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Serum Th1/Th2 Cytokine Levels During Acute Mumps Infection: Prediction To Infertility Patients with History of Mumps Infection

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Abstract:
Background: *Mumps is the best-known cause inflammation of the testicles, reduced testosterone level, failure of sperm production and lead to infertility. The hypothesis of this study is to know mumps virus may be the cause of infertility in patients who have infertility problem with history of mumps infection. It is with this notion we aimed to compare and link the Th1/Th2 cytokine levels between acute mumps patients and infertility patients with history of mumps.*
Methods: *Mumps specific IgM, MMR specific IgG antibody and Th1/Th2 cytokines, namely interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-4 and IL-10 were measured simultaneously in serum from acute mumps cases, infertility cases, control subjects and supernatant of PBMCs stimulated with mumps virus. Hormonal evaluation (testosterone, FSH and LH) and spermatograms were measured for infertility cases. Statistical significance was analysed between each group by means of Mann-Whitney U test, Kruskal–Wallis test and Spearman's rank correlation coefficient test.*
Result: *Significant reduction of testosterone, an increased FSH & LH levels, greatly reduced sperm count, and severe abnormalities in motility and morphology seen in infertility patients with history of mumps. Also IFN-γ and IL-2 showed a significant increase in groups, acute mumps pediatrics cases (Group A), adult cases (Group B), and infertile with history of mumps infection (Group C). IL-10 level significantly deceased in group C when compared to group A and B.*
Conclusion: *Upon evaluation of hormonal levels and spermatograms confirms the mumps virus can be the possible reason for the infertility in group C patients and Th1 cells play important roles during the acute phase in the pathogenesis of mumps and infertility with history of mumps.* ***Key word:*** *Infertility, Mumps infection, Th1/Th2 cytokines*

I. Introduction

Mumps infection in children less than 5 years of age frequently presents as upper respiratory tract symptoms with or without parotitis, which is usually the clinical manifestation. Mumps orchitis usually follows 4 to 8 days after parotitis, but takes upto 6 weeks post primary infection [1]. Upto 30 - 40% of mumps infections are subclinical, and orchitis can occur without parotid involvement [2]. Recent data show that primary mumps infection among adults directly leads to infertility [3, 4]. The consequence of the physiological events that occur between mumps virus primary infection and development of infertility is not fully known [5]. It is not known whether mumps orchitis is a purely a viral disease or a virus induced immunopathological disorder [6, 7, 8]. There are reports that mumps virus causes infertility without involvement of orchitis [9, 10, 11, 12, 13] and we find many male infertility cases with decreased testosterone and history of mumps infection and mild testicular pain during their mumps infection. Hence the present study was aimed to predict the link between acute mumps infection and infertility with history of mumps to know whether infertility is due to mumps virus in non orchitis mumps patients by analysis of Th1/Th2 cytokine levels in acute mumps infection and semen, hormones and Th1/Th2 cytokine levels in infertility cases.

II. Materials And Method

Case definition and data collection:

WHO guidelines were adopted for selection of mumps cases and infertility cases [14, 15]. A proforma containing following information was obtained from each patient (mumps and infertility cases), which included name, age, date of birth, gender, residence, occupation, date of disease onset, signs and symptoms, date of diagnosis, current complication, MMR immunization status, history of infertility problems, other complications and reinfections etc. An informed consent was obtained from each patient and control; human ethical clearance for sample collection was obtained.

Acute mumps patients and controls:

Acute mumps pediatrics cases (Group A) and adult cases (Group B) samples were collected over a period of 32 months from July 2010 to February 2013. Blood for antibody and cytokine analysis were collected. The negative control subjects were afebrile, non-infected and non-MMR vaccinated 20 pediatric (Group E) aged from 5 years to 12 years. Patients and control subjects having a history of recent other infections, having received vaccine within the last 4 weeks, those who received blood, plasma or immunoglobulin within the last 3 months were excluded from the study.

Infertility patients and controls:

The study population comprised of those are infertile with history of mumps infection (Group C) and patients with infertility who never had mumps (Group D) served as the positive control and fertile adult male blood donors who never had mumps (Group F) served as the negative control group. Samples were collected between February 2011 to November 2012 to analyze, persistence of mumps specific IgG antibody, semen, testosterone and Th1/Th2 cytokine levels. Semen was collected and spermatogram was evaluated immediately and blood (for antibody, testosterone and cytokine study) was collected and stored at -86°C.

Virus positive controls:

Human blood obtained from healthy donors (Blood Bank, Voluntary Health Services, Chennai, India). PBMC isolation was done by using Histopaque (Sigma Cat No. 1077) and the procedure used was as directed by the manufacturer [17]. Isolated PBMCs were plated at a concentration of 2 $x10^6$ cells/ml/well in 24 well tissue culture plates and infected with 50µl of 5000 $TCID_{50}$ mumps virus (Group G) as described [18, 21]. Plates were incubated in a CO_2 incubator at 37ºC for 72 hours. After 72 hours culture supernatants were collected, aliquoted and immediately stored at -86°C until use.

Determination of antibody, hormones and cytokine concentrations:

Mumps serum samples were tested for mumps specific IgM antibody by ELISA (Labor Diagnostika Nord GmbH & Co. KG, Germany) and Measles, Mumps and Rubella specific quantitative IgG ELISA (Techno Genetics, Italy) was done to check MMR induced specific IgG antibody in all patients and controls, in order to confirm that they had been vaccinated. IgG antibody titers were calculated from the sample absorbance and reference standard curve generated from the reference sera provided with the kit. IgG antibody titers of > 115 mU/ml for mumps, > 115 mIU/ml for measles and >15 IU/ml for rubella were defined as seropositivity. Hormones namely, testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) estimation was done for diagnostic analysis as part of infertility investigations; this value was used for this study. The reference range of the hormonal levels used in this study: testosterone = 249 – 836 ng/dL, LH = 1.2 - 8.6 mIU/mL, FSH = 1.3 – 9.9 mIU/mL.

Serum samples and culture supernatants were tested for Th1 cytokines and Th2 cytokines by standard ELISA as described before [18]. Cytokines evaluated in this study were IFN-γ (BD Pharmingen Cat. No. 555142) and IL-2 (Cat. No. 555190) (Th1 cytokines), IL-10 (Cat. No.555157) and IL-4 (Cat.No.555194) (Th2 cytokines). Cytokine evaluation was done as per manufacturer's instructions. Concentration of each cytokine of sample was calculated based on the standard curve [19].

Collection and analysis of semen samples:

Semen was collected for diagnostic analysis as part of infertility investigations; this value was used for this study. The semen sample was collected into a standard plastic container and analysed according to a protocol based upon the techniques outlined by the World Health Organization as described previously [16, 20]. Spermatograms (sperm count (millions/ml), motility/progression, volume (ml), and morphology (normal, abnormal)) were performed and the absence of spermatozoa confirmed by centrifugation of the semen at 3,000 rpm for 15 min and microscopic examination of the pellet.

III. Statistical analysis

Values are reported as the median with range when normally distributed. The differences in the results between two groups were analyzed by means of student T-test, non-parametric Mann-Whitney U test. Difference between more than two groups was analyzed by means of One-way ANOVA, non-parametric Kruskal–Wallis test. A $p < 0.05$ was considered to be statistically significant. Correlations were analyzed with use of Spearman's rank correlation coefficient test. For all the above analyses, a GraphPad Prism 6, version 6.01 (GraphPad Software, Inc, USA) was used.

IV. Result

Patient demographic and antibody analysis:

A total of 74 mumps parotitis cases were collected. Of the 74 samples collected from various age groups, 42 (57%) were from group A with a median age of 6 years and 32 (43%) were from group B with a median age of 21 years. Of these, 54% were males were and 46% were females. There was no significant difference in patients with regard to age and gender. Of the 74, 67 (91%) had been vaccinated and tests for mumps IgM in vaccinated cases indicated that an alarming 67/67 (100%) samples tested positive, whereas 57/67 (85%) samples were negative for mumps IgG. These facts inevitably state that MMR vaccine failed to offer protection in vaccinated individuals against mumps infection. 67 (100%) samples tested positive for rubella specific IgG and 65 (97%) samples tested positive for measles specific IgG, suggesting that the mumps component in the MMR vaccine had low efficacy. Of the 74 acute mumps cases 7 of them were non-vaccinated, mumps IgM was positive for these samples; however they were negative for both mumps and rubella IgG [52] Measles IgG was positive for these samples because a separate measles vaccine is given at 9 months of age, prior to the MMR vaccine.

A total of 56 cases in group C aged 24 to 48 with a median age of 33 years, 93 % positivity to mumps specific IgG and complete negative of rubella specific IgG confirms the non vaccinated infertile male with history of mumps infection. A total of 115 cases in group D aged 23 to 50 with a median age of 32 years, complete absence of mumps and rubella specific IgG confirms the non vaccinated infertile male without history of mumps infection and 20 subjects in group F aged 18 to 25 with a median age of 22 years, negative for mumps and rubella IgG. Measles IgG was positive for these samples because a separate measles vaccine is given at 9 months of age, prior to the MMR vaccine (**Table 1**). The median value for mumps, measles, rubella specific IgG was 422 mU/ml, 1009 mIU/ml, 298 IU/ml respectively.

Spermatograms and hormone levels:

Group C patients were diagnosed as 52% & 48% of primary infertility and primary sub fertility respectively. Upon semen analysis 40% were azoospermia, 100 % sperm counts were decreased (< 20 millions/ml) with median range of 10 (1 - 18) millions/ml. Sperm morphology and motility was abnormal with median range of 98 (93 - 100) and 45 (25 - 95) percentage respectively. The majority characteristics of the semen analysis in both groups group C and D showed poor quality and classified into the oligoasthenoteratzoospermia category (greatly reduced sperm count, and severe abnormalities in motility and morphology) (**Table 2**). There was significant difference in motility and morphology of the sperm ($p = 0.0118$, $p < 0.0001$; $p < 0.0001$, $p < 0.0001$) in both groups C and D.

Of the 56 cases in group C, 83 % of them showed reduced levels of testosterone below the reference value when compared to controls group D and F were within the normal reference range; except 4% in group D. The median range of testosterone values in group C were 235 (180 - 735) ng/ml while in group D and group F were 352 (346 - 966) and 548 (196 – 802) ng/ml respectively. Of the 56 patients in group C, 75% of them showed elevated levels of FSH and LH above the reference value when compared to controls group D and F were within the normal reference range; except 7% elevated in group F. The median range FSH and LH levels (mIU/ml) in group C were 6.9 (1.2 – 11) and 6.7 (2.6 – 10.9) respectively, whereas in group D and F, 5.9 (3.4 – 9.3) and 4.9 (2.7 – 8.1); 3.6 (2.9 – 9.1) and 3.7 (2.7 – 9.2) respectively (**Table 3**). The statistical differences of the reduced testosterone levels in group C were compared with group D and F; also between group D and F ($p = 0.0485$ and $p = 0.0600$; $p = 0.0970$ respectively). While the increased FSH and LH levels were compared between as mentioned above groups ($p < 0.0001$ and $p = 0.0821$; $p = 0.0620$).

Th1/Th2 Cytokine profiles:

The lower detection limit of the IFN-γ, IL-2, IL-4 and IL-10 cytokines evaluated in this study was 2.35 pg/ml, 1.95 pg/ml, 0.9 pg/ml and 1.95 pg/ml respectively. The concentrations below the detection limit were taken as 0 pg/ml. The median concentration and range of group E were 11.2 (9.6 – 17.61 pg/ml), 2.7 (1.26 – 6.15 pg/ml), 0 (0 – 3.01 pg/ml) and 2 (0 – 3.2 pg/ml) respectively and in group F were 15.1 (9.74 – 23.6 pg/ml), 6 (1.98 – 8.91 pg/ml), 0.9 (0 – 3.24 pg/ml), 2.2 (0 – 3.68 pg/ml) respectively. Genotype C of mumps virus

isolated from our previous study and quantified by $TCID_{50}$ [21]. The quantified virus was used for PBMC infection as group G. The average concentrations of group G were 217.1 (210 – 223 pg/ml), 210.5 (192.6 – 206.4 pg/ml), 2 (1.94 – 2.12 pg/ml) and 194.2 (192.3 – 199.4 pg/ml) respectively (**Table 4**). IFN-γ and IL-2 showed a statistically significant increase in group C when compared to group A and B ($p < 0.001$, $p = 0.0008$ and $p < 0.0001$, $p < 0.0001$, respectively). IL-10 level significantly deceased in group C when compared to group A and B ($p < 0.0001$, $p < 0.0001$). IL-4 levels were slightly reduced which were no significant $p = 0.515$, $p = 0.7263$. IFN-γ, IL-2 and IL-10 levels significantly higher in group D when compared to group C ($p = 0.0076$, $p < 0.0001$ and $p < 0.0001$). All the test groups were compared with control, which are represented in **table 4, 5a.**

Correlation between Immunological variables:

Table 5b, shows the correlation among the pairs of measures of IFN-γ, IL-2, IL-4 and IL-10 as represented by a Spearman's correlation matrix. The IFN-γ and IL-2 were correlated to IL-10 in both group C and D (group C: $r = 0{\cdot}331$, $p = 0.013$; $r = 0{\cdot}029$, $p = 0.0831$, group D: $r = 0{\cdot}148$, $p = 0.114$; $r = 0{\cdot}161$, $p = 0.085$ respectively), but lacked any correlation with the measures of IL-4 secretion.

V. Discussion

Viral infections can cause inflammation of the testicles, failure of sperm production and lead to infertility. Mumps is the best-known cause for infertility. After primary mumps virus infection mostly in children, patients are cured without any trace but later on in age, they develop mumps mediated infertility without any symptom of orchitis and in adults directly leads to symptomatic orchitis [2, 5, 9, 10, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31], which physically and psychologically affects the patients during their adolescence. We undertook this study to know mumps virus may be cause of infertility in patients who had asymptomatic mumps orchitis, for this we compared group A and B with group C. There have been no reports on serum cytokine levels in acute mumps infection and infertility patients with history of mumps infection and here we compared cytokine levels to find any link between acute mumps and infertility patients with history of mumps. We showed here the significant reduction of serum testosterone (83%) and an increased FSH and LH levels (75%) in groups C confirms the primary hypogonadism. Upon evaluation of testicular involvement, the semen analysis showed 100 % greatly reduced sperm count, and severe abnormalities in motility and morphology which confirms the mumps virus may be the possible reason for the infertility in non orchitis mumps patients. Also IFN-γ and IL-2 showed a statistically significant increase in group C and group A and B. IL-10 level significantly deceased in group C when compared A and B.

Infertility is in fact a rare complication, but subfertility occurs in an estimated 13% of mumps patients, and can occur in patients with no signs of testicular atrophy or orchitis [3, 24, 32, 33, 34, 35, 36]. In this study we showed 52% & 48% of primary infertility and primary sub fertility respectively without testicular atrophy except 9 patients gave history of testicular pain during acute mumps. Infertility in male patients resulting from mumps orchitis is a controversial and topical issue, as there is conflicting evidence on the effect of mumps orchitis on the endocrine function of the testes. FSH, LH and testosterone evaluation is useful in the management of male infertility. In the infertile men, higher concentration of FSH is considered to be a reliable indicator of germinal epithelial damage, and was shown to be associated with azoospermia [22, 37, 38, 39]. Studies have shown decreased testosterone levels and increased FSH and LH levels in mumps orchitis and infertility [40, 41, 42, 43]. However, other studies have shown both normal and only transient changes in testosterone and FSH as a result of mumps orchitis [11, 35]. In this study we find that group C showed 40% azoospermia, 100 % sperm counts were decreased < 20 millions/ml and 83 % of reduced levels of testosterone below the reference value when compared to both controls, group D and group F were within the normal reference range and 75% of them showed elevated levels of FSH and LH above the reference value when compared to group D and group F were within the normal reference range; except 7% elevated in group B.

With respect to cytokine the previous studies demonstrated that an increased IFN-γ, IL-2, IL-6, and suggested that Th1 cells play an important role in CSF of mumps meningitis [44, 45, 46]. In this study we demonstrated an increased level of IFN-γ, IL-2 and IL-10 in group A & B and group C. An increased level of IL-10, which is mainly produced by CD4+ Th2 cells, inhibits cytokine production by CD4+ Th1 cells [47]. Therefore, we suggest that IL-10 is induced in response to higher production of IFN-γ to modulate the balance of Th1 and Th2. The levels of Th1 cytokine concentration were higher in group C when compared to group A and B and lower in group C when compared to group D. The level of IL-4 was less in group A, B, C and D; IL-10 levels were higher in group A, B and group D when compared to group C. The studies were documented mumps epididymo orchitis and infertility problem in MMR vaccinated patients [3, 48, 49, 24, 2, 50, 51]. In this study we find 91 % of group A and B were MMR vaccinated and increased cytokine (IFN-γ, IL-2 and IL-10) levels; according to our results, we predict that in near future there is a possibility of getting infertility problem if the virus would have infected the testis asymptomatically.

In addition, there is no family history and other know cause of infertility in the cases studied and hence the infertility in these cases might be attributed to decreased testosterone level where mumps virus would have infected the leydig cell asymptomatically. In summary results clearly indicate significant increase in gonadotropins (FSH and LH) in group C with reduction of testosterone level might have disrupted the spermatogenic process leading to the decline in the sperm count and infertility. IFN-γ, IL-2 and IL-10 concentration were significantly increased in group C when compared to group D and F with strong positive correlations and little reduced level of IL-4 reflected raised Th-1 cytokines. Th1 cells play important roles during the acute phase in the pathogenesis of mumps and infertility with history of mumps, which could be involved in characterized principally by cell, mediated immune responses.

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Table 1: Details of patients and control subjects

	Acute Mumps – Pediatric cases	**Acute Mumps – Adult cases**	**Infertility with mumps**	**Infertility without mumps**	**Pediatric Control**	**Adult Control**
Number	42	32	56	115	20	20
Age (Median age)	< 12 years (6)	13 – 42 years (21)	24 – 48 (33)	23 – 50 (32)	5 – 12 years (8)	18 – 25 years (22)
Sex (Male : Female)	26:16	14:18	56:0	115:0	10:10	10:10
Onset of parotitis to sampling (average)	3 days	3 days	-	-	-	-
Duration of fever (average)	5 days	6 days	-	-	-	-
MMR vaccination	35/42 (83%)	32	0	0	0	0
No. positive for Mumps IgM antibody	42	32	0	0	-	-
No. positive for Mumps IgG antibody	2/35 (6%)	8/32 (27%)	52 (93%)	0	0	0
No. positive for	33/35	32/32	47	83	19	16

Measles IgG antibody						
No. positive for Rubella IgG antibody	35/35	25/32	0	0	0	0

Table shows the case distribution along with controls. A total of 74 patients with acute mumps were recruited in this study and divided into paediatrics and adults. Of 74 cases all of them were positive for parotitis, mumps IgM. The infertility cases comprised of those are infertile with history of mumps infection and patients with unexplained infertility who never had mumps served as the positive control group. Pediatric control and fertile male blood donors (adult control) who never had mumps served as the negative control group.

Table 4: Levels of Cytokine concentration (pg/ml) observed in this study

Groups (no. of cases and control)	**IFN - □**			**IL - 2**		
	Range (pg/ml)	**Mean (pg/ml)**	**Median (pg/ml)**	**Range (pg/ml)**	**Mean (pg/ml)**	**Median (pg/ml)**
A. Acute Mumps – Pediatric (42)	86.2 – 329.4	172.2	164.4	85.2 – 248.7	141.6	132.7
B. Acute Mumps – Adult (32)	110.1 – 329	209	200.4	89.8 – 325.5	143.1	138.6
C. Infertility with mumps (56)	115.6 – 316.2	244.9	205.1	111.4 – 250.4	178.5	175.4
D. Infertility without mumps (115)	108.6 – 389.2	266.2	268.2	138.9 – 522.6	262.4	245.7
E. Pediatric Control (20)	9.6 – 17.61	11.6	11.2	1.26 – 6.15	3.2	2.7
F. Adult Control (20)	9.74 – 23.6	15.5	15.1	1.98 – 8.91	5.5	6
G. Virus positive control	210 – 223	217.1	217.1	192.6 – 206.4	210.1	210.5
Groups (no. of cases and control)	**IL - 4**			**IL - 10**		
	Range (pg/ml)	**Mean (pg/ml)**	**Median (pg/ml)**	**Range (pg/ml)**	**Mean (pg/ml)**	**Median (pg/ml)**
A. Acute Mumps – Pediatric (42)	0 – 2.5	0.26	0	96.1 – 322.7	170.5	156.5
B. Acute Mumps – Adult (32)	0 – 3.2	0.22	0	27.4 – 365.1	229.7	254
C. Infertility with mumps (56)	0 – 2.1	0.05	0	3.2 – 112.5	62.3	65.01
D. Infertility without mumps (115)	0 – 34.1	0.86	0	22.3 – 245.3	133.5	134.56
E. Pediatric Control (20)	0 – 3.01	0.91	0	0 – 3.2	1.3	2
F. Adult Control (20)	0 – 3.24	1.2	0.9	0 – 3.68	1.8	2.234
G. Virus positive control	1.94 – 2.12	2.0	2.0	192.3 – 199.4	195.3	194.2

Table 5a: Comparison of significance differences (*p*-values) between the groups

Cytokines	**C** vs **A**	**C** vs **B**	**D** vs **C**	**A** vs **B** vs **C vs D**	**A** vs **B** vs **C vs D vs E vs F**	**C** vs **D vs E vs F**
IFN - □	$p < 0.001$	$p = 0.0008$	$p = 0.0076$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
IL - 2	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
IL - 4	$p = 0.5150$	$p = 0.7263$	$p = 0.6706$	$p = 0.6936$	$p < 0.0001$	$p < 0.0001$
IL - 10	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$

Table 5b: Correlation between IFN-γ, IL-2, IL-4, and IL-10 responses in study subjects as represented by a Spearman's correlation matrix

Group C					**Group D**				
Variable	**IFN - □**	**IL - 2**	**IL - 4**	**IL - 10**	**Variable**	**IFN - □**	**IL - 2**	**IL - 4**	**IL - 10**
IFN - □	-	$p = 0.773$	$p = 0.464$	$\mathbf{p = 0.013}$	**IFN - □**	-	$p = 0.881$	$p = 0.141$	$p = 0.114$
IL - 2	$p = 0.773$	-	$p = 0.735$	$p = 0.831$	**IL - 2**	$p = 0.881$	-	$p = 0.458$	$p = 0.085$
IL - 4	$p = 0.464$	$p = 0.735$	-	$p = 0.163$	**IL - 4**	$p = 0.141$	$p = 0.458$	-	$p = 0.471$
IL - 10	$\mathbf{p = 0.013}$	$p = 0.831$	$p = 0.163$	-	**IL - 10**	$p = 0.114$	$p = 0.085$	$p = 0.471$	-

Table 2: Spermatograms of infertility cases:

Groups	Disease diagnosed (%)		Percentage of Azoospermia	Sperm count millions/ml (median with range)	Percentage of motility (median with range)		Percentage Morphology (median with range)	
	Primary infertility	Primary sub fertility			motile	immotile	normal	abnormal
Group C (56)	52 %	48 %	40 %	10 (1 - 18)	40 (5 - 60)	45 (25 - 95)	2 (1 - 37)	98 (93 - 100)
Group D (115)	45 %	55 %	37 %	8 (1 – 18)	25 (5 - 65)	60 (25 – 99)	2 (1 – 8)	98 (92 - 100)

Table 3: Hormonal evaluation of infertility cases:

Groups	Testosterone level (ng/dL) (median with range)	Follicle-stimulating hormone level (mIU/mL) (median with range)	Luteinizing hormone level (mIU/mL) (median with range)
Group C (56)	235 (180 - 735)	6.9 (1.2 – 11)	6.7 (2.6 – 10.9)
Group D (115)	352 (346 - 966)	5.9 (3.4 – 9.3)	4.9 (2.7 – 8.1)
Group F (32)	548 (196 – 802)	3.6 (2.9 – 9.1)	3.7 (2.7 – 9.2)