the plate. The magnetic washer and plate unit were held securely together and turned upside down to decant plate fluids (step 2), followed by gently blotting the plate on dry paper towel (step 3). Blotting the plate removed excess liquid which could cause cross contamination of assay wells. The magnetic washer and plate unit was gently turned to face up and the plate was removed from the handheld magnetic washer before adding a wash solution or any other reagent into assay wells (step 4) (222). These steps are repeated to wash the plate twice.

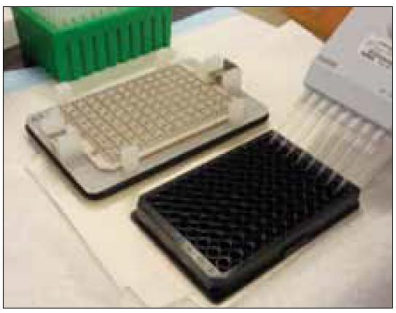


Step 1

Step 2



Step 3



Step 4

Figure 4.Bio-Rad. "Bio-Plex® Handheld Magnetic Washer". Retrieved 2016/09/23, 2016, from http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10023087.pdf

Appendix F: Luminex® 200™ instrument setup

The Luminex® 200™ daily maintenance and calibration was done during the first 45 minutes of cytokine detection incubation period before each assay could be acquired. The machine and computer components were turned-on and the BioPlex Manager 5.0 software was launched. The instrument lasers were warmed up prior to instrument calibration and the user was notified by the system at the end of laser warming session. This was followed by a start-up session which included the priming of the instrument to remove any air bubbles that could be trapped within the fluidics system, and flushing the fluidics with 70% ethanol and distilled water.

The Luminex® 200™ was calibrated with CAL1 and CAL2 (BioRad, USA) control beads on MCV plate IV (BioRad, USA) following the instructions elicited by the software. CAL1 calibrates the classification of magnetic beads while CAL2 calibrates the reporter fluorescence. The instrument was kept in an air-cooled room of approximately 24 °C because the calibration standards are maintained at room temperature changes of not more or less than 2 °C; severe temperature deviation could require the calibration to be redone. One assay plate was acquired daily and the instrument was primed, flushed with alcohol, bleach and distilled water before instrument and computer shutdown.

A once-off Luminex® plate protocol was prepared and saved for the assay acquisition using the reference card provided in the kit. The details of Table 5 and Table 6 below were logged into the instrument to form part of the protocol to be used during each plate acquisition; furthermore the concentration of S1 in Table 2 appendix D were also logged into the protocol to facilitate the generation of the cytokine standard curve with concentrations of varying magnitude. The lot numbers of antibody bead concentrate, biotinylated antibody, and cytokine standards were logged into the assay protocol and saved; the logging of assay reagents lot numbers was verified during each assay. Kits of a single batch number were purchased to minimise inter-assay variation.

Table 5. Instrument setup details for detection and quantitation of cytokines

Luminex ® instrument protocol setup details	
1	Appropriate Bead Region assigned according to Table 3.5 below.
2	Count 100 events/bead regions.
3	Set Minimum Events to 0.
4	Set Sample Size to 75 µL.
5	Set Flow Rate to 60 µL/ minute.
6	Double Discriminator (DD) gate setting of 7,800-20,000.
7.a	Final plasma dilution 2 X at sample acquisition
7.b	Final NPA dilution 2.14 X at sample acquisition
8	Enter S1 concentration (according to Table 3.2)
9	Standard dilution 3 X

Table 6.Cytokines and corresponding bead regions used during instrument setup.

Cytokines and corresponding bead regions										
Cytokine	IL-8	IL-1β	IL-4	IL-10	IL-13	IL-6	TNF-α	RANTES	IL-9	IL-12 (p70)
Bead Region	78	13	77	15	18	19	52	21	44	42
Cytokine	MIP-1α	IFN-γ	IL-1α	MCP-1	IL-5					
Bead Region	26	38	37	29	34					

Appendix G: Calculating Limit of detection for cytokines in plasma and nasopharyngeal aspirate

An average standard curve equation estimated by the Bio-Plex software manager was selected and used to calculate the limit of detection (LOD) of each cytokine. The LOD was determined for cytokines in both the plasma and nasopharyngeal aspirates. The LOD for each of the 15 cytokines was determined as the concentration at the "average background MFI +2SD". For each cytokine in the plasma or nasopharyngeal aspirate, the "average background MFI" was calculated as the average of MFI of all the blank samples. The LOD concentration was obtained by solving the 5 parameter logistic regression equation for "X" following substitution of "average background MFI +2SD" into the same equation as shown below in Table 8.

Table 8.LOD (pg/ml) calculations for plasma and nasopharyngeal aspirate assays

	Bio-Plex Estimated equation: FI=d+ (a-d)/⟦[1+(X/C)^b]⟧^g	**FI+2SD**	**x = LOD**
Plasma cytokines			
IFN-γ	FI = 2.03368 + (23363.0 - 2.03368) / ((1 + (x / 1874.89)^-1.00197))^1.01634	12.856	0.998
IL-1α	FI = 5.45600 + (169838 - 5.456) / ((1 + (x / 5.07881)^-0.188936))^10	283.792	8.918
IL1-β	FI = -6.72648 + (7971.38 + 6.72648) / ((1 + (x / 1503.09)^-1.33452))^0.659796	259.255	31.723
IL-4	FI = -1.20993 + (12416.2 + 1.20993) / ((1 + (x / 4563.27)^-2.06149))^0.436223	16.798	3.182
IL-5	FI = 5.22173 + (25892.9 - 5.22173) / ((1 + (x / 944.779)^-1.48111))^0.692957	9.905	0.213
IL-6	FI = 3.88705 + (14411.9 - 3.88705) / ((1 + (x / 676.063)^-1.57927))^0.608209	20.634	0.595
IL-8	FI = 8.37847 + (13624.2 - 8.37847) / ((1 + (x / 1314.55)^-1.73642))^0.523127	64.103	3.09
IL-9	FI = 15.5693 + (20424.7 - 15.5693) / ((1 + (x / 296.792)^-1.06792))^0.929186	159.457	2.023
IL-10	FI = -7.00664 + (22161.2 + 7.00664) / ((1 + (x / 2224.86)^-2.19310))^0.38537	13.021	0.557
IL12p70	FI = 1.87292 + (10441.8 - 1.87292) / ((1 + (x / 2253.02)^-1.20309))^1.00452	16.884	10.033
IL-13	FI = -1.42387 + (8444.02 + 1.42387) / ((1 + (x / 541.585)^-1.43615))^0.779843	11.105	1.614
MCP-1	FI = 3.91277 + (12511.0 - 3.91277) / ((1 + (x / 5712.15)^-2.72118))^0.392096	53.14	31.826
MIP-1α	FI = 1.70973 + (7506.49 - 1.70973) / ((1 + (x / 685.627)^-1.29031))^1.00738	18.473	6.272

RANTES	FI = -17.3548 + (9790.39 + 17.3548) / ((1 + (x / 406.862)^-10.5747))^0.0999999	108.112	6.596
TNF-α	FI = 6.46345 + (16615.7 - 6.46345) / ((1 + (x / 1160.09)^-1.76227))^0.57047	12.725	0.456
Nasopharyngeal aspirate cytokines			
IFN-γ	FI = 4.04801 + (1.77129E+007 - 4.04801) / ((1 + (x / 2519.85)^-0.152825))^10	12.534	1.051
IL-1α	FI = 177.297 + (74361.6 - 177.297) / ((1 + (x / 108.618)^-0.287646))^4.94589	437.678	7.769
IL-1β	FI = 108.415 + (9270.49 - 108.415) / ((1 + (x / 1433.07)^-1.42917))^0.716802	207.829	17.343
IL-4	FI = 6.25544 + (14678.4 - 6.25544) / ((1 + (x / 2743.37)^-1.11492))^1.04461	14.735	4.553
IL-5	FI = 3.45616 + (38529.8 - 3.45616) / ((1 + (x / 658.428)^-0.768026))^1.65900	8.732	0.615
IL-6	FI = 1.35022 + (17586.5 - 1.35022) / ((1 + (x / 559.403)^-1.06364))^1.01951	14.417	0.729
IL-8	FI = 41.3175 + (18682.2 - 41.3175) / ((1 + (x / 1114.66)^-0.901495))^1.20898	66.835	2.641
IL-9	FI = 94.4956 + (23623.7 - 94.4956) / ((1 + (x / 308.194)^-0.968606))^1.10066	159.952	1.241
IL-10	FI = -1.59637 + (26787.6 + 1.59637) / ((1 + (x / 1789.14)^-1.13173))^0.873295	11.903	0.824
IL12p70	FI = 3.12984 + (14251.3 - 3.12984) / ((1 + (x / 1902.3)^-0.922135))^1.60872	14.484	15.708
IL-13	FI = 1.05806 + (13009.9 - 1.05806) / ((1 + (x / 186.693)^-0.633489))^2.65011	10.913	2.88
MCP-1	FI = 16.2316 + (12795.1 - 16.2316) / ((1 + (x / 5331.18)^-1.87259))^0.688083	35.952	35.057
MIP-1α	FI = 8.53779 + (8389.62 - 8.53779) / ((1 + (x / 379.757)^-0.940666))^1.76453	18.49	6.719
RANTES	FI = 23.8345 + (10475.8 - 23.8345) / ((1 + (x / 225.173)^-1.47845))^1.03919	57.079	5.347
TNF-α	FI = 1.47873 + (21487.1 - 1.47873) / ((1 + (x / 1270.06)^-1.14732))^0.928141	11.585	0.953

Appendix H: Cytokine standard curves produced during plasma and NPA assays

The standard curves generated during the plasma and NPA assays are shown in the diagrams below. During plate 7 NPA assay the S1 was mistakenly not serially diluted; as a consequence the full reading of the S1-S9 curve were available and only S1 concentration and FI reading were available. The obtained S1 concentration and FI from plate 7 NPA assay for each and every cytokine were used to find the best representative curve among the 6 NPA assays that were already conducted. The NPA plate 7 standard curves for each cytokine were substituted with a standard curve from another NPA assay plate which had similar S1 concentration and FI. The NPA assay/plate number that was used to substitute and interpolate the concentrations of plate 7 NPA assay are shown in the Figure of the respective cytokine standard curve. The substituted NPA plate 7 assays are shown in a dotted black line.

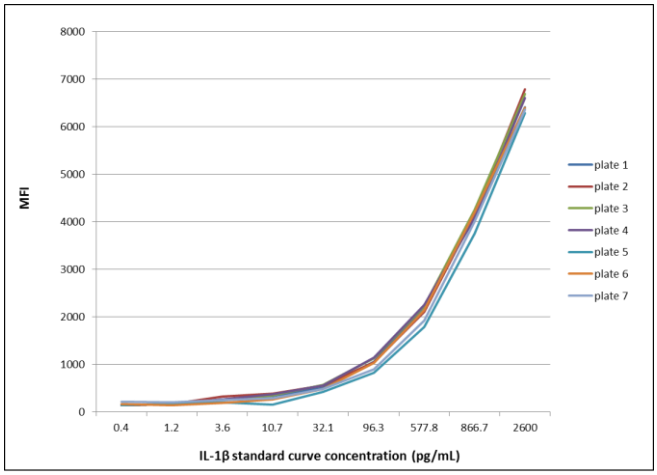


Figure 5.1. Plasma assays IL-1β standard curves

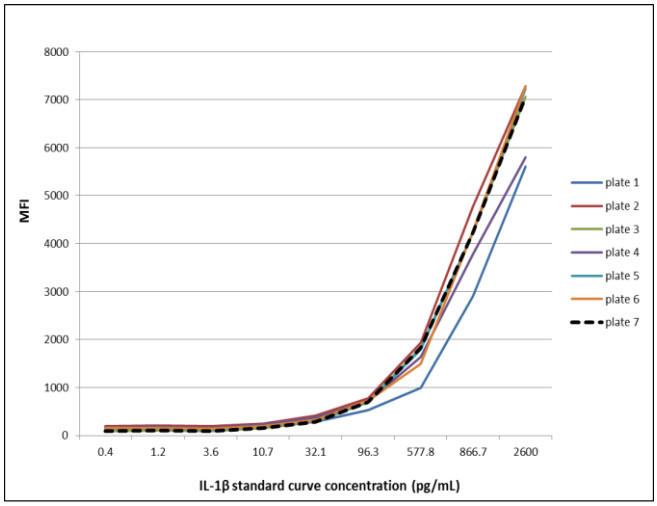


Figure 5.2.NPA assays IL-1β standard curves.

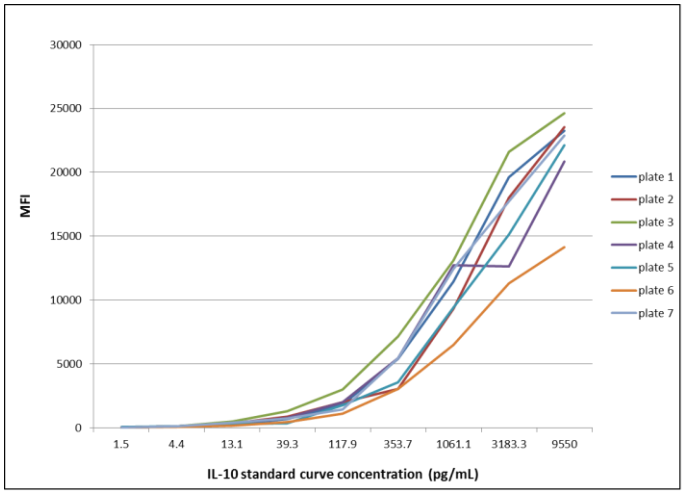


Figure 6.1. Plasma assays IL-10 standard curves.

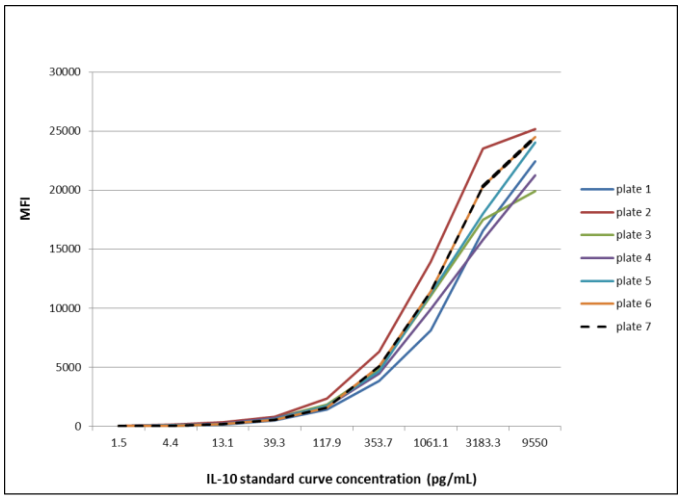


Figure 6.2.NPA assays IL-10 standard curves.

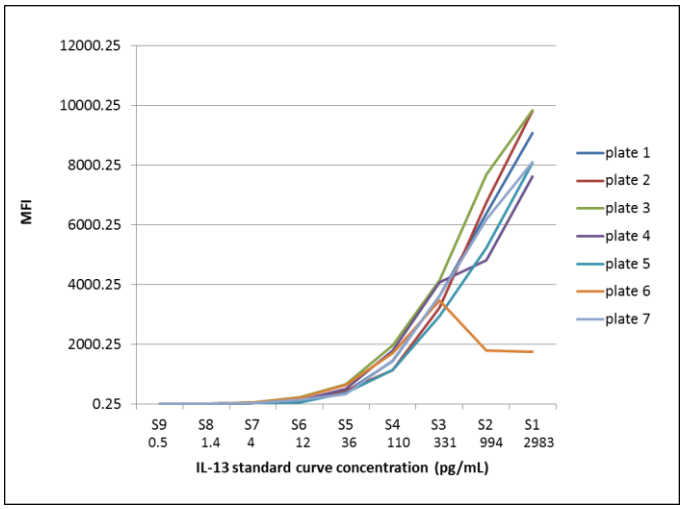


Figure 7.1. Plasma assays IL-13 standard curves

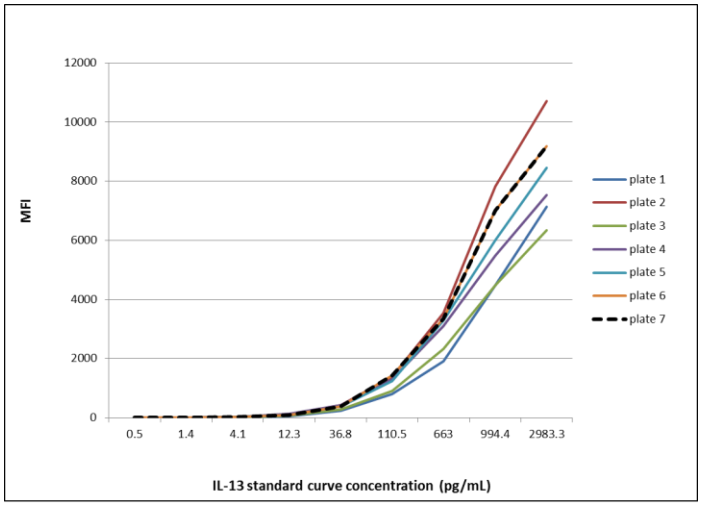


Figure 7.2.NPA assays IL-13 standard curves.

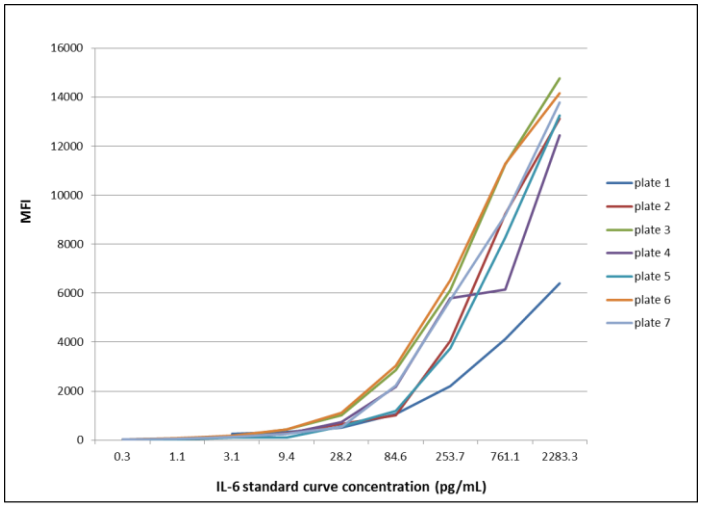


Figure 8.1. Plasma assays IL-6 standard curves.

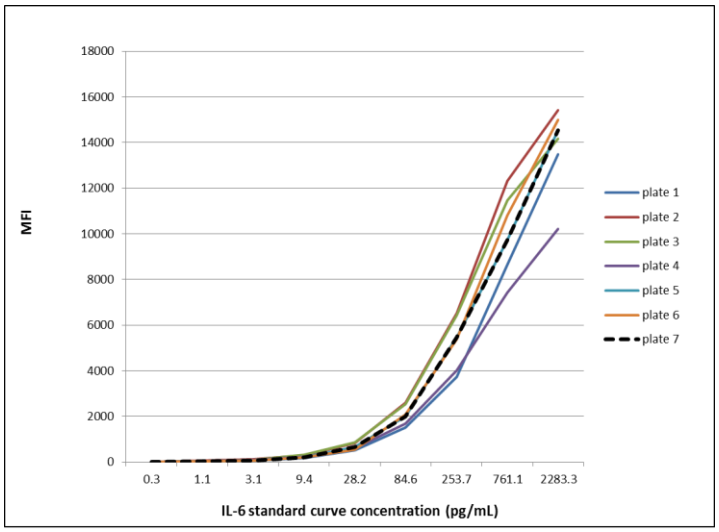


Figure 8.2.NPA assays IL-6 standard curves.

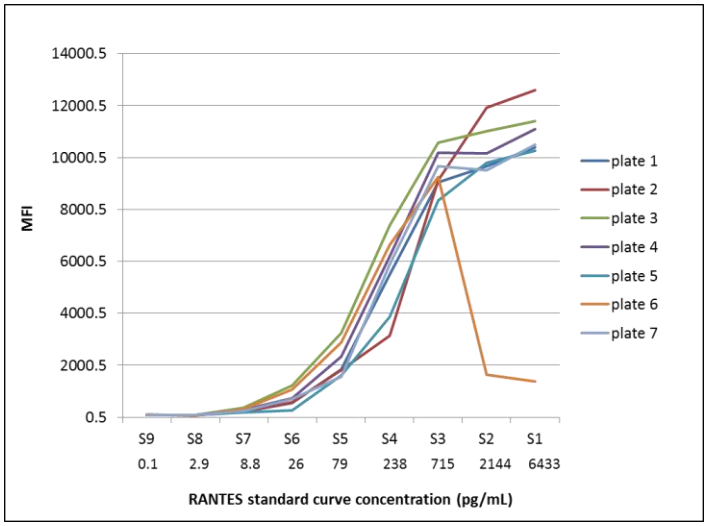


Figure 9.1. Plasma assays RANTES standard curves.

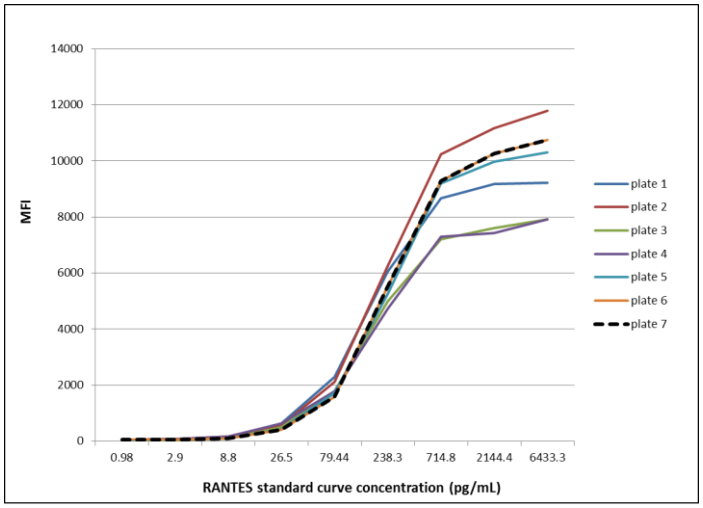


Figure 9.2.NPA assays RANTES standard curves.

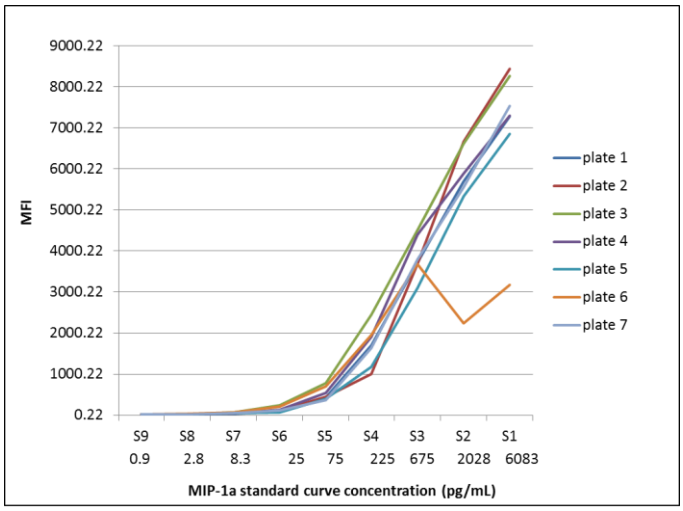


Figure 10.1. Plasma assays MIP-1α standard curves.

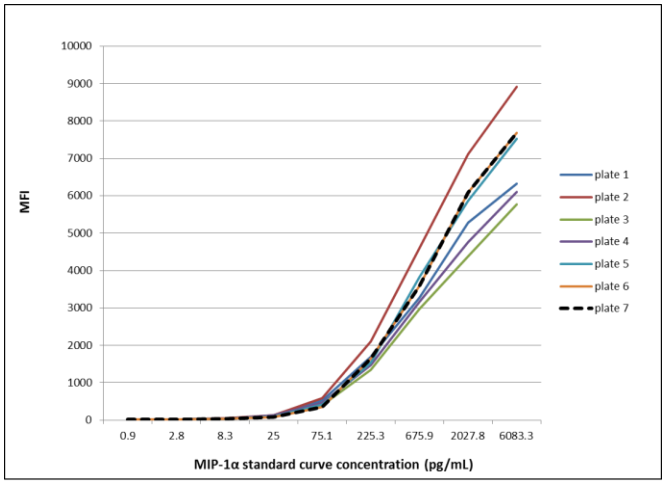


Figure 10.2.NPA assays MIP-1α standard curves.

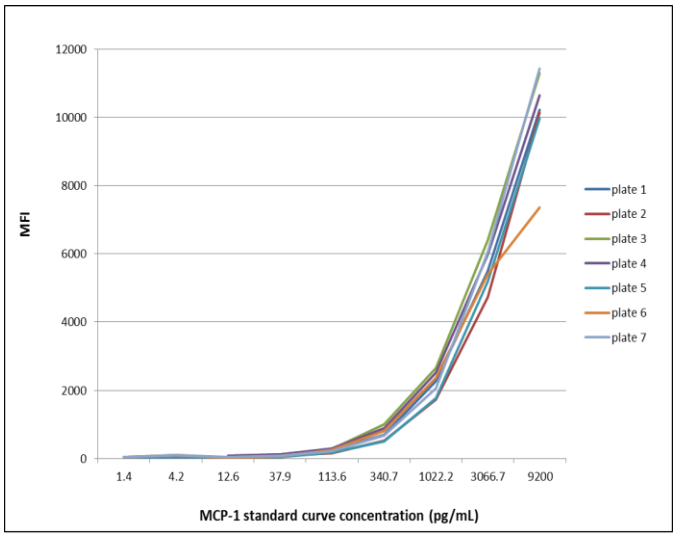


Figure 11.1. Plasma assays MCP-1 standard curves.

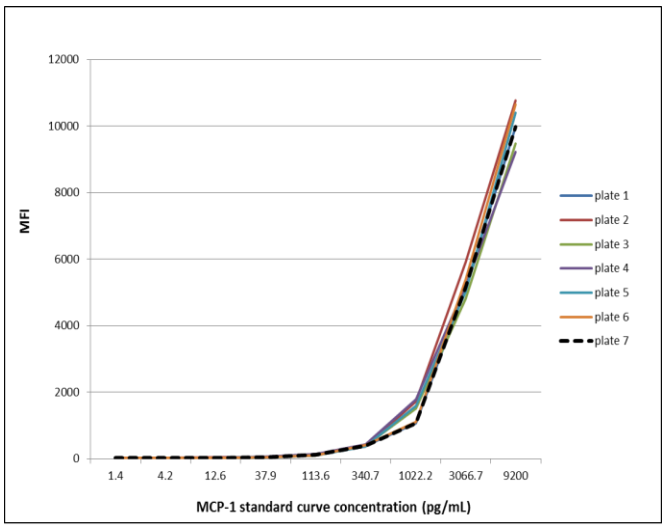


Figure 11.2.NPA assays MCP-1 standard curves.

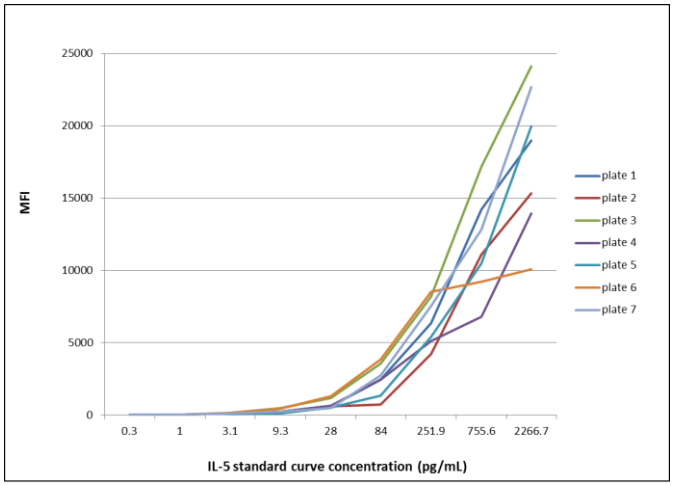


Figure 12.1. Plasma assays IL-5 standard curves.

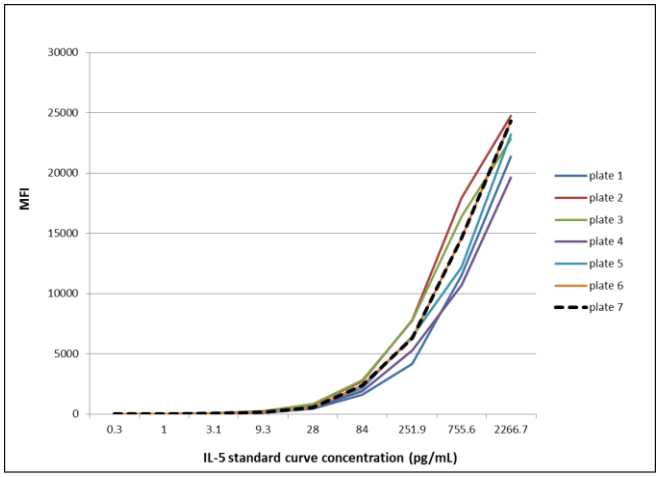


Figure 12.2.NPA assays IL-5 standard curves.

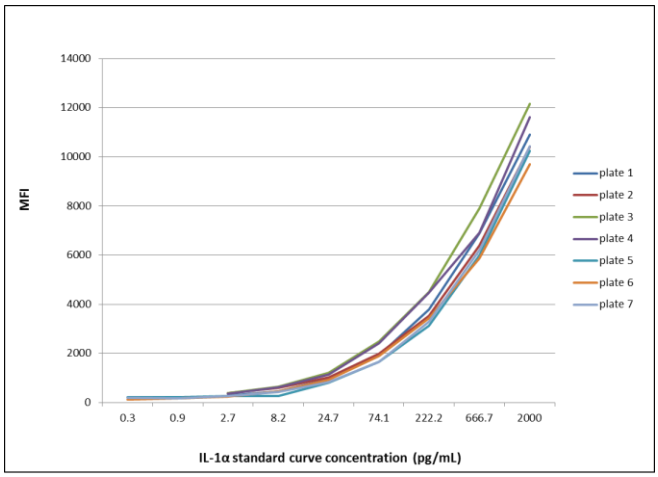


Figure 13.1. Plasma assays IL-1α standard curves.

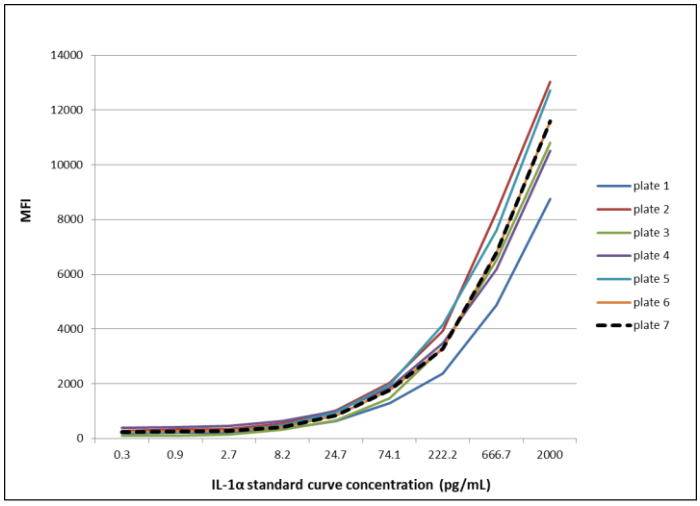


Figure 13.2.NPA assays IL-1α standard curves.

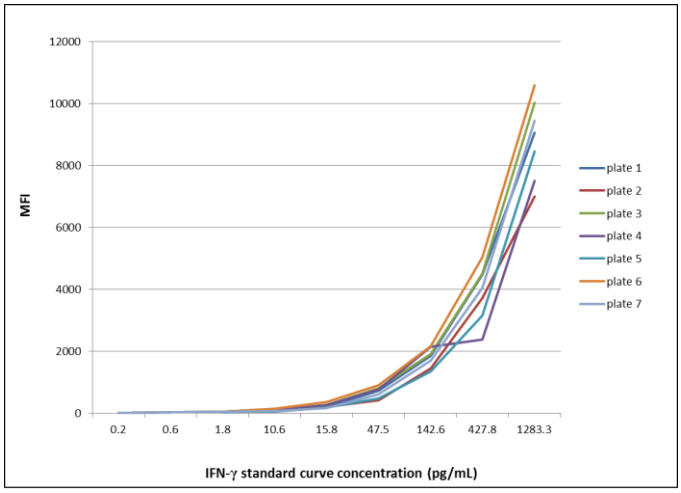


Figure 14.1. Plasma assays IFN-γ standard curves.

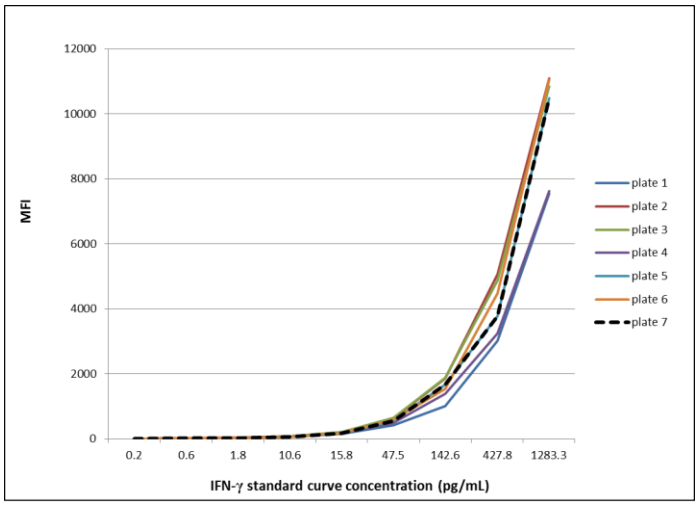


Figure 14.2.NPA assays IFN-γ standard curves.

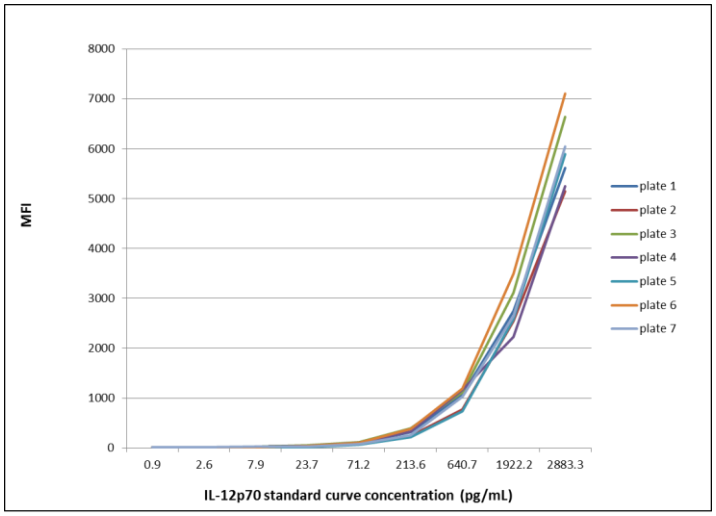


Figure 15.1. Plasma assays IL-12p70 standard curves.

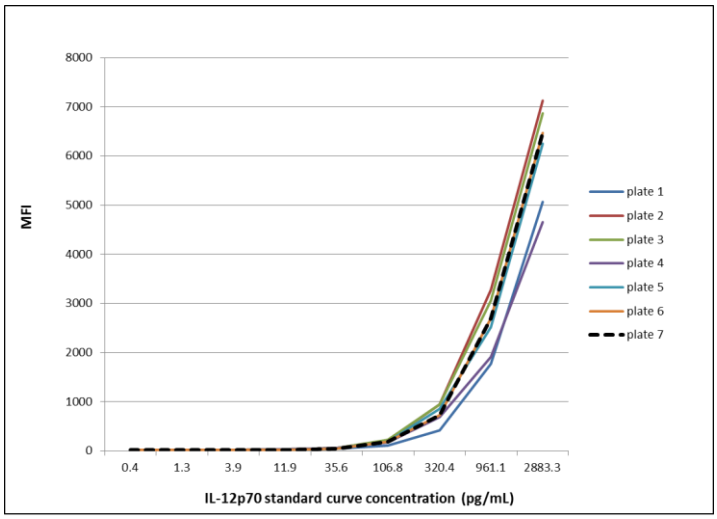


Figure 15.2.NPA assays IL-12p70 standard curves.

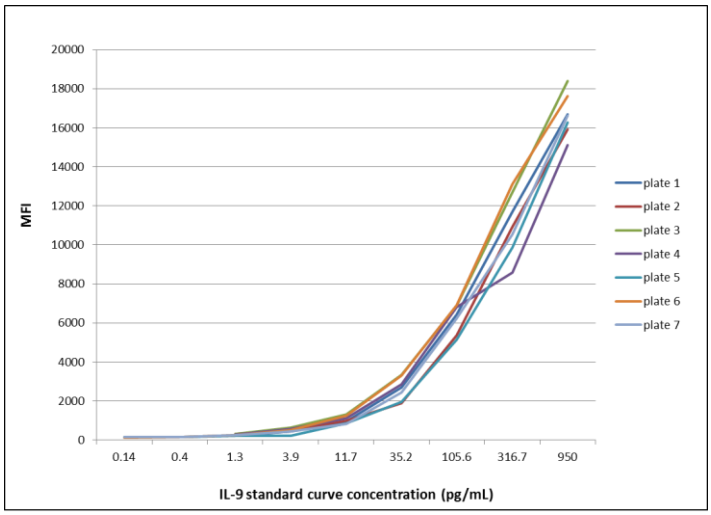


Figure 16.1. Plasma assays IL-9 standard curves.

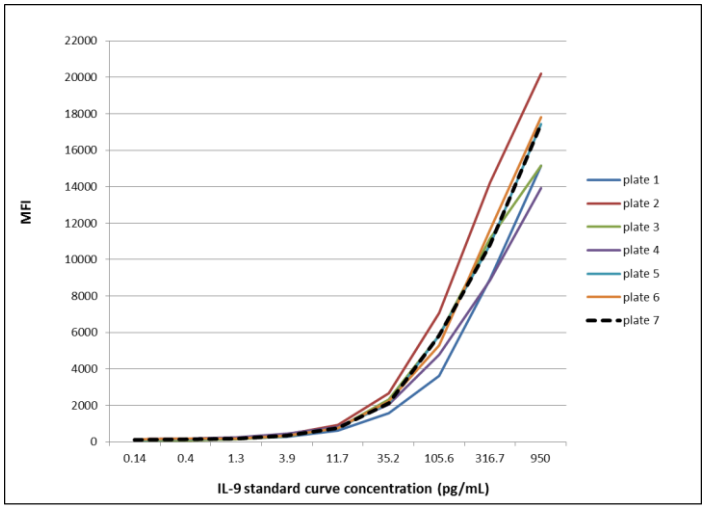


Figure 16.2.NPA assays IL-9 standard curves.

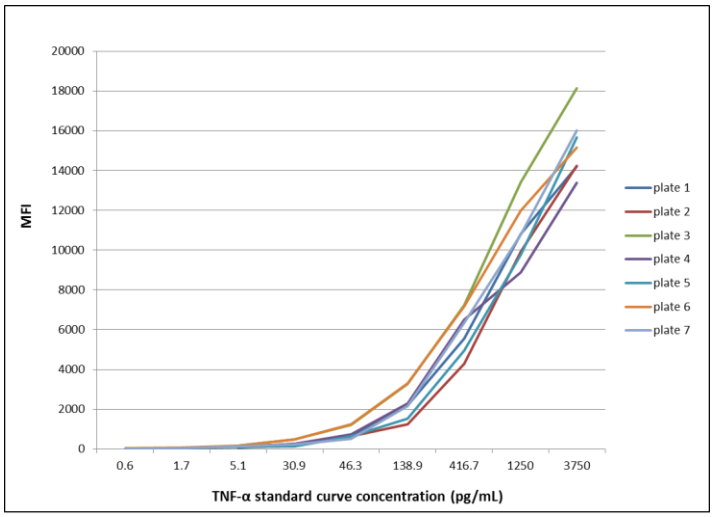


Figure 17.1. Plasma assays TNF-α standard curves.

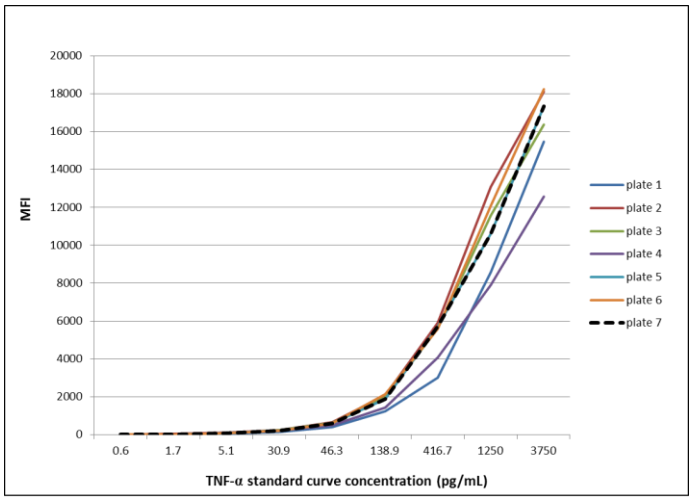


Figure 17.2.NPA assays TNF-α standard curves.

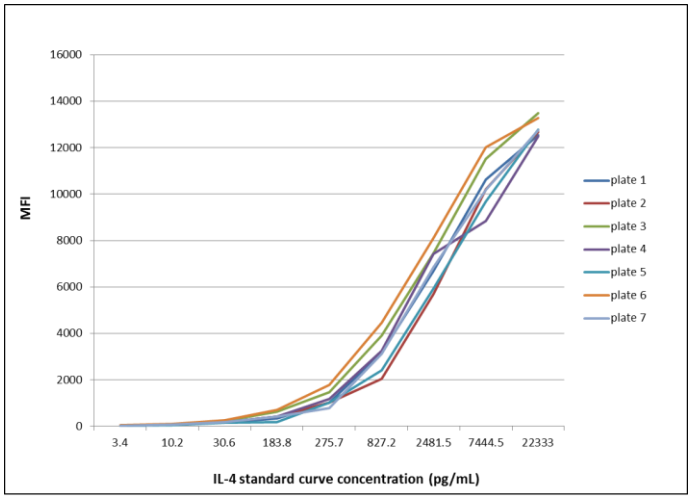


Figure 18.1. Plasma assays IL-4 standard curves.

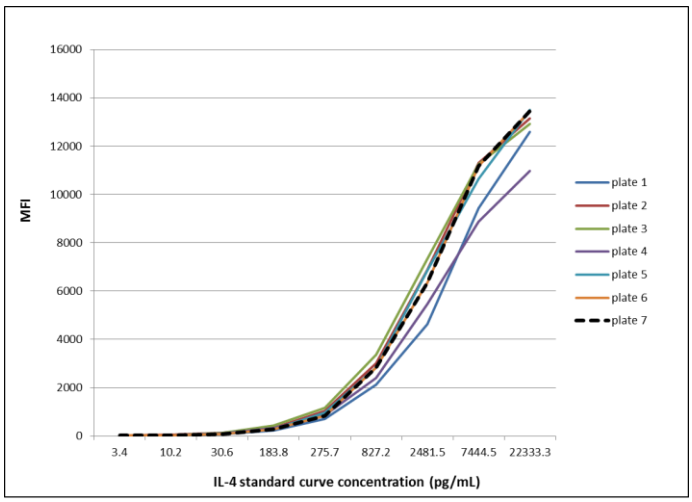


Figure 18.2.NPA assays IL-4 standard curves.

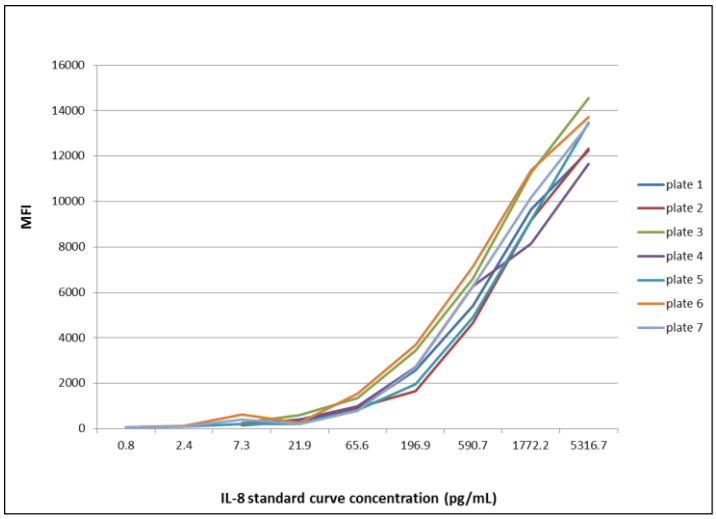


Figure 19.1. Plasma assays IL-8 standard curves.

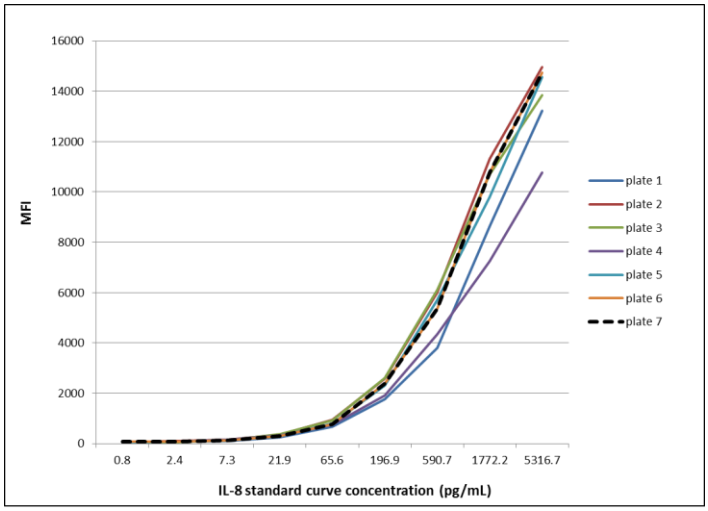


Figure 19.2.NPA assays IL-8 standard curves.

Appendix I: Levey Jennings Charts for Inter assay variability

In all the Levey Jennings charts below; the blue squares show the observed fluorescence intensity, and the orange squares show observed concentration for the particular cytokine within the control sample that was included on each assay plate. All control samples were stored as single use aliquot and each aliquot was freshly thawed and included on each assay plate. Inter-assays that are not within the limits are discussed in the results and discussion section. Plate 1 control plasma sample had reduced concentration in all the cytokines of interest because it was not spiked. After analysis of plate 1 results, it was decided to create high concentration control and previously frozen plasma samples were quickly retrieved and spiked without being thawed (procedure performed on dry-ice). According to Westgard (1981), the assay results can be accepted provided that the control observations were affected by a justifiable event such as changing the control sample.

Levey Jennings Charts: Inter assay variability for 15 cytokines during plasma assays

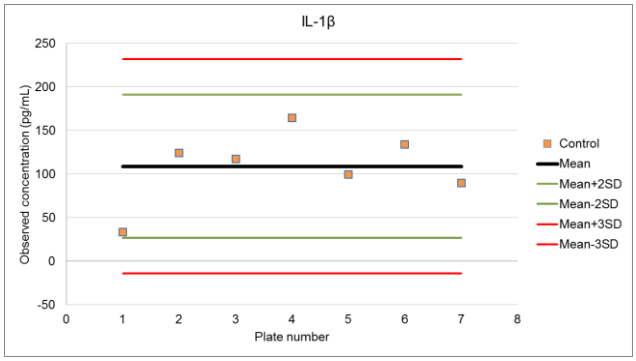


Figure 20.1.Observed concentrations: inter assay variability for IL-1β during 7 plasma assays.

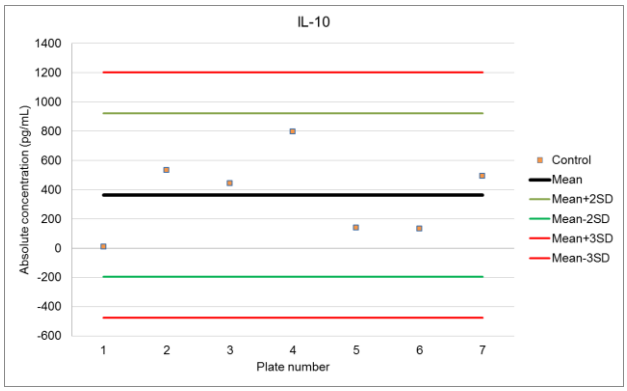


Figure 20.2.Observed concentrations: inter assay variability for IL-1β during 7 plasma assays.

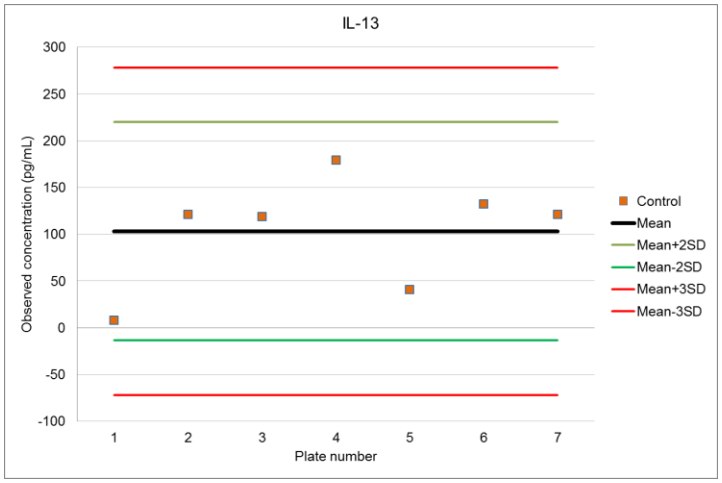


Figure 20.3.Observed concentrations: inter assay variability for IL-13 during 7 plasma assays.

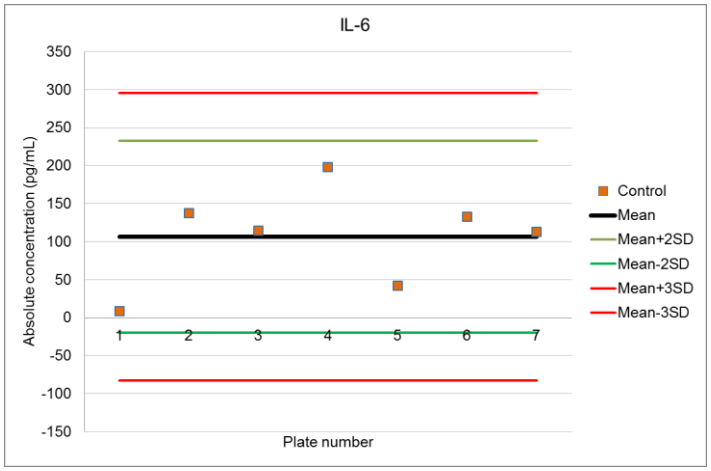


Figure 20.4.Observed concentrations: inter assay variability for IL-6 during 7 plasma assays.

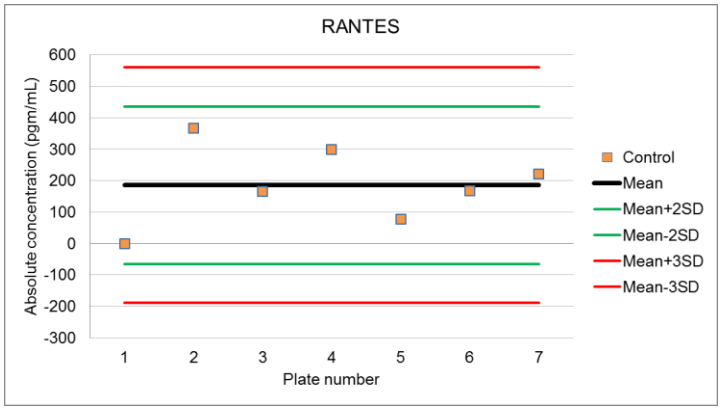


Figure 20.5.Observed concentrations: inter assay variability for RANTES during 7 plasma assays.

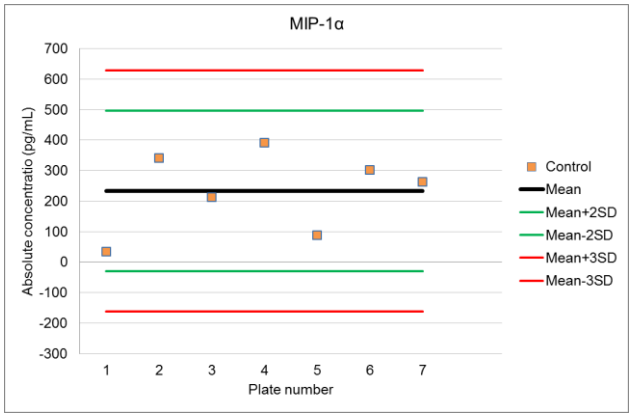


Figure 20.6.Observed concentrations: inter assay variability for MIP-1α during 7 plasma assays.

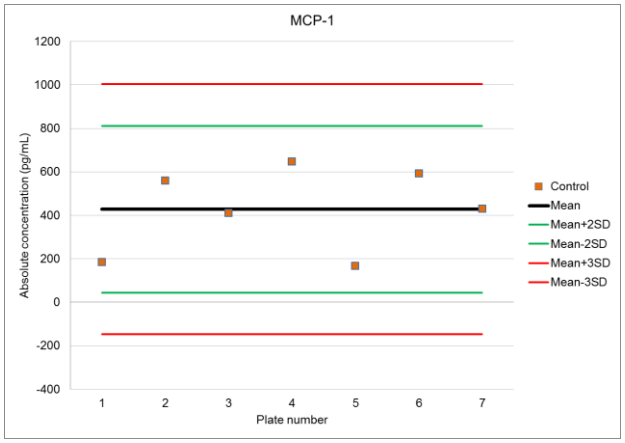


Figure 20.7.Observed concentrations: inter assay variability for MCP-1 during 7 plasma assays.

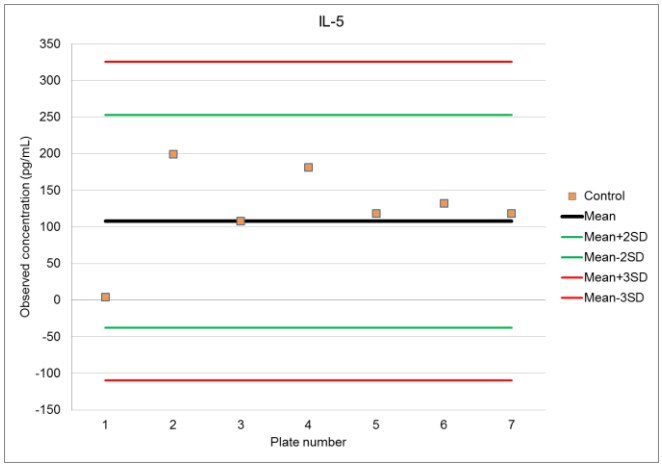


Figure 20.8.Observed concentrations: inter assay variability for IL-5 during 7 plasma assays.

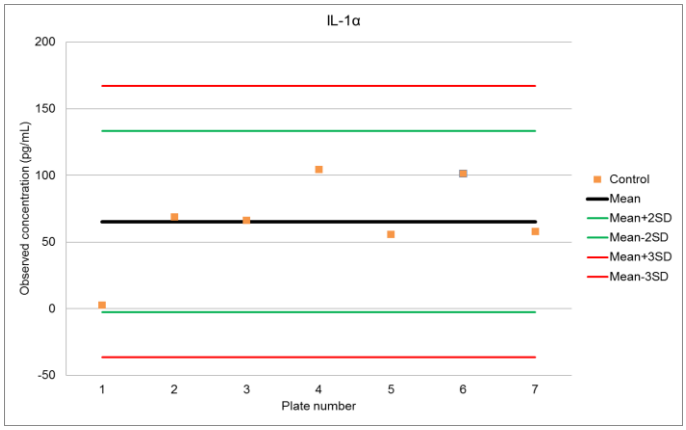


Figure 20.9.Observed concentrations: inter assay variability for IL-1α during 7 plasma assays.

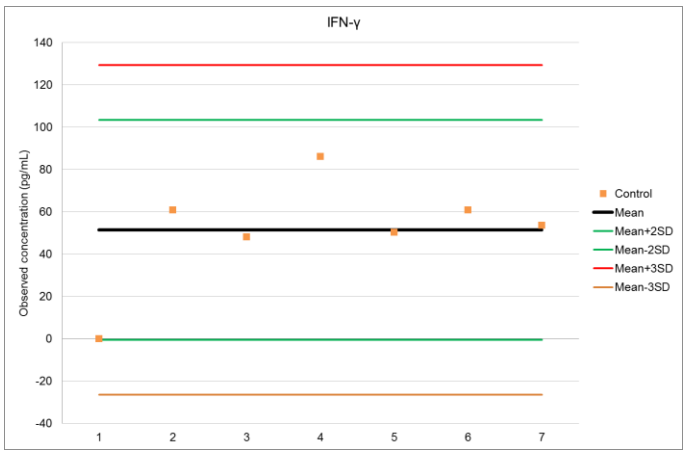


Figure 20.10.Observed concentrations: inter assay variability for IFN-γ during 7 plasma assays.

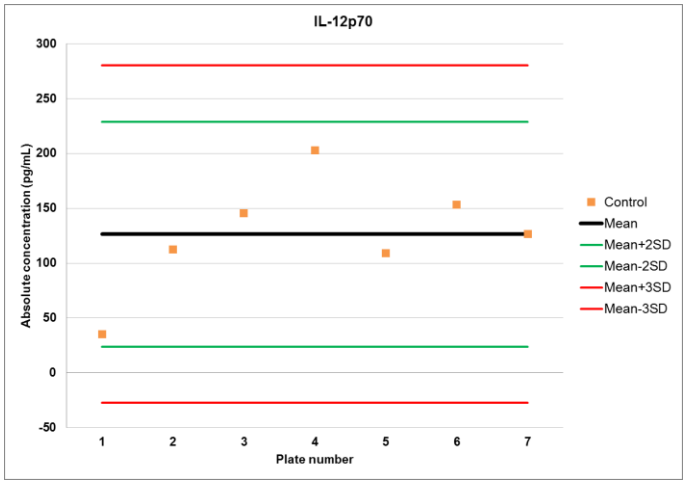


Figure 20.11.Observed concentrations: inter assay variability for IL-12p70 during 7 plasma assays.

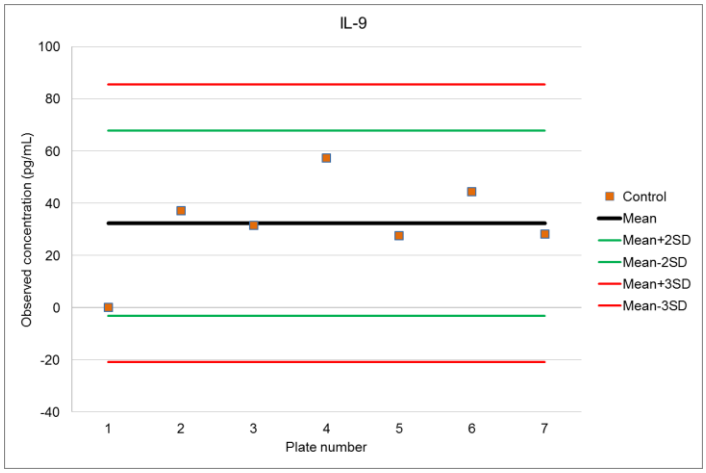


Figure 20.12.Observed concentrations: inter assay variability for IL-9 during 7 plasma assays.

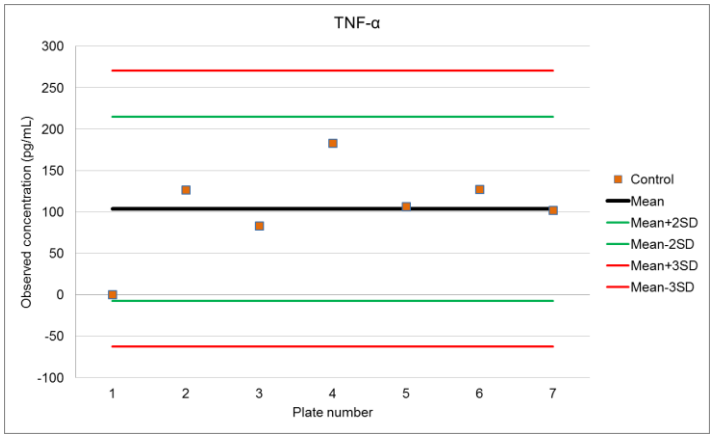


Figure 20.13.Observed concentrations: inter assay variability for TNF-α during 7 plasma assays.

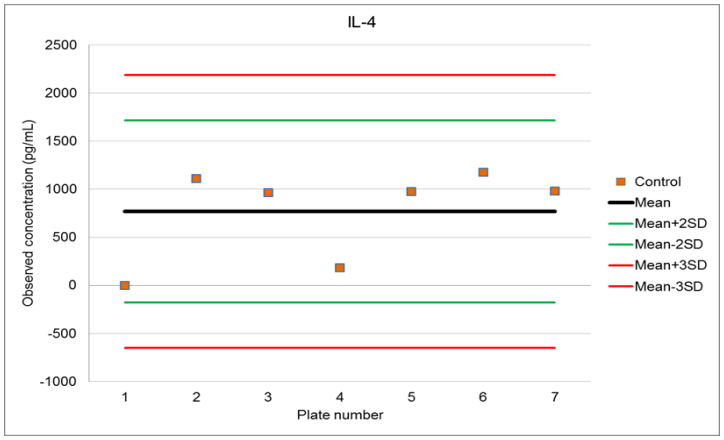


Figure 20.14. Observed concentrations: inter assay variability for IL-4 during 7 plasma assays.

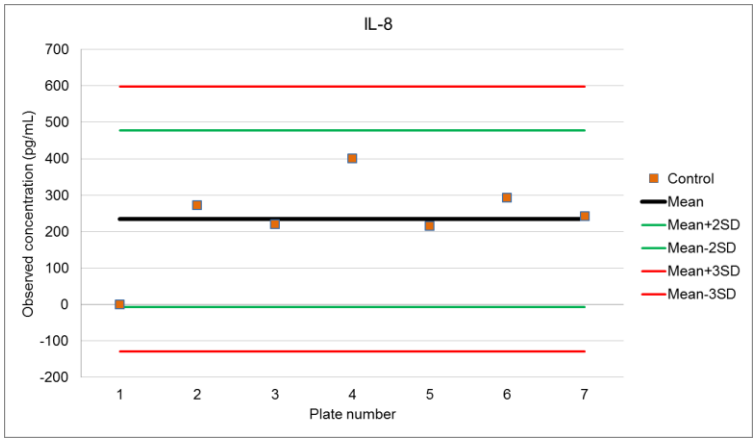


Figure 20.15. Observed concentrations: inter assay variability for IL-8 during 7 plasma assays.

Levey Jennings Charts: Inter assay variability for 15 cytokines during NPA assays

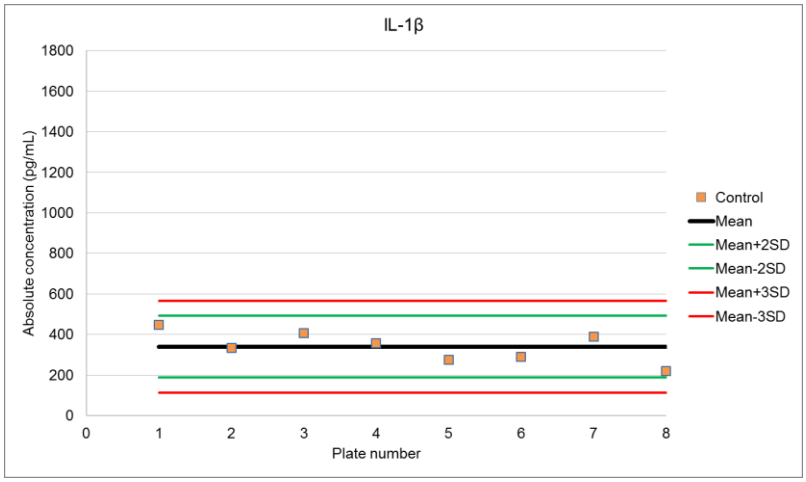


Figure 21.1. Observed concentrations: inter assay variability for IL-1β during 7 NPA assays.

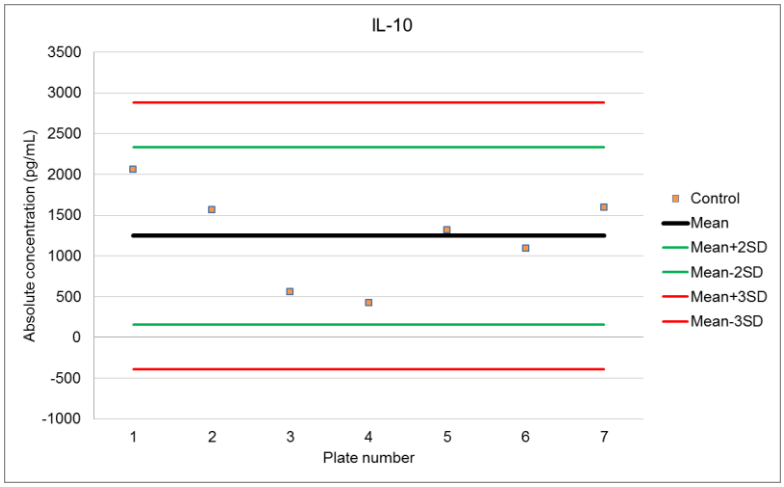


Figure 21.2. Observed concentrations: inter assay variability for IL-10 during 7 NPA assays.

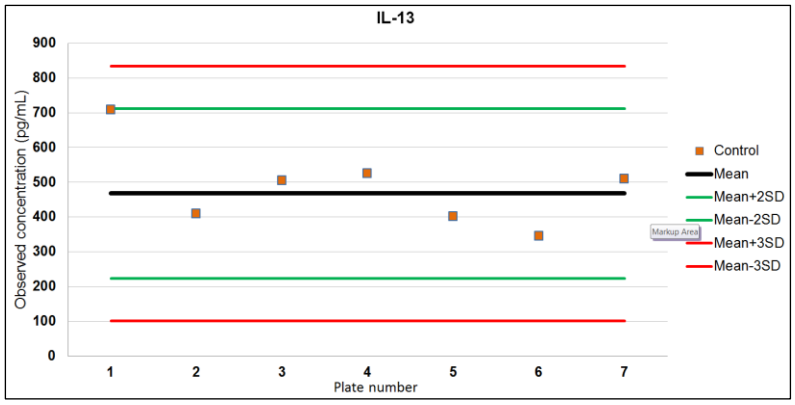


Figure 21.3. Observed concentrations: inter assay variability for IL-13 during 7 NPA assays.

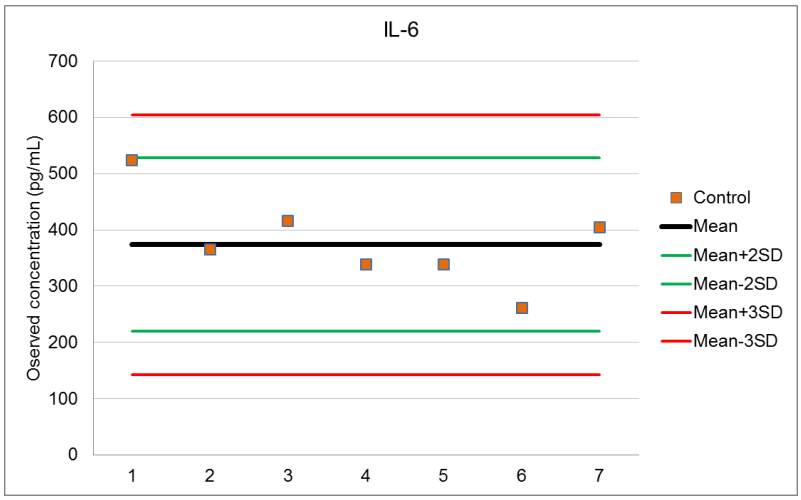


Figure 21.4. Observed concentrations: inter assay variability for IL-6 during 7 NPA assays.

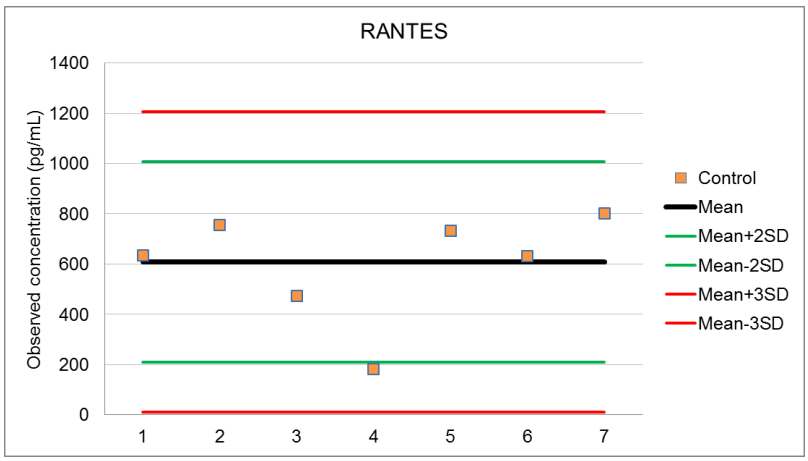


Figure 21.5. Observed concentrations: inter assay variability for RANTES during 7 NPA assays.

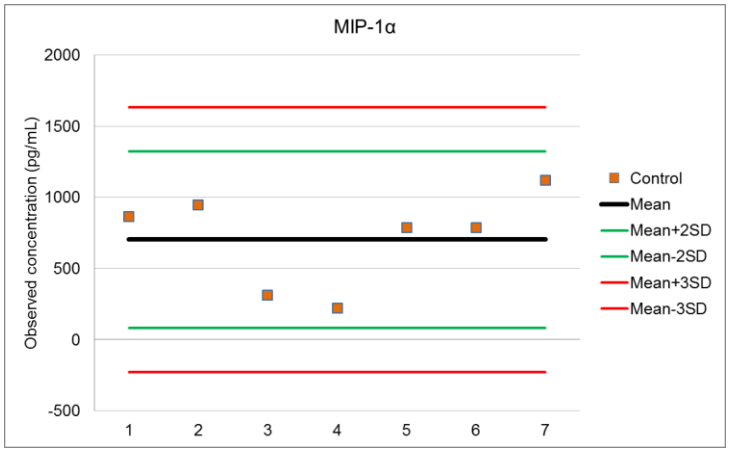


Figure 21.6. Observed concentrations: inter assay variability for MIP-1α during 7 NPA assays.

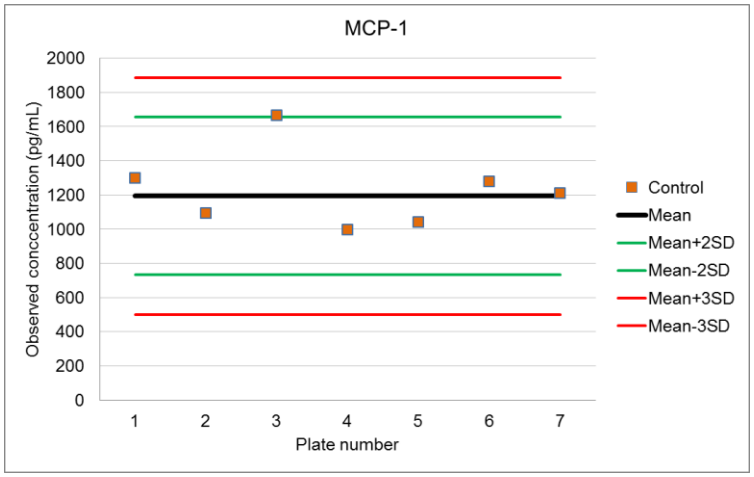


Figure 21.7. Observed concentrations: inter assay variability for MCP-1 during 7 NPA assays.

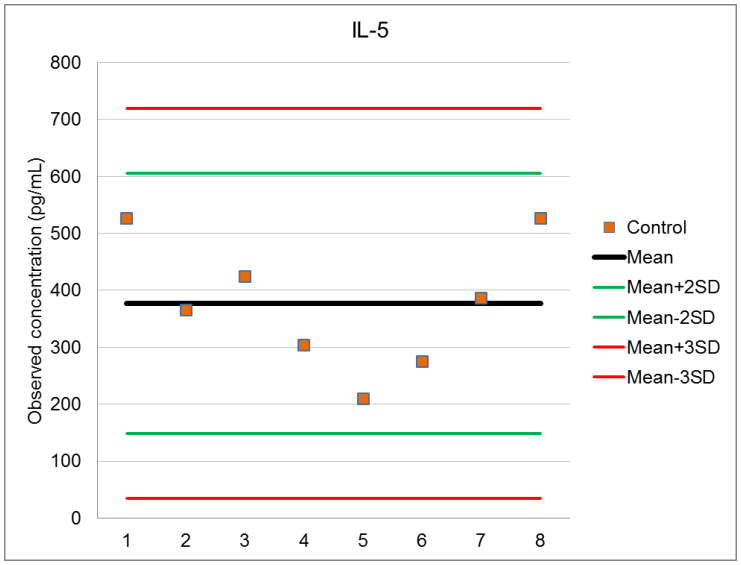


Figure 21.8. Observed concentrations: inter assay variability for IL-5 during 7 NPA assays.

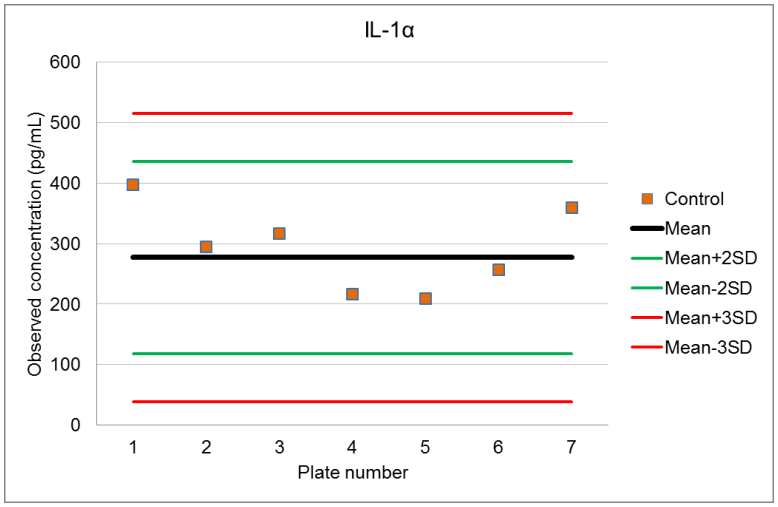


Figure 21.9. Observed concentrations: inter assay variability for IL-1α during 7 NPA assays

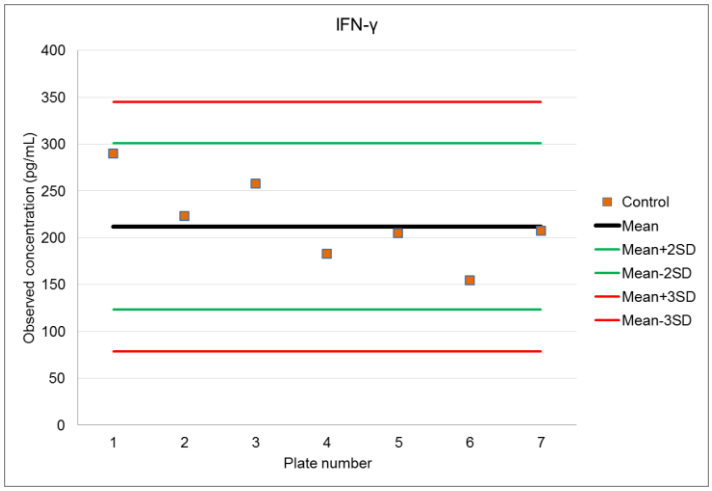


Figure 21.10. Observed concentrations: inter assay variability for IFN-γ during 7 NPA assays

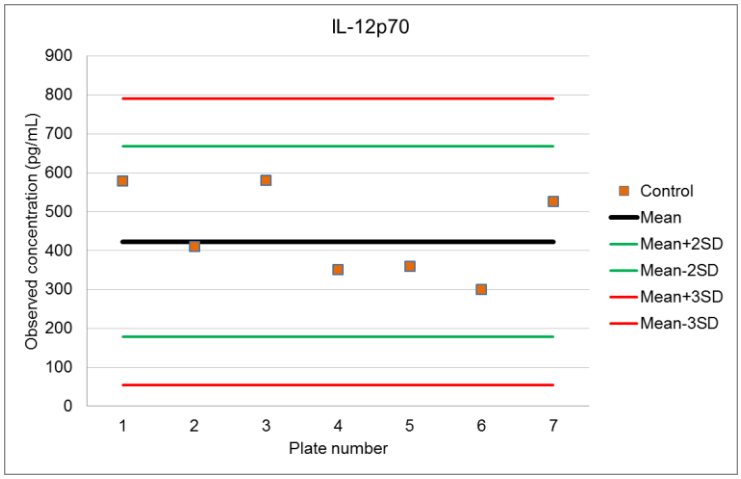


Figure 21.11. Observed concentrations: inter assay variability for IL-12p70 during 7 NPA assays.

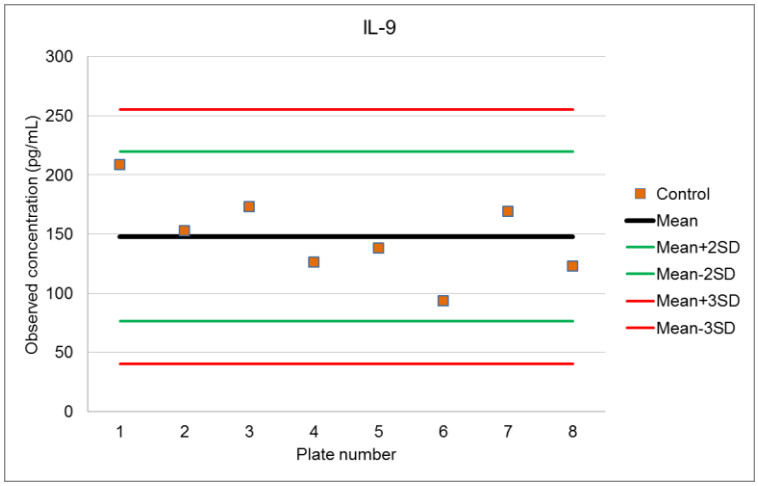


Figure 21.12. Observed concentrations: inter assay variability for IL-9 during 7 NPA assays

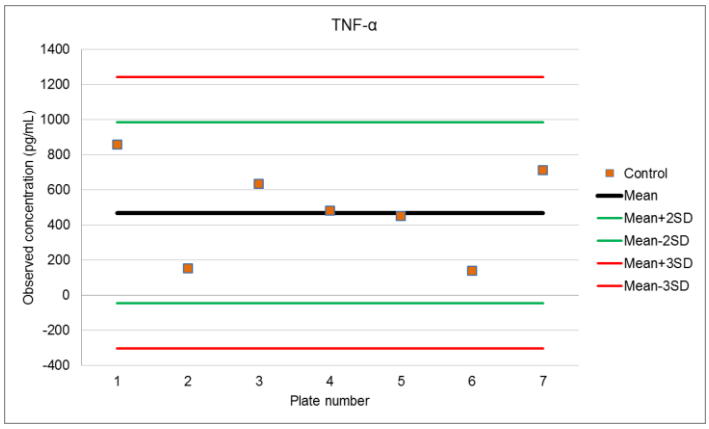


Figure 21.13. Observed concentrations: inter assay variability for TNF-α during 7 NPA assays

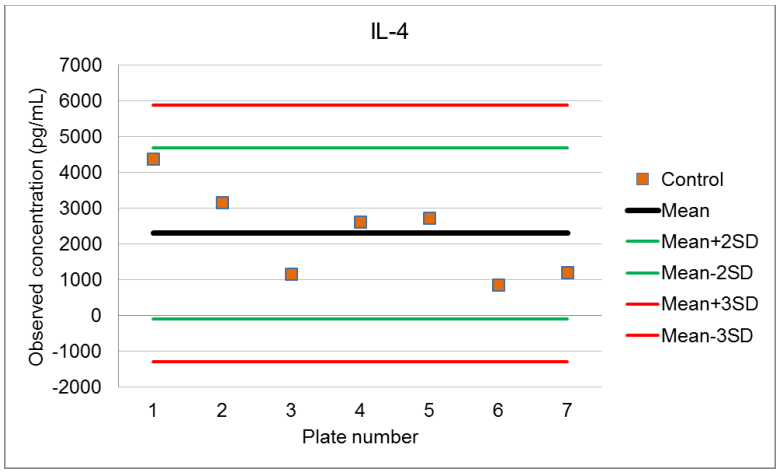


Figure 21.14. Observed concentrations: inter assay variability for IL-4 during 7 NPA assays

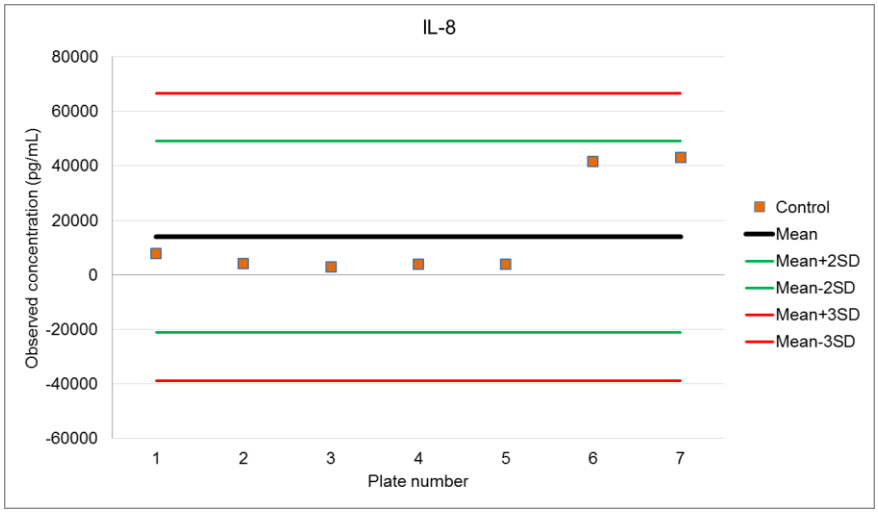


Figure 21.15. Observed concentrations: inter assay variability for IL-8 during 7 NPA assays