Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Revello MG, Lazzarotto T, Guerra B, et al. A randomized trial of hyperimmune globulin to prevent congenital cytomegalovirus. N Engl J Med 2014;370:1316-26. DOI: 10.1056/NEJMoa1310214

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Supplementary Appendix

Supplement to: Revello, Lazzarotto, Guerra et al. A Randomized Trial of Hyperimmune Globulin to Prevent Congenital Cytomegalovirus

Methods

Results

A Randomized Trial of Hyperimmune Globulin to Prevent Congenital Cytomegalovirus

METHODS

Identification of women with primary HCMV infection

HCMV screening is not recommended in Italy. However, about 40% pregnant women are routinely tested for virus-specific IgG and IgM during the first trimester of pregnancy. In case of absence of antibody, the woman is usually monitored every 4-6 weeks. In case of seroconversion or positive IgM result, the woman is referred to a reference center for further testing.

In Italy, the two major reference centers for HCMV infection in pregnancy are the Policlinico San Matteo in Pavia and the Ospedale Sant'Orsola Malpighi in Bologna. Altogether, hundreds of women with suspected primary HCMV infection are yearly referred at either center. A battery of serologic and virologic (commercial and non-commercial) assays are available in either center for diagnosis of primary HCMV infection including: ELISA for IgG, IgM, IgG avidity, immunoblot, neutralization assays on different cell substrates, rapid virus isolation (shell-vial), pp65 antigenemia determination, real-time PCR for viral DNA detection in blood and other clinical samples (amniotic fluid, urine etc), as well as HCMV-specific T cell determination.

The above assays are used in a step-wise manner depending on the complexity of the single case. In both centers, firm diagnosis of primary HCMV infection requires presence of IgG seroconversion, or two or more of the following: IgM, low IgG avidity index, positive pp65 antigenemia, DNAemia. Moreover, all pregnant women with suspected primary HCMV infection are interviewed and anamnestic data (clinical and laboratory) are collected.

ADMISSION CRITERIA

A. Inclusion Criteria

1) Pregnant women, >18 years of age, including pregnancies after *in vitro* fertilization
2) Primary HCMV infection acquired between 5 and 26 week of pregnancy
3) < 6 weeks from presumed onset of infection
4) Gestational age >5 and <32 weeks
5) Willing to continue pregnancy beyond 12 week of gestation
6) Written informed consent

B. Exclusion Criteria

1) Multiple pregnancy.
2) Maternal HIV, HBV, HCV infection
3) Known immune deficiency (i.e. congenital agammaglobulinemia or hypogammaglobulinemia, common variable immune deficiency, Wiskott Aldrich syndrome), or immune suppression (i.e. transplanted patients), or congenital/acquired autoimmune disease
4) Known intolerance to immunoglobulins or proteins of human origin, or history of vaccine reactions
5) IgA deficiency
6) Preexisting risk factors for thrombotic disorders
7) Renal function defects
8) Lack of motivation or inability to comply with the requirements of the study
9) Suspected psychiatric or organic disease
10) Not willing to give written consent

CYTOTECT PREPARATIONS

A single lot of 20 ml ampoules of Cytotect was used throughout the study to approximate the dosage based on body weight. Fine adjustment of the final volume to be infused was achieved with 10 ml ampoules from 5 different lots. Overall, 77% of the total volume of HIG administered in the study was covered by a single lot (lot A144069). The remaining 23% was covered by 5 additional lots (A144048, A144058, A144079, A144106, B144010). Testing of Cytotect preparations for HCMV-specific neutralizing antibody was not foreseen by the study protocol.

CONTRIBUTORS AND CONFIDENTIALITY AGREEMENT

Umberto Nicolini (deceased), Maria Grazia Revello, and Catherine Klersy (statistician) designed the study. All coauthors contributed to data gathering. Catherine Klersy analyzed the data. Maria Grazia Revello and Catherine Klersy vouched for the data and the analysis. Maria Grazia Revello and Giuseppe Gerna wrote and decided to publish the paper. Maria Grazia Revello wrote the first draft of the paper.

There was no confidentiality agreement between the authors and the Agenzia Italiana del Farmaco.

INVESTIGATORS

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Table S1. Maternal Immunological and Virological Findings at Enrollment and Study End(*).

Parameter	HIG (N=61)	Placebo (N=62)	P Value
Antibody			
ELISA IgG AU (0.4 neg; >0.4 pos)			
enrollment	1.43 (0.86-1.96)	1.68 (0.96-2.19)	0.36
end of study	1.97 (1.33-2.86)	1.91 (1.46-3.11)	0.83
ELISA IgM ratio (<0.9 neg; >1.1 pos)			
enrollment	3.5 (2.16-4.54)	2.86 (1.55-4.63)	0.18
end of study	0.6 (0.41-0.97)	0.56 (0.40-0.93)	0.62
IgG Avidity Index (<35 low; >45 high)			
enrollment	15 (12.78-19.33)	16 (13.20-20.60)	0.35
end of study	38 (31.55-44.55)	37 (31.10-44.80)	0.76
VR1814 neutralizing Ab titer (reciprocal) in ARPE-19 (>40 pos)			
enrollment	640 (320-2,560)	1,280 (640-2,560)	0.15
end of study	2,560 (2,240-10,240)	5,120 (2,560-10,240)	0.27
AD169 neutralizing Ab titer (reciprocal) in human fibroblasts (>5 pos)			
enrollment	5 (<5-20)	10 (<5-40)	0.35
end of study	160 (80-320)	160 (80-320)	0.85
Lymphocyte subpopulations (no.cells/µl)			
$CD3^+$			
enrollment	1,663.5 (1,378-2,036)	1,605.5 (1,411-2,049)	0.74
end of study	1,676.5 (1,348-2,208)	1,573 (1,207-2,070)	0.17
$CD4^+$			
enrollment	773 (547-906)	703.5 (570-910)	0.79
end of study	893.5 (774-1,203)	879.5 (635-1,121)	0.27
$CD8^+$			
enrollment	817 (611-1,046)	830 (577-1,060.5)	0.78
end of study	669 (525-939)	693 (514-857.5)	0.60

Table S1 (cont'd)

$CD56^+$			
enrollment	165 (119-300)	167 (92-295)	0.81
end of study	96 (60-165)	97.5 (59-149)	0.85
$CD19^+$			
enrollment	146 (98-184)	133 (92-176)	0.43
end of study	180 (136-260)	153.5 (109-225.5)	0.14
$CD4^+/CD8^+$ (ratio)			
enrollment	0.86 (0.64-1.32)	0.86 (0.73-1.03)	0.94
end of study	1.38 (1.03-1.73)	1.23 (1.05-1.55)	0.26
HCMV-specific T cells† (no. cells/µl)			
$CD4^+$ $IFN\gamma^+$			
enrollment	1.90 (1.01-4.74)	4.88 (0.99-9.17)	0.21
end of study	3.78 (2.42-5.99)	6.19 (4.41-22.23)	0.08
$CD8^+$ $IFN\gamma^+$			
enrollment	30.83 (12.93-46.18)	27.36 (11.86-46.58)	0.79
end of study	15.12 (7.71-17.15)	26.32 (12.48-45.33)	0.11
DNAemia			
No. women positive/tested (%)			
enrollment	37/61 (61)	33/62 (53)	0.41
end of study	2‡/60 (6)	0/61 (0)	
Viral load (IU/ml)			
enrollment	200 (0-200)	200 (0-200)	0.48
end of study	0 (0-0)	0 (0-0)	

(*) Data are reported as median and in brackets interquartile range or percentage as appropriate.

†20 women (10 transmitters and 10 non-transmitters) were examined in each arm.

‡One woman was DNAemia-positive at the time HCMV was detected at amniocentesis and one woman was positive when she had preterm labour at 25 weeks' gestation.

Table S2. Baseline Characteristics of Transmitter and Non-Transmitter Mothers (*).

Variable	HIG-treated mothers		P Value†	Placebo-treated mothers		P Value†
	Transmitters (N = 18)	Non-transmitters (N = 43)		Transmitters (N = 27)	Non-transmitters (N = 35)	
Week of gestation at maternal infection	12 (10-17)	13 (10-18)	0.96	14 (8-22)	13 (8-18)	0.58
Weeks between maternal infection and treatment	5 (3-5)	5 (4-5)	0.77	5 (4-5)	5 (3-5)	0.24
Weeks between enrollment and study end	10 (9-19)	22 (16-25)	0.0014	12 (8-16)	21 (16-27)	<0.001
Treatments – no. per woman	3 (2-5)	5 (3-6)	0.014	3 (2-4)	5 (4-6)	<0.001

(*) Data are reported as median and in brackets interquartile range or percentage as appropriate.

† Bonferroni significance if <0.025.

Table S3. Immunological and Virological Findings in Transmitter and Non-transmitter Mothers at Enrollment and Study End (*).

Variable	HIG-treated mothers			Placebo-treated mothers		
	Transmitters (N = 18)	Non-transmitters (N = 43)	P Value†	Transmitters (N = 27)	Non-transmitters (N = 35)	P Value†
Antibody						
ELISA IgG AU/ml (0.4 neg; >0.4 pos)						
enrollment	1.25 (0.97-1.66)	1.54 (0.79-2.51)	0.30	1.67 (0.94-2.01)	1.7 (0.96-2.29)	0.85
end of study	2.47 (1.55-3.47)	1.81 (1.30-2.63)	0.15	2.37 (1.56-3.58)	1.7 (1.43-2.71)	0.14
ELISA IgM ratio (<0.9 neg; >1.1 pos)						
enrollment	4.18 (2.41-5.59)	3.34 (1.89-4.32)	0.24	3.2 (1.49-4.71)	2.75 (1.70-4.48)	0.88
end of study	0.85 (0.54-1.40)	0.58 (0.37-0.89)	0.04	0.63 (0.32-0.94)	0.51 (0.44-0.95)	0.87
IgG Avidity Index (<35 low; >45 high)						
enrollment	15.5 (13.95-18.18)	15 (12.50-20.25)	0.75	16.4 (14.28-20.23)	16 (12.50-20.85)	0.61
end of study	34 (26.63-39.85)	41.2 (32.70-46.70)	0.029	35 (31.00-41.75)	38.75 (31.48-45.05)	0.55
VR1814 neutralizing Ab titer (reciprocal) in ARPE-19 (≥ 40 pos)						
enrollment	1,280 (560-2,560)	640 (320-2,560)	0.48	1,280 (640-2,560)	640 (640-2,560)	0.27
end of study	2,560 (1,280-10,240)	2,560 (2,560-8,960)	0.82	5,120 (2,560-10,240)	5,120 (2,560-10,240)	0.38
AD169 neutralizing antibody titer (reciprocal) in HELF (≥ 5 pos)						
enrollment	7.5 (<5-40)	5 (<5-20)	0.41	10 (<5-80)	5 (<5-20)	0.38
end of study	160 (40-640)	160 (80-320)	0.87	160 (80-320)	80 (80-320)	0.23

Lymphocyte subpopulations (no. cells /µl)

$CD3^+$ (no. cells /µl)						
enrollment	1,822 (1,446-2,721)	1,639.5 (1,363-1,996.5)	0.24	1,521 (1,396-1,834)	1,747 (1,427-2,152)	0.31
end of study	1,636 (1,433-1,853)	1,722.5 (1,305.5 -2,304.5)	0.68	1,997 (1,100-2,111)	1,391 (1,242-1,641)	0.20
$CD4^+$ (no. cells /µl)						
enrollment	776.5 (547-994)	748 (542-903.5)	0.77	725.5 (486-887)	679.5 (573-933)	0.98
end of study	825 (759-1,376)	898.5 (793-1,154)	0.50	898.5 (793-1,154)	871 (635-983)	0.27
$CD8^+$ (no. cells /µl)						
enrollment	824.5 (692-1,821)	817 (565.5-1,043.5)	0.37	783.5 (576-859)	885 (606-1,137)	0.43
end of study	668 (568-848)	690 (499-1,024)	0.90	700 (482-990)	686 (520-824)	0.47
$CD56^+$ (no. cells /µl)						
enrollment	223.5 (128-462)	146.5 (98.5-229)	0.049	154.5 (87-278)	194 (122-313)	0.35
end of study	91 (52-198)	96 (71-154)	0.91	130 (93-171)	90 (52-139)	0.09
$CD19^+$ (no. cells /µl)						
enrollment	165.5 (122-195)	140 (97-179)	0.40	109.5 (71-150)	155 (97-189)	0.16
end of study	180.5 (128-209)	175 (136-278)	0.71	135 (74-220)	164 (122-273)	0.09
$CD4^+/CD8^+$ (ratio)						
enrollment	0.78 (0.55-1.13)	0.88 (0.7-1.33)	0.16	0.86 (0.75-1.17)	0.86 (0.72-1.0)	0.35
end of study	1.34 (1.10-1.63)	1.42 (1.02-1.93)	0.65	1.31 (1.01-1.58)	1.22 (1.10-1.52)	0.74

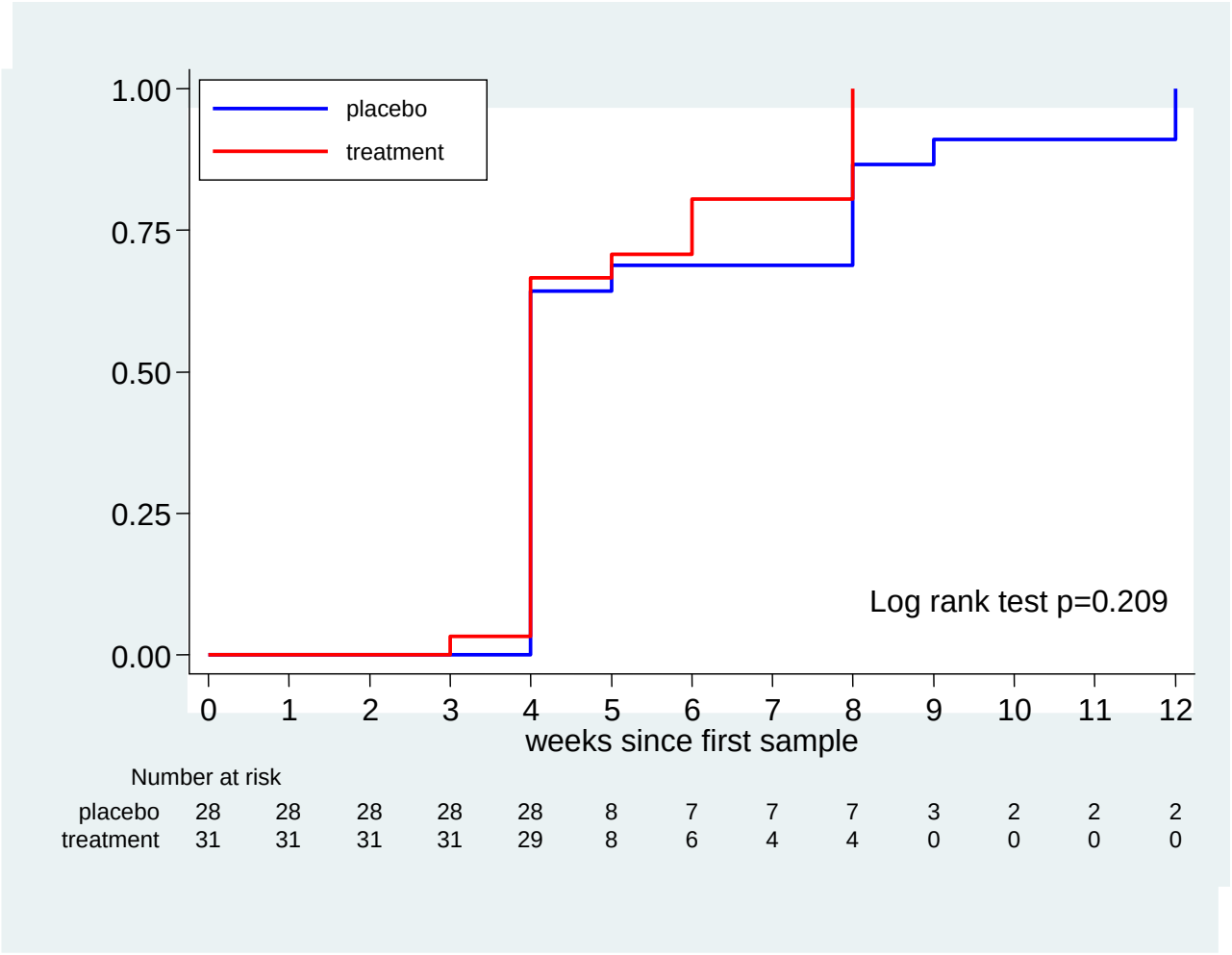
Table S3 (cont'd)

HCMV-specific T cells‡(no. cells/µl)						
$CD4^+IFN\gamma^+$						
enrollment	2.08 (1.9-4.74)	1.86 (1.57-2.06)	0.74	4.88 (3.41-9.17)	3.10 (0.84-7.52)	0.53
end of study	2.43 (1.79-2.77)	5.29 (4.79-7.79)	0.41	8.36 (6.01-15.35)	5.34 (4.85-29.8)	0.93
$CD8^+IFN\gamma^+$						
enrollment	14.04 (11.52-44.28)	35.34 (30.83-55.25)	0.17	31.08 (27.36-43.58)	16.63 (11.17-44.14)	0.32
end of study	9.13 (6.29-13.88)	16.77 (15.12-18.01)	0.06	39.96 (12.57-65.33)	24.89 (12.48-26.32)	0.53
DNAemia-positive women						
enrollment	11 (61)	26 (60)	1	14 (52)	19 (54)	1
end of study	1 (6)	1 (2)		0	0	
Viral load (IU/ml)						
enrollment	200 (0-801.5)	200 (0-200)	0.43	200 (0-253.8)	200 (0-200)	0.87
end of study	0 (0-0)	0 (0-0)		0 (0-0)	0 (0-0)	

(*) Data are reported as median and in brackets interquartile range or percentage as appropriate.

† Bonferroni significance if <0.025.

‡ 10 women were examined in each subgroup.



CUMULATIVE HCMV DNAEMIA CLEARANCE

Figure S1. Kaplan-Meier estimate by treatment.

Power evaluation

The sample size for our study was computed based on the published observational study by Nigro et al, where a relative decrease in transmission of 60% was shown in treated patients (absolute difference of 24%, from 40% to 16%). In our study the observed relative decrease was 32% (absolute difference 14%, from 44% to 30%). With the available sample size the power to show such an effect is only 33%. Based on the same alpha of 5%, 2-tailed, and power 80%, 174 patient per group would have been needed to show such a difference.