

Comparisons of Vitreal Angiogenic, Inflammatory, Profibrotic Cytokines, and Chemokines Profile Between Patients With Epiretinal Membrane and Macular Hole

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Research Article

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Abstract

Objectives: Idiopathic epiretinal membrane (iERM) or idiopathic macular hole (iMH) is frequently used as “healthy” control in comparison of vitreous cytokines with other vitreoretinal diseases. This study aimed to investigate if there is difference in vitreal cytokines expression between patients with iERM and iMH.

Methods: In this prospective study, all subjects received standard pars plana vitrectomy surgery and 0.5 ml of native vitreous sample was extracted during the vitrectomy. Luminex technology and enzyme-linked immunosorbent assay were used to profile the concentration of 52 classic angiogenic, inflammatory, and profibrotic cytokines, and chemokines. Statistical analyses were performed by Mann-Whitney U test, followed with multiple comparisons by Bonferroni correction.

Results: Vitreal samples from 13 iERM and from 24 iMH were studied. Of the 52 tested cytokines, 41 were similar in expression, 5 were under the detection limit, while 6 cytokines ((MMP-8, Eotaxin, MIP-1a, RANTES, TGF- β 2, IL-4)) were differently expressed between two groups ($p < 0.05$). Nevertheless, these significances disappeared after adjustment of Bonferroni correction.

Conclusion: The tested cytokines showed similar expression between iERM and iMH patients. This indicates that eyes with iERM or iMH can be together served as “healthy” controls.

1. Introduction

Human vitreous, a complex and transparent extracellular matrix, is composed of approximately 98% water and 2% solid material, including collagen fibrils, hyaluronic acid molecules and a small amount of proteins and cytokines[1, 2]. Cytokines are important cell-signaling mediators and participate in many conditions[3]. The abnormal expression of vitreal cytokines is acknowledged to be associated with pathogenesis process, including immunological regulations, inflammation, fibrous hyperplasia, neovascularization, repair processes and growth mechanisms[4]. As reported, a number of cytokines in vitreous, such as tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), interleukin-1 β and so on, are involved in different vitreoretinal disease[5–7]. To quantify the cytokines in diseased vitreous, most previous studies employed idiopathic epiretinal membrane (iERM) or idiopathic macular hole (iMH) as negative, or “healthy” controls because the two diseases are typically localized in macular zone[5, 7, 8].

iERM may occur idiopathically or secondly to other vitreoretinal diseases, leading to metamorphopsia and vision loss. Retinal glia, retinal pigment epithelial cells, and hyalocytes altogether consist of the fibrocellular tissue[9, 10]. The occurrence of iERM is closely related with posterior vitreous detachment (PVD), which can interrupt the ILM, permitting glial cell and other cells to migrate and proliferate[9, 10]. The above cellular proliferation and migration is actually promoted by cytokines interaction. Researchers have demonstrated that several cytokines are associated with the pathological membranes in iERM[11]. As for iMH, a full thickness foveal defect, is also responsible for blurred vision, especially central vision

deterioration. It is generally accepted that the development of iMH is secondary to abnormal vitreous-retinal traction[12, 13]. Both proliferation and cell migration play a dominant role in iMH development[14].

Despite patients with iMH or iERM were usually selected as “healthy” control, there remains a paucity of evidence on its reasonability and validity. In fact, publications concerning the cytokine levels in the vitreous of patients with iERM and iMH are still rare and controversial. In 2013, Nakul Mandal *et al*[15] detected none of the 330 tested proteins different significantly between the iERM and iMH vitreous groups, while another study[16] conducted in 2016 found the vitreal levels were significantly higher in iERM than in iMH for 72% of the tested cytokines, indicating that eyes with iERM necessitate a cautious approach to assessing its suitability as a healthy control group[16].

This prospective study was designed to investigate the expression profiles of angiogenic, inflammatory, profibrotic cytokines, and chemokines in the vitreous humor of patients with iERM compared to patients with iMH to evaluate their rationality as a control group. Our results provide a theoretical basis for designing ophthalmology clinical research and may shed light on future etiological study and therapeutic strategies of iERM and iMH.

2. Methods

2.1 Subjects

The study protocol is available at <https://clinicaltrials.gov/> (Registration date: 24/04/2018; Registration ID NCT03506750). The study adhered to the tenets of the Declaration of Helsinki, and was approved by Ethic Committee of First Affiliated Hospital of Nanjing Medical University (2017-SR-283). Informed written consent was obtained from all patients prior to enrollment.

These patients were treated by standard pars plana vitrectomy surgery under local anesthesia at the First Affiliated Hospital with Nanjing Medical University in the period between March and June 2018. The inclusion criteria were as follows: (1) patients were diagnosed with idiopathic ERM and MH by optical coherence tomography (OCT) scan (Carl Zeiss Meditec), and (2) patients with iERM or iMH received standard pars plana vitrectomy surgery and consent was informed. The exclusion criteria were as follows: (1) patients with a history vitrectomy or other eye surgery, and (2) patients with other ocular lesions, such as ocular trauma, uveitis, glaucoma, diabetic retinopathy; and (3) patients with high myopia (< - 6D) or pathological myopia, and (4) patients combined with peripheral retinopathy after thorough examination of the peripheral retinas during surgery.

2.2 Sample collection

All native undiluted vitreous samples (approximately 500 ul) were collected with a vitreous cutter at the start of vitrectomy before intraocular infusion. For patients receiving combined phacoemulsification, vitreous cut and harvest was performed first, following with compensation of balanced salt solution and

phacoemulsification. The vitreous fluids were centrifuged at 1500 rpm for 5 min and then immediately frozen at a temperature of -80°C until assay.

2.3 Luminex

The target cytokines measured in this study were classified into four types, angiogenic, inflammatory, profibrotic cytokines, and chemokines (Fig. 1). The concentrations of 48 cytokines were measured with the assistance of Wayen Biotechnologies (Shanghai), Inc., using Luminex multiplex technology (Bio-Rad) according to the manufacturer's protocol. Using this technology, an array of cytokines can be tested and quantified in a single small volume sample. In brief, 50 μl of provided standard or test sample was added to each well of a 96-well micro-titer plate. After incubation at room temperature, the samples were incubated with diluted biotin antibody, then with Streptavidin-PE followed by wash buffer. Finally, the plate was read on the Bio-Plex MAGPIX System (Bio-Rad).

2.4 Enzyme linked immunosorbent assay (ELISA)

NLRP3, VASH2, VEGF-B, and CTGF in the vitreous samples were evaluated using sandwich enzyme linked immunosorbent assay (ELISA) (MyBioSource ELISA kit, San Diego, CA and RayBiotech ELISA kit, Norcross, GA) according to each manufacturer's protocol. After color development, the optical density was assayed at 450 nm using a Varioskan flash multifunction plate reader (Thermo Fisher Scientific, Waltham, MA, USA). This experiment was independently repeated three times.

2.5 Statistical analysis

Normally, the concentrations of cytokines below the limit of detection are given a value at 0.5 times the limit of detection for the respective cytokine for data analyses[17, 18]. The statistical software SPSS 22.0 (IBM SPSS Statistics for Macintosh, version 22.0.) was used for statistical analyses. Data are reported as mean, standard deviation (SD). First, the normal distribution of data was tested using the Shapiro–Wilk test. Because the assumption of normal distribution was not satisfied, Mann-Whitney U test was applied for comparisons between groups. P-values from multiple comparisons were adjusted to 0.001 by the Bonferroni correction.

3. Result

3.1 Clinical characteristics of subjects

Thirty-eight eyes from 37 patients with iMH or iERM (24 iMH and 13 iERM) in the CONCEPT trial were recruited in this study (Fig. 2). The clinical characteristics of the subjects are presented in Table 1. The iMH group includes eight males and sixteen females, and the iERM group includes six males and seven females. Though the two groups are mainly composed of females, the distribution of gender do not differ significantly between the two groups ($p = 0.73$). The average patient age is similar and no significant difference is observed in the two groups (iMH: 67.77 ± 8.72 , iERM: 63.54 ± 6.00 , $p = 0.1$). Furthermore, the mean preoperative vision of iERM group is 0.83 ± 0.25 , better than that in the iMH group, 1.14 ± 0.40 ($p =$

0.02). The intervals between the first diagnosis by OCT and vitrectomy was 2.07 months in iMH group and 1.95 months in iERM group ($p = 0.74$). Seven cases (54%) in iERM group and ten cases (42%) in iMH group underwent combined cataract surgery ($p = 0.51$).

Table 1
Clinic characteristics of patients with iMH and iERM.

	iMH	iERM	P value
Number	24	13	-
Gender (m/f)	8/16	5/8	0.73
Age (years)	67.77 ± 8.72	63.54 ± 6.00	0.1
Smoking status	7	5	0.56
Hypertension	10	5	0.85
Diabetes	4	2	0.58
Vision (LogMAR)	1.14 ± 0.40	0.83 ± 0.25	0.02
Axial length	23.9 ± 0.99	23.9 ± 0.81	0.95
Duration (months)	16.3	13.2	0.72
Combination-surgery	14	6	0.51

m: male; f: female;

Data are expressed as mean ± SD.

3.2 Vitreal levels of cytokines

Using ELISA and Luminex multiplex technology, we determined the profiles of cytokines in the vitreous of subjects. Then comparison of vitreal cytokine profiles from patients affected by iERMs and iMH was performed. Up to 52 cytokines were measured and the mean concentrations of these factor in each group were shown in Table 2 **and** Fig. 3.

Table 2
Comparisons of 52 vitreal cytokines between iERMs and iMHs.

Cytokine	iERM			iMH			P value
	Mean	SD	Se	Mean	SD	Se	
Angiopoietin-2	93.17	49.34	17.44	123.87	116.90	33.75	0.97
CXCL12	539.87	126.12	44.59	582.49	157.83	45.56	0.53
CXCL9	135.57	94.06	42.06	114.76	51.79	14.95	0.96
Endostatin	62975.80	12018.00	4249.01	57561.62	17190.33	4962.42	0.45
FGF acidic	36.96	3.98	1.41	37.45	4.15	1.20	0.80
FGF basic	4.41	2.27	0.63	5.83	1.19	0.24	0.08
G-CSF	3.73	0.28	0.10	3.79	0.43	0.12	0.91
GM-CSF [#]							
M-CSF	154.86	62.47	22.08	141.57	98.68	28.49	0.74
MIF	7995.97	3911.00	1382.75	11903.57	12912.18	3727.43	0.85
MMP-1	50.97	18.08	6.39	41.24	21.25	6.13	0.43
MMP-3	190.62	178.07	62.96	160.49	112.80	32.56	0.65
MMP-7	196.21	69.45	24.56	281.02	215.86	62.31	0.57
MMP-8	342.45	42.58	15.05	221.51	93.32	26.94	0.00*
PDGF-AB	41.87	17.61	6.23	27.66	17.71	5.11	0.07
PDGF-DD [#]							
PDGF-bb	5.63	2.12	0.75	6.66	2.14	0.62	0.52
PLGF	2.97	2.84	0.79	2.34	1.53	0.31	0.91
Periostin	2615.99	4663.18	2085.44	582.38	344.35	99.41	0.65
Eotaxin	4.12	1.05	0.37	5.14	1.94	0.56	0.03*
IFN-g	43.06	9.75	2.70	51.32	24.47	5.00	0.20
IP-10	884.75	1071.70	297.24	1032.97	920.38	187.87	0.34
MCP-1	136.27	27.54	9.74	149.56	46.91	13.54	0.48
MIP-1a	0.25	0.05	0.02	0.29	0.05	0.01	0.04*
MIP-1b	1.70	0.74	0.20	2.04	1.25	0.36	0.68

Cytokine	iERM			iMH			P value
	Mean	SD	Se	Mean	SD	Se	
RANTES	1.30	0.19	0.07	1.71	0.35	0.10	0.00*
TNF-a	1.54	0.51	0.18	1.65	0.51	0.15	0.68
TGF-β1	363.34	155.10	43.02	362.57	155.24	31.69	0.98
TGF-β2	587.05	88.89	24.65	458.65	117.30	23.94	0.01*
TGF-β3	1.91	0.70	0.19	1.60	0.57	0.12	0.15
VASH2	0.08	0.01	0.01	0.08	0.02	0.01	0.77
CTGF	13.30	4.43	1.23	12.90	6.17	1.32	0.60
VEGF-A	24.06	21.43	9.59	17.69	6.99	2.02	0.36
VEGF-B	0.04	0.02	0.01	1.00	2.68	0.95	0.82
VEGF-C	446.85	290.81	80.66	488.22	202.90	41.42	0.62
VEGF-D	113.07	61.02	16.92	101.15	85.30	17.48	0.28
IL-1β	0.07	0.01	0.00	0.10	0.05	0.01	0.06
IL-1ra	10.14	5.33	1.48	17.07	20.70	4.22	0.91
IL-2	0.19	0.10	0.04	0.26	0.09	0.03	0.15
IL-4	0.11	0.02	0.01	0.18	0.07	0.02	0.00*
IL-5 [#]							
IL-6	13.72	22.66	10.13	9.64	17.93	5.18	0.65
IL-7	16.13	6.36	2.25	15.48	5.43	1.57	0.68
IL-8	113.07	61.02	16.92	101.15	85.63	17.48	0.28
IL-9	0.08	0.10	0.06	0.27	0.24	0.07	0.23
IL-10 [#]							
IL-12	0.16	0.00	0.00	0.23	0.14	0.04	0.38
IL-13	0.48	0.38	0.14	0.48	0.33	0.11	1.00
IL-15 [#]							
IL-17A	0.89	0.70	0.19	0.78	0.39	0.08	0.89
IL-18	69.45	35.09	9.73	57.16	15.62	3.19	0.31

Cytokine	iERM			iMH			P value
	Mean	SD	Se	Mean	SD	Se	
NLRP3	5.35	2.03	1.01	4.72	1.97	0.74	0.65

#: the cytokine concentrations lay below the limits of detection

*: $p < 0.05$, however, these differences were not significant after Bonferroni's correction ($p < 0.001$ as statistically significant)

A statistically significant difference was obtained in the expression of 6 cytokines between two groups using $p < 0.05$ significance level. These cytokines are TGF- β 2 (587.05 vs 458.65, $p = 0.01$), IL-4 (0.11 vs 0.18, $p = 0.002$), MMP-8 (342.45 vs 221.52, $p = 0.002$), Eotaxin (4.12 vs 5.14, $p = 0.03$), MIP-1a (0.25 vs 0.29, $p = 0.04$) and RANTES (1.3 vs 1.71, $p = 0.002$). However, these differences were not significant after Bonferroni's correction ($p < 0.001$ as statistically significant).

Thirteen of the 52 tested cytokine levels were less than the lowest detected concentration of the standard, and, thus, the multiparameter standard curve was used to calculate the concentration of these cytokines (Fig. 3). However, the concentrations levels of five of them lay below the linear range of the standard curve, which means that the content of GM-CSF, PDGF-DD, IL-5, IL-10 and IL-15 in vitreous of patients with iERM and iMH is too low to be detected.

4. Discussion

Idiopathic epiretinal membrane and idiopathic macular hole are two of the most common vitreomacular interface disease. Many studies, involving the measurements of intraocular cytokines, usually employed iERM and iMH as controls for retinal pathologies such as proliferative diabetic retinopathy, uveitis, retinal vein occlusion, and high myopia[5, 7, 8, 19]. However, the comparative data of the vitreal cytokine levels in patients with ERMs and MHs are very sparse. In present study, we found the no difference in the vitreal cytokine profiles between Asian patients with iERM and those with iMH. According to our study results, the vitreous fluid in the eyes of patients with either iERM or iMH can be served as controls in further researches.

iERM, as one of the vitreous macular traction diseases, its pathophysiology mechanism remains unclear. A generally received opinion is that age-related vitreoretinal interface change and pathologic cell proliferation result in vertical or horizontal traction force to the macula[20, 21]. A number of cellular factors are involved in cell recognition and signal transduction during the occurrence and development of iERM formation, among which, TGF- β 2 is the most extensively studied factor[6, 20, 22]. The concentration of vitreal TGF- β 2 level we measured is 587.05 ± 88.89 pg/ml, while already published data is 327.98 ± 99.58 and 951.06 ± 593.25 [6]. The discrepancy between this data could be due to differences in the tested methods and test kits.

Our data suggested that before Bonferroni correction, the TGF- β 2 levels in the vitreous fluids of the patients with iERM were significantly higher than those in iMH ($p = 0.01$). These findings were similar to those reported by Ludovico Iannetti, whose research found that TGF- β 2 and nerve growth factor (NGF) were associated with idiopathic ERMs[6]. However, in our research, the statistical difference disappeared after Bonferroni correction. In addition to iERM or iMH, Iannetti *et al* once used primary retinal detachment (within 72 hours of retinal detachment onset) as control group[6]. Ideally then, the human vitreous of healthy subjects should be used as the control. However, in clinical practice, vitreous sample from healthy subjects cannot be obtained because of the ethic issue. The present study demonstrates that the cytokine expression profile between patients with iERM and patients with iMH is similar, which means that like iMH, iERM can be reliably used as control groups.

Before our CONCEPT trial, we thoroughly retrieved the published literatures on vitreous cytokines during the past decade. Considering the compatibility of different cytokines in single commercial bead array, we finally investigated 48 cytokines using multiplex bead array method and other 4 cytokines with ELISA. Here, a total of 52 cytokines were included in the present study, covering most of the published factors associated with intraocular diseases and some new cytokines in other related diseases, such as asthma and Alzheimer's disease. And these cytokines can be classified into four main groups: the inflammatory factors and chemokines, the promoting angiogenesis factors, and the fibrogenic cytokines, which corresponding respectively to inflammatory, neovascular, and fibrotic retinopathy.

There were published studies focusing on the protein or cytokine profiles of vitreous from patients with iERM and iMH but yielding opposite conclusion[15, 16, 23]. Nakul Mandal *et al* demonstrated that none of the 330 protein spots changed significantly between the iERM and iMH groups using comparative proteomic experiments. On the contrary, Souska Zandi *et al* employed ELISA and multiplex technology while Zhang *et al* employed one-dimensional gel fractionation and liquid chromatography–tandem mass spectrometry analyses, and both found some of the differently expressed cytokines between ERM and MH. Several issues should be kept in mind to interpret the controversial findings between ours and others. Firstly, the expression of cytokines may be duration related. The average course of iERM in our study was 1.95 months, while the duration of iERM in Souska Zandi's study was unknown. What we know is that the subjects whose courses were more than 6 months constitute 94% of the ERM group in the Souska Zandi's study and 62% of those in our study. Besides, in 31.9% of the measured patients in Souska Zandi's study, the cytokine concentrations lay below the lower cutoff level. Though half of the lower cutoff value was used for subsequent calculation, the result would be still in error. Third, the race might also be involved in the difference of cytokine levels in the vitreous. The above mentioned studies were conducted in Switzerland, in Europe, and in USA, while our patients were all Asian populations.

We acknowledge that this study has several limitations. First, the number of patients included is limited. More cases are required in future to confirm the results. Second, we did not compare the vitreous cytokines among the two macular diseases and vitreous floaters. However, the safety and efficiency of vitrectomy over Nd:YAG laser for floaters remains to be demonstrated. Thus, most studies involving the cytokines measurements still employed iERM or iMH as “negative” controls. [5, 7, 8, 19]. Third, we did not

include ERM or MH eyes with high myopia. ERM and MH are two of the main complications of pathological myopia. It will be interesting to investigate the vitreous cytokines between emmetropic and myopic eyes in conditions of ERM or MH.

In conclusion, the current research provides substantial evidence that the cytokine in the vitreous show similar expression in iERM group and iMH group. This prospective control study is the first one that demonstrates Asian patients with iERM and iMH can be chosen as “negative” control. These results provide theoretical foundation for the future clinical design methods. Further investigations with more participants should be conducted to verify these findings.

Declarations

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Ethics approval and consent to participate

This study was performed following the guidelines of the Declaration of Helsinki and Tokyo for humans, and approved by Institutional Review Board (IRB) at the Nanjing First Hospital, Nanjing Medical University. The study protocol is available at <https://clinicaltrials.gov/> (Registration date: 24/04/2018; Registration ID: NCT03506750). Informed written consent was obtained from all patients prior to enrollment.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZH and LQ generalized the idea of the new technique, performed the surgery, writing and revising the manuscript. LC and ZH analyzed and interpreted the patient data. LC and WZ performed the examination

followed the patients. PX, JJ, HQ and SY contributed in the design and discussion of the work. All authors read and approved the final manuscript

Authors' information (optional)

Not applicable.

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Figures

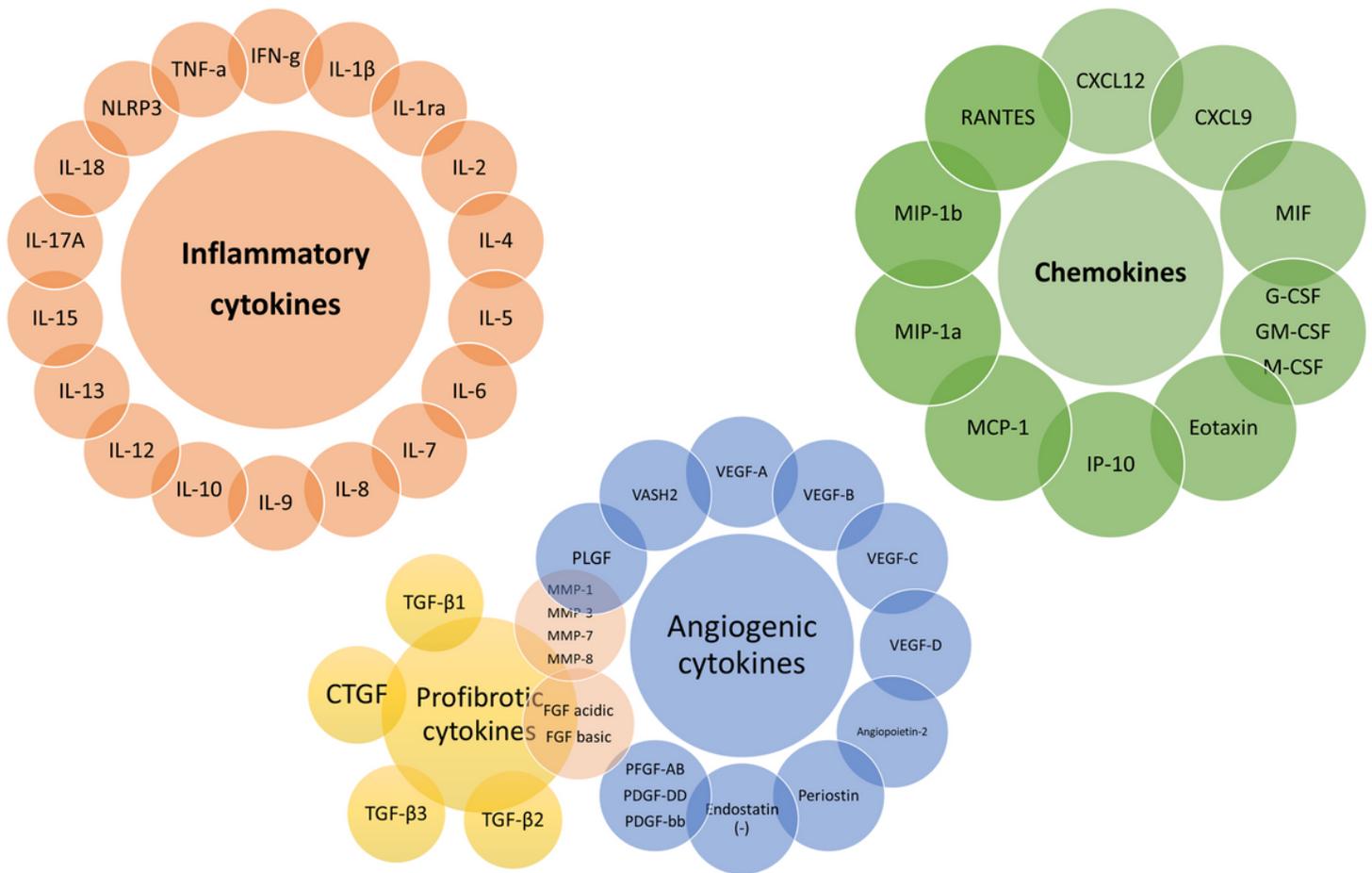


Figure 1

Cytokines measured in this study. The cytokines can be classified into angiogenic (blue), inflammatory (orange), profibrotic cytokines (yellow), and chemokines (green). Such MMP and FGF cytokines were both profibrotic and angiogenic cytokines.

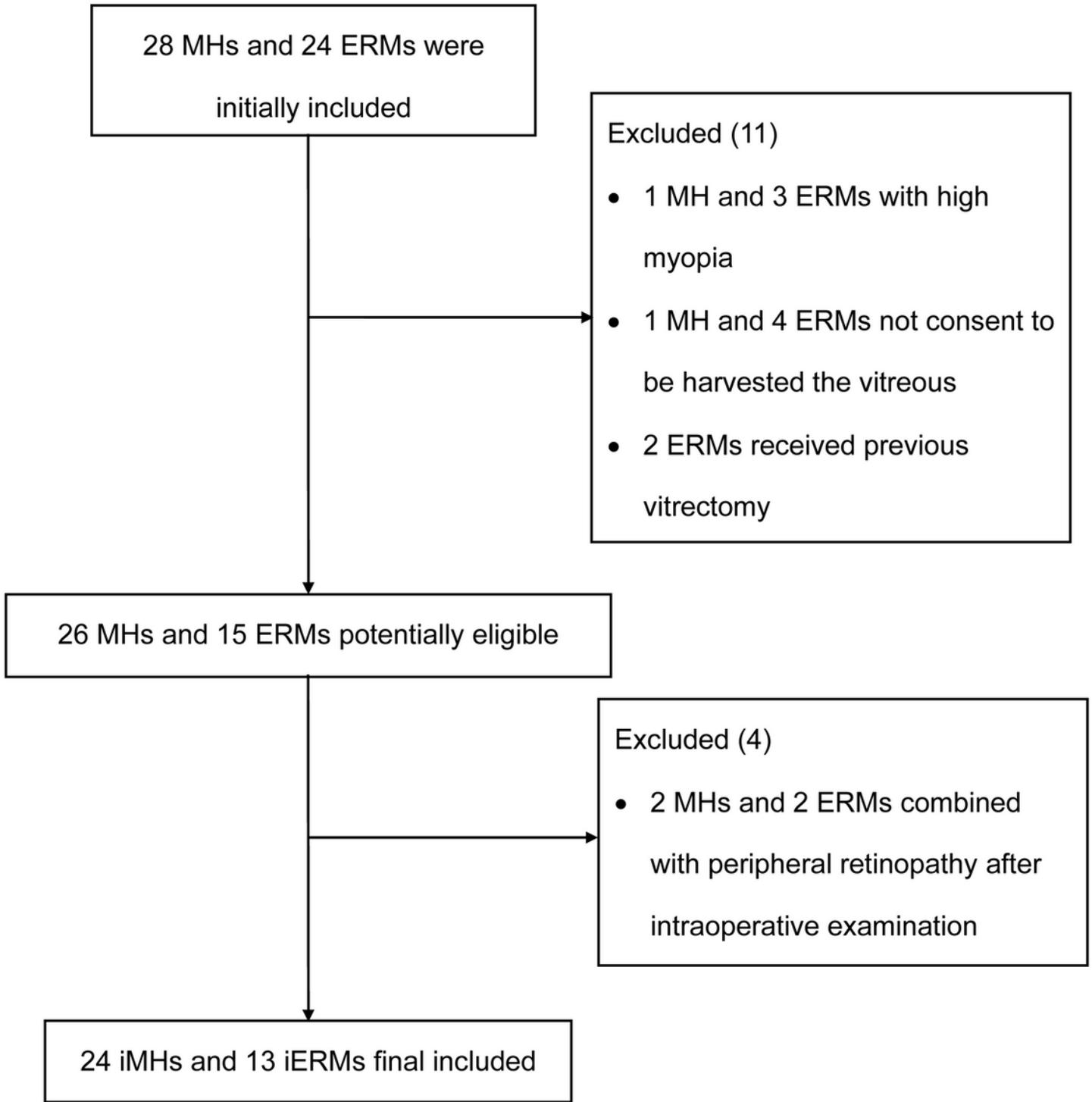


Figure 2

Flow diagram of patients included in this study.

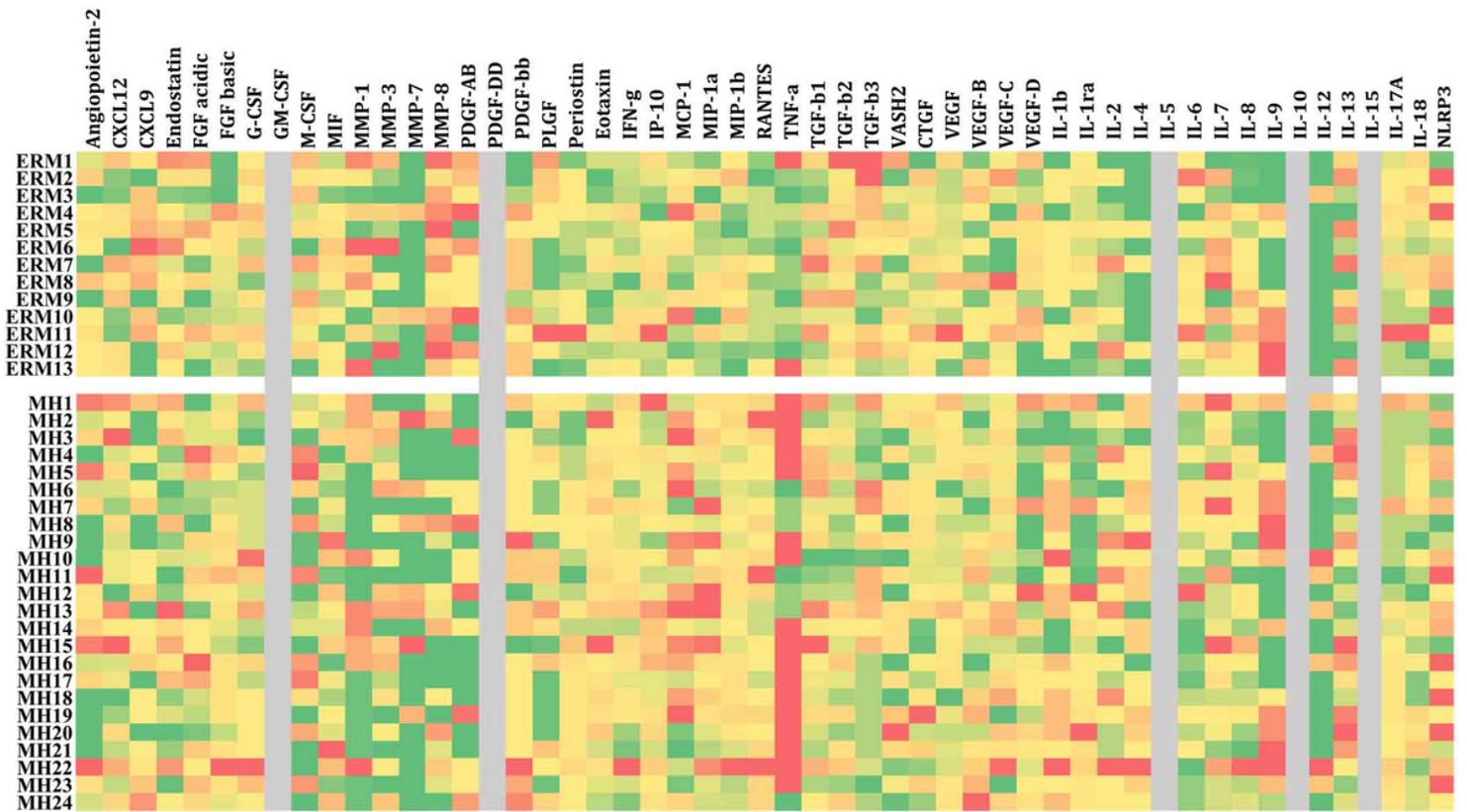


Figure 3

Visualization of the intergroup differences using a heat map chart for all 52 cytokines. Red indicates high expression and green indicates low expression. GM-CSF, PDGF-DD, IL-5, IL-10 and IL-15 in vitreous is too low to be detected (Grey).