Evaluation of UL99 transcript as a target for antiviral treatment efficacy.

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SUMMARY

Human Cytomegalovirus (HCMV) is a virus belonging to the Beta Herpes virus family. Its genome contains many different genes that can be clustered in immediate early, early and late genes. In this last cluster is included UL99, a late gene that encodes for a tegument protein called pp28. In immunocompetent patients HCMV infection occurs asymptomatically, while in immunocompromised patients its reactivation can be cause of pneumonia, retinitis and gastrointestinal diseases. To prevent or to contrast HCMV infection, several drugs (such as Ganciclovir, Acyclovir, Foscarnet) are administered and HCMV DNA load is usually evaluated for therapy monitoring, but also for antiviral resistance onset that may occur after the therapy. In this study is described the production of a Real Time PCR for the detection and quantification of UL99 transcript and the rapid clearance of this target compared to HCMV DNA, both in vitro and in vivo on bronchoalveolar lavage samples.

KEYWORDS: Human Cytomegalovirus, UL99, Ganciclovir, Real Time PCR, Transcript Quantification, Antiviral Resistance.

EVALUATION OF UL99 TRANSCRIPT AS A TARGET FOR ANTIVIRAL TREATMENT EFFICACY.

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Human Cytomegalovirus (HCMV) is a virus belonging to the Beta Herpes virus family. Its genome, ~235 kb long, is organized in two region flanked by inverted repeats that define a unique long (UL) and a unique short (US) region. Based on the region and the position in which the ORFs are located, HCMV's genes are named with abbreviations, such as UL54 DNA polymerase or UL97 protein kinase. UL99 ORF encodes for a 190 amino acids long protein, which is expressed in the late phase of viral replication and belongs to the proteins of tegument (Britt and Boppana, 2004). Studies on this protein have described its essentiality in the production of infectious virus, in fact deletion of the UL99 ORF resulted in the production of non enveloped and non infectious cytoplasmic particles (Silva et al., 2003; Jones and Lee, 2004). HCMV infects world's human population in a range that varies from 60% to 90% depending on the socio-economic class and geographic location (Pass, 2001). Primary infection in immunocompetent patients is usually asymptomatic, with few cases of mononucleosis like syndrome. Once infected the human host, HCMV persists in the organism in latent form with the possibility to rapidly reactivate in particular conditions, such critical illness and immune system deficit (Cook and Trgovcich, 2011). Immunocompromised patients, and in particular transplanted patients, are one of the most subjected to HCMV reactivation with the risk of pneumonia, retinitis and gastrointestinal disease developing (Griffiths and Walter, 2005). The principal therapy is based on the antiviral drugs administration, which exert their activity on UL54 DNA polymerase (Acyclovir, Ganciclovir, Valganciclovir, Cidofovir, Foscarnet) or UL97 protein kinase (Maribavir) inhibiting the DNA viral replication (Mercorelli et al., 2008; De Clercq, 2013). Despite antiviral treatment is usually able to block the HCMV replication, the prolonged use of antiviral drugs, such as Ganciclovir, by critically ill or immunosuppressed patients can act as a promoting factor for the antiviral drugs resistances onset (Erice et al., 1989). Furthermore, HCMV DNA represents the principal target for the monitoring after antiviral and preemptive therapy (Yoshida et al., 2001; Gerna et al., 2011). In this work, UL99 viral transcript has been taken in consideration as a rapid marker of therapy effectiveness and a quantitative RT Real Time PCR has been developed as a tool for antiviral therapy control. The RT Real Time PCR was tested both in vitro and in vivo on 18 bronchoalveolar lavages samples belonging to patients who were or were not subjected to an antiviral treatment.

The antiviral susceptibility test in vitro has been performed including AD169 HCMV and ganciclovir resistant VR5438 HCMV viruses in presence of raising antiviral drug concentration in order to evaluate HCMV DNA and UL99 RNA concentration. The virus production was achieved through a prior propagation in Helf cells. Two T150 flasks were plated in order to obtain about 4×10^6 cells. The cells were then infected with AD169 CMV virus strain (ATCC VR-538) and with VR5438 HCMV ganciclovir resistant virus, MOI 0,01 and the flasks were left for 1 hour at room temperature and 1 hour at 37°C. After this incubation period the viruses from both the flasks were removed, MEM 10% FCS was added and the flasks were incubated at 37°C 5% CO_2 till approximately all the cells resulted infected. The virus recovery was achieved with an initial cell medium removal and a following plate scraping. The cells recovered were then subjected to three rapid cycles of freezing and thawing with liquid nitrogen. A following centrifugation at 1500 rpm for 5 minutes at 4°C occurred and the supernatant collected together with the flask medium.

Subsequently, ten fold viral dilutions till 10^{-7} were produced from both the virus propagated. Helf cells, dispensed in a 96 wells microplate, were then infected with 50 µl of viral dilutions and incubated at 37°C for 2 hours. Cell medium was removed and substituted with 100 µl of Methyl-Cellulose 1% (Sigma Aldrich, St. Louis (MO) USA) and left at 37°C for 8 days. The Methyl-Cellulose was then removed and 50 µl of Crystal violet 0,1% (Sigma Aldrich, St. Louis (MO) USA) added. The microplate was incubated 30 minutes in dark room, removed the crystal violet and unstained the plate with fresh water. The plaque forming unit (PFU) was then calculated in order to evaluate the viral titer.

The nucleic acids extraction was then performed using automatic extractor NucliSens EasyMag (Biomérieux, Marcy L'Etoile France), according to manufacturer's instructions, to obtain 50 µl of eluate containing both DNA and RNA from 1 ml of sample. A first DNAse step to eliminate the correspondent DNA target was executed using Deoxyribonuclease I Amplification Grade (Life technologies, Carlsbad, California USA), following manufacturer's instructions. The 11 µl of DNAse product were then purified by a second extraction in a 25 µl final volume. A subsequent retrotranscription was performed using a first mix composed by random primers (600 ng/µl) and dNTPs (10 mM). After 5 minutes of incubation at 70°C with 10 µl of sample RNA, a second mix composed by $MgCl_2$ (25 mM), ImProm Buffer 5x [200 mMTris-HCl (pH8.4), 500 mMKCl], ImProm-II Reverse Transcriptase™ (1 U/µl) and recombinant Rnasin Ribonuclease Inhibitor (40 U/µl) (Promega, Madison (WI) USA) was added to obtain a 20 µl final volume. The total volume mixture was then incubated for 5 min at 25°C, 60 min at 42°C and 15 min at 70°C using 9800 Fast Thermal Cycler (Life technologies, Carlsbad, California USA).

UL99 cloning primers and Real Time primers and probes were designed using Primer express 3.0 (Life technologies, Carlsbad, California USA). A subsequent analysis with BLAST (www.ncbi.nlm.nih.gov/blast/) occurred in order to confirm the design and to exclude possible cross recognitions with other targets.

An initial Fast PCR was performed in order to amplify UL99 target region containing 20 pmol of cloning primers (UL99 clonF 5'-CGGGGGAAACGACAGTAGTA-3' and UL99 clonR 5'-AGTCGCTTAGCCACCACCT-3'), 6 µl of GoTaq® HotStart Polymerase buffer 5x (Promega, Madison (WI) USA), 200 µM of each dNTP, 6mM of $MgCl_2$ and 1 unit of GoTaq® HotStart Polymerase (Promega, Madison (WI) USA). The thermal profile used was: 94° C for 3 min, followed by 35 cycles of 95°C for 0' sec, 60°C for 15 sec and 72° C for 10 sec, with a final step at 72°C for 7 min. The Fast PCR was performed on 9800 Fast Thermal Cycler (Life technologies, Carlsbad, California USA). The 260 bp DNA fragment was cloned into a pCR 2.1-TOPO plasmid vector, using pTOPO-TA cloning technology (Life technologies, Carlsbad, California USA). Once produced the plasmid, it transfected in competent Escherichia coli TOP10 cells, which were subsequently propagated overnight. The selected transformed clones were amplified through culture in liquid LB medium (10 g BACTOTRYPTONE, 5 g yeast extract, 171 mM NaCl, 15 g/l agar, pH 7.5) containing 50 µg/ml of ampicillin. Plasmid DNA was extracted and purified using the PureLink™ HiPure Plasmid Midiprep Kit (Life technologies, Carlsbad, California USA). The plasmid concentration was estimated on spectrophotometric reading at OD260.

The UL99 cRNA production was performed as described in Terlizzi et al. (2011) in order to obtain scalar cDNA standard dilutions for the RT Real Time PCR absolute quantification.

UL99 Real Time PCR mix was produced adding to the 1×Master Mix (Platinum qPCR supermix—UDG with ROX, Life technologies, Carlsbad, California USA), UL99 primers (UL99 F 5'-CGACGTTTCGCGAGGACAA-3', UL99 R 5'-GTGGTGATGTTTTGAGGGTTTCTTT-3'), UL99 Taqman probe (5'-FAM-CGAAACCGAGCAAGC-MGB-3') and 2 µl of cDNA, for a total volume of 20 µl. Primers and Taqman probe were tested in four different concentrations (900/250 nM, 900/100 nM, 250/250 nM, 250/100 nM primers/probe concentrations) in order to find the best amplification conditions. RPL27 ribosomal gene was used as internal control (RPL27 F 5'-GGGTGGTTGCTGCCGAAATG-3', RPL27 R 5'-CTTTGCGTCCGGAGTAGCG-3', RPL27 Taqman probe 5'-VIC-GGAAGGTGGTGCTTGTCCTGGCT-TAMRA-3') with a 60/50 nM primers/probe concentration. The dynamic range, defined as the range of dilutions in which a linear regression curve can be constructed, and the sensitivity, expressed as the lowest target concentration detectable at a frequency of 100%, were obtained using 10 fold dilutions of UL99 plasmid, ranging from 10^{10} to 10^{0} copies/reaction.

cDNA standards produced from UL99 cRNA and ranging from 10^{5} to 10^{2} copies per reaction were used to create a standard curve for the absolute quantification. The UL99 load raw result was then corrected taking in consideration extraction, DNAse and retrotranscription previous steps and multiplying this load, expressed as copies per reaction, for 1375 in order to obtain UL99 load expressed as copies per millilitre of sample.

In order to develop the antiviral susceptibility test in vitro, Helf cells were propagated on a 24 wells plate and then infected with the two virus strains described above. Briefly, 1 ml of Helf cells suspension in a medium composed by MEM 10% FCS (PAA Laboratories GmbH, Pasching Austria), 1% glutamine (L-Glutamine, Life technologies, Carlsbad, California USA), 0.15% Pen-Strep solution (Sigma Aldrich, St. Louis (MO) USA), 0.2% Fungizone (Bristol-Myers Squibb, New York (NY) USA) were added in each well and the plate has been incubated at 37°C 5% CO_2 until the cell confluence reached the 80-90%. The Helf cells were then infected adding 10 PFU/ml of HCMV AD169 strain and HCMV drug resistant virus VR5438 and incubating the plate at 37°C 5% CO_2 for 3 hours. One well was not infected as negative control. The medium containing the virus was then removed and were added separately in each well 100 µl of Ganciclovir and Cidofovir in different concentrations. Ganciclovir concentrations tested were 5, 50 and 500 µM, while Cidofovir concentrations were 0.5, 5 and 50 µM, according to what has been described in previous works (Drew et al., 1993; Landry et al., 1999; Drew et al., 2006). The final volume was brought up to 1 ml with cell medium MEM 2% FCS, 1% glutamine, 0.15% Pen-Strep solution, 0.2% Fungizone. Ganciclovir or Cidofovir were not added in two wells, one for each virus, to obtain positive controls wells. The plate was then incubated for 10 days at 37°C 5% CO_2. After the incubation period, the cell medium suspension of each well was collected and the Helf cells were subjected to a trypsin-EDTA digestion with 200 µl (trypsin 1%, 0.05mM EDTA pH 8.0) for 5 minutes at 37°C and then collected. The collected material was centrifuged at 13000 rpm for 2 minutes. 900 µl of Lysis buffer (Biomérieux, Marcy L'Etoile France) were added before the nucleic acids extractions with NucliSens EasyMag (Biomérieux, Marcy L'Etoile France) in a 50 µl final volume elute. Subsequently were executed the DNAse and retrotranscripition steps described before.

HCMV-DNA was quantified using Q-CMV Real Time Complete Kit (ELITech Group, Puteaux, France), targeting the exon 4 region of the HCMV MIEA gene (major immediate early antigen, HCMVUL123). The amplification was carried out on 7500 Real Time PCR System (Life technologies, Carlsbad, California USA) adding 5 µl of sample DNA to 20 µl of mix with the following profile: one cycle of decontamination at 50°C for 2 min, one cycle of denaturation at 95°C for 10 min followed by 45 cycles of amplification - 95°C for 15 s, 60°C for 60 s. Ten-fold scalar standard dilutions ranging from 10^2 to 10^5 were employed for the standard curve construction and target quantification. Inner control for the beta actin gene was used to confirm negative results and AD169 HCMV strain DNA and double distilled water were used, respectively, as positive and negative controls. Amplification data were analysed by sequence detection system software (Life technologies, Carlsbad, California USA). Lower limit of detection and quantification were respectively equal to 8.5 and 15.8 gEq/reaction.

To confirm that possible negative results were not caused by RNA degradation, all the samples were amplified in order to verify the presence of endogenous RNA on GAPDH gene. The amplification mix was produced with 10×Master Mix (Platinum qPCR supermix—UDG with ROX, Life technologies, Carlsbad, California USA), GAPDH

primers 60 nM (GAPDH F 5'-GCCAAAAGGGTCATCATCTC-3', GAPDH R 5'-GGGGCCATCCACAGTCTTCT-3'), GAPDH Taqman probe 50 nM (5'-VIC-TGGTATCGTGGAAGGA-MGB-3'), while the thermal profile has been kept the same used for UL99 Real Time PCR.

Eighteen bronchoalveolar lavages from 14 patients (7/7 M/F; mean age 59.64 years, range 21-80) positive to HCMV infection were considered for the detection of UL99 transcript. All the samples were originated from the virology Unit of Azienda Ospedaliero-Universitaria and come from Pulmonology and Intensive care units. Twelve samples belonging to 9 patients undergone to antiviral treatment (Foscarnet, Acyclovir or Ganciclovir), while 6 samples belonging to 5 patients were not subjected to an antiviral treatment. RNA and DNA were extracted from 500 µl volume of bronchoalveolar lavage, liquefied 1:1 with N-acetylcisteine, and eluted to a final volume of 110 µl with the extraction method described above. The DNAse, retrotranscription and UL99 Real Time PCR were executed as referred above. Each sample was tested in three repetitions and HCMV load was evaluated with Q-CMV Real Time Complete Kit (ELITech Group, Puteaux, France).

Standard cDNA dilutions ranging from 10^5 to 10^2 were amplified in 5 repetitions. The results obtained were used in order to calculate trueness, precision, accuracy of the test, as described in Hauck et al., 2008 and in Menditto et al., 2007. SPSS Statistics 18 software (IBM© Company, New York, USA) was used for statistical analysis, including Shapiro-Wilk and Dixon tests. Mann Whitney and Chi square test were used to evaluate statistically significant differences between "treated" and "not treated" group.

Taking in consideration the results achieved in the analysis of the dynamic range and the precision and sensitivity evaluation, the best amplification performance was obtained with a 900/100 nM respectively of primers and probe concentration (data not shown).

Amplification linearity was evaluated by standard UL99 cDNA dilutions ranging from 10^{10} to 1 copies/reaction amplification, taking in consideration an approximate equal gap in terms of Ct between the dilutions tested as linearity evidence. The range of linearity observed was from 10^{10} to 10 copies/reaction, while sensitivity was attested at 10 copies/reaction.

Validation and standardization of the method was obtained by statistical analysis including the evaluation of trueness, precision and accuracy. Five repetitions of standard cDNA (from 10^5 to 10^2 copies per reaction) were used for analysis, mean values and standard deviation are reported in table 1. Dixon's test showed the absence of outlier values in the results obtained, both for upper and lower limit, while Shapiro-Wilk's test confirmed the values normal distribution (data not shown). Trueness, consisting in the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value, was verified by the comparison between t_{calc} and t_{tab} values of the five repetitions. The test precision, defined as the closeness of agreement between independent test results obtained

under stipulated conditions, was evaluated by repeatability limit, Intra and Inter test variability, and accuracy (table 1).

In the test in vitro, different antiviral drugs concentrations were inoculated in Helf cells infected with HCMV AD169. Cidofovir and Ganciclovir, both DNA polymerase inhibitor, were tested at different concentrations: the first one at concentrations from 0.5 up to 50 µM, the second one was tested at concentrations from 5 up to 500 µM. The evaluation of HCMV load and UL99 transcript with Real Time PCR showed a progressive reduction of viral load and UL99 transcript levels with the increasing of antiviral drugs concentration (figure 1). While, no substantial differences in terms of viral load and UL99 transcript levels were observed for antiviral resistant viral isolate VR5438 (figure 1).

Considering clinical samples,18 bronchoalveolar lavages from 14 patients and positive to HCMV were used for UL99 transcript detection in vivo. 6 samples belonged to 5 patients who did not undergo to an antiviral treatment, while 12 samples came from 9 patients subjected to antiviral therapy. UL99 viral transcript was detected in 5 of 6 (83.3%) samples of not treated patients with a mean load of 102714 copies/ml. For what concerns the treated patients group, 3 of 12 (25%) samples were found positive to UL99 transcript with a mean load of 159720 copies/ml. The results are summarized in table 2 and figures 2 and 3. Chi square test showed a statistically significant higher UL99 transcript detection rate in the “not treated” group compared to “treated” group ($p<0.05$), but no statistically significant differences in terms of UL99 load were observed (figure 4).

HCMV infection occurs in the 60-90% of world population, depending on the socio-economic class and geographic dislocation, with a higher prevalence in developing countries (Pass, 2001). Once infected the organism, HCMV replicates within different cellular types, such as monocytes, macrophages, epithelial and endothelial cells (Revello and Gerna, 2010), by means of the expression of well defined group of genes that are clustered in 3 categories depending on their sequential expression: Immediate Early (IE), Early (E), Late (L) (Mocarski, 1996). UL99 is an ORF that encodes for a tegument protein pp28 essential for the virus maturation (Silva et al., 2003) and constitutes one of the so called “Late” genes, group of genes expressed during the final steps of viral replication. While in healthy patients the infection is mostly asymptomatic, in immunodeficient patients the infection can lead to several different pathologies (Drew, 1992).To overcome or prevent HCMV infection, antiviral drugs are administered in immunodeficient patients. However, is demonstrated that the prolonged antiviral drugs administration can be cause of antiviral resistance onset (Lurain and Chou, 2010).

In this study is described the production of a RT Real time PCR for the detection of UL99 transcript. UL99 transcript was chosen as target for its late expression in the viral replication cycle permitting to associate its detection with virus effective replication.

Firstly, a test in vitro was developed to evaluate the HCMV DNA and UL99 RNA loads in antiviral drug presence. As displayed in figure 1, with the increasing antiviral drug concentration a clear reduction on both HCMV DNA and UL99 RNA is observable. In particular, this reduction is more significant in the UL99 RNA load with a total absence at maximal antiviral drug concentration, while HCMV DNA, though considerably reduced, is still present in several copies at the maximal concentration. Anyway, when considering a viral isolate with a strong resistance to both Ganciclovir and Cidofovir, UL99 RNA and HCMV DNA levels didn't decrease, confirming the HCMV DNA and UL99 RNA drug dependent reduction.

Broncho alveolar lavage samples of patients found as positive to HCMV DNA were then tested for the presence of UL99 transcript with the same assay. Of 18 samples, 6 samples belonged to patients who didn't undergo to an antiviral treatment, while 12 samples belonged to patients treated with Acyclovir, Ganciclovir or Foscarnet. The HCMV DNA load and the UL99 transcript load are reported in table 2. Five out of 6 samples tested in the not treated group were found as positive to UL99 RNA while in the treated group only 3 samples were found as positive to UL99 RNA and 9 were negative. The difference between the two groups was statistically significant ($p=0.0037$), with a clear association between positivity to UL99 RNA and absence of antiviral treatment.

The results obtained with test in vitro and clinical samples test showed a clear difference between detection of UL99 transcript and HCMV DNA. Even though the presence of an antiviral drug, such as Ganciclovir or Cidofovir, caused the reduction of HCMV DNA load in the test in vitro, only UL99 transcript levels were zeroed (figure 1). Furthermore, considering clinical samples test, UL99 transcript has been found negative mainly in patients treated with an antiviral drug than in not treated patients, suggesting an interaction that blocked the fundamental steps that lead to late genes expression and in particular UL99 RNA production.

This test could be, indeed, a useful assay for drug effectiveness confirmation, as observable in the clinical sample test where, even in presence of extremely high levels of HCMV DNA, UL99 transcript was not detected in most of samples of patients undergone to an antiviral treatment.

Besides, this test could be helpful as a further tool to suspect a drug resistance onset, as a UL99 transcript positive result after antiviral therapy could be an important sign to investigate. In this study, three samples were found as positive despite the therapy, but no antiviral resistance investigation was performed.

Taking in consideration the results obtained, this procedure could be a useful tool for the evaluation of the therapy response compared to evaluation of HCMV DNA and a further tool for the evaluation of a suspected resistance onset.

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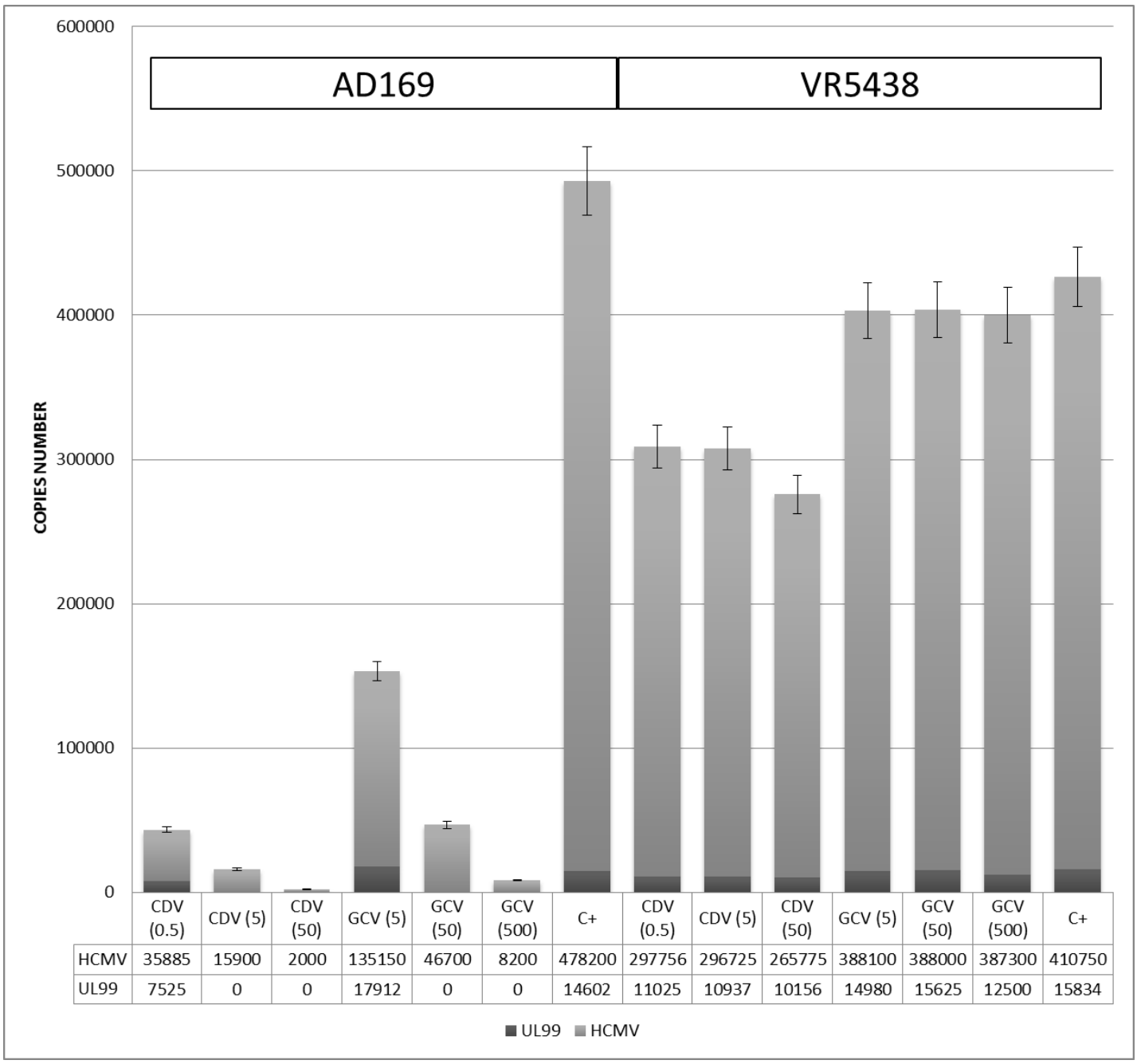
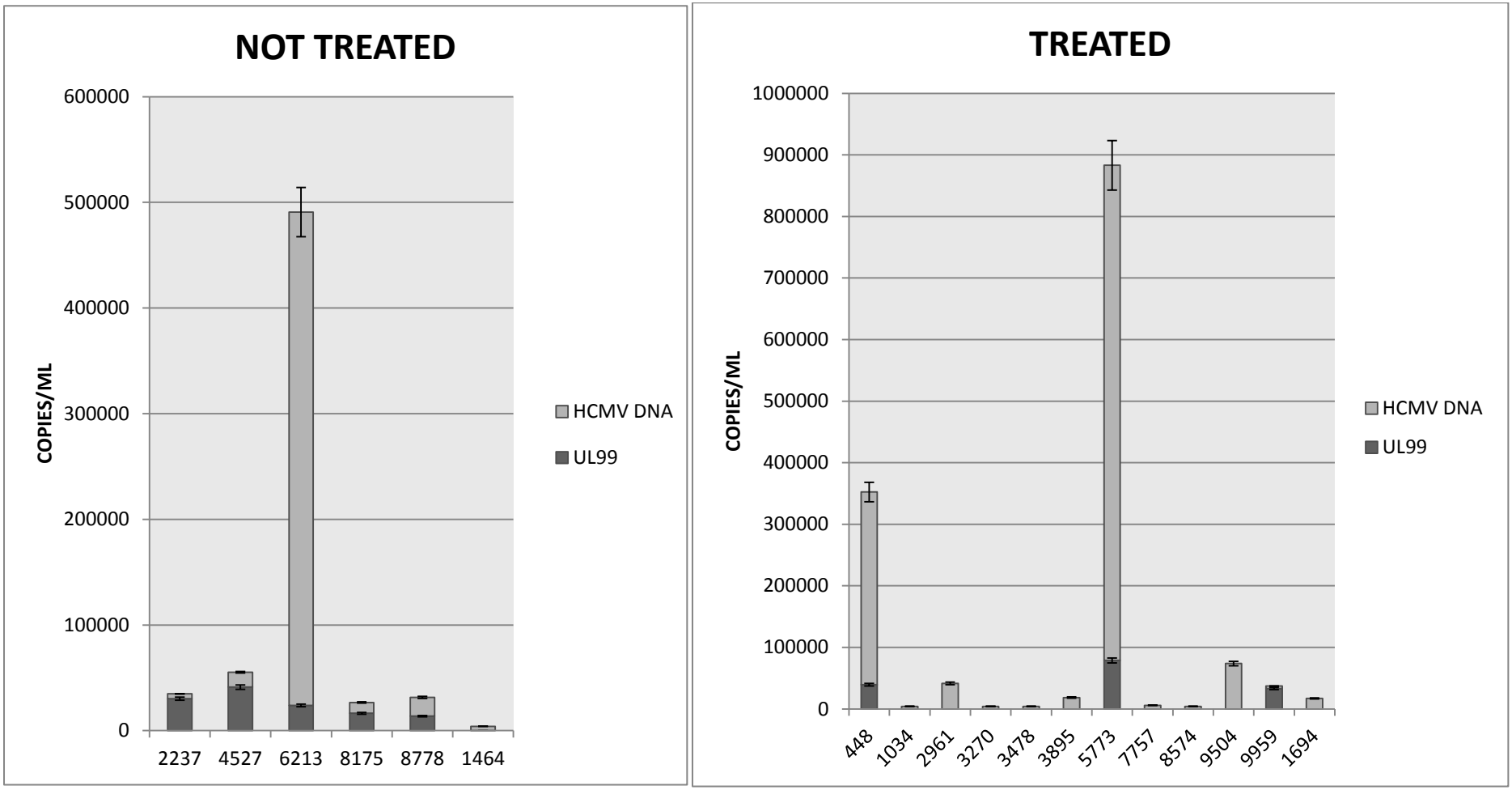


	CDV (0.5)	CDV (5)	CDV (50)	GCV (5)	GCV (50)	GCV (500)	C+	CDV (0.5)	CDV (5)	CDV (50)	GCV (5)	GCV (50)	GCV (500)	C+
HCMV	35885	15900	2000	135150	46700	8200	478200	297756	296725	265775	388100	388000	387300	410750
UL99	7525	0	0	17912	0	0	14602	11025	10937	10156	14980	15625	12500	15834

Figure 1. Susceptibility test. HCMV and UL99 transcript loads for AD169 strain and VR5438 isolate in presence of increasing antiviral drug concentrations. The concentrations reported are expressed as micromolar concentrations and viral load as copies number. CDV: Cidofovir; GCV: Ganciclovir; C+: positive control.



Figures 2 and 3. HCMV and UL99 loads in “Treated” and “Not treated” groups by Real Time PCR. Light grey columns show HCMV DNA load, while dark grey columns show the UL99 transcript load. The results are expressed as copies/ml.

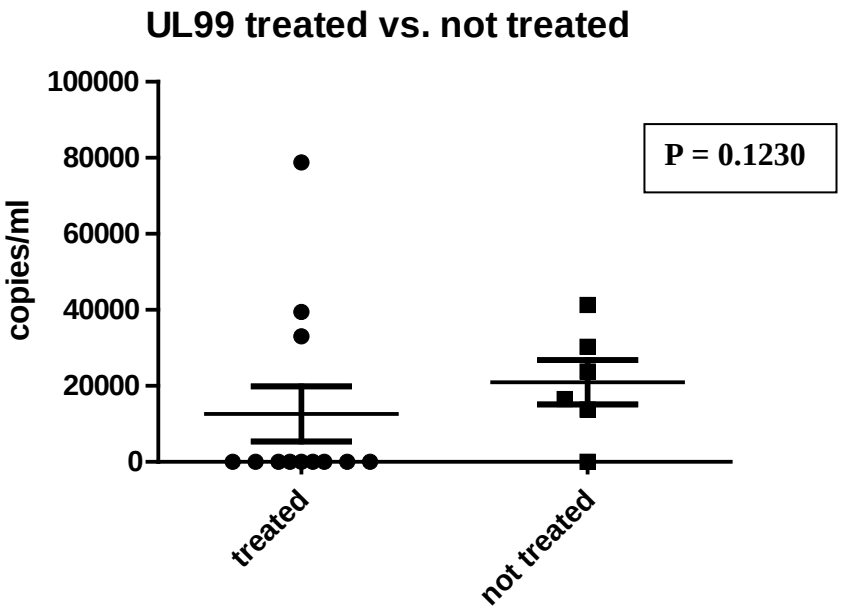


Figure 4. Scatter plot of UL99 transcript loads, expressed as copies/ml, in “treated” and “not treated” patients. The mean and the standard error of mean are displayed in the figure. Mann Whitney test for not normally distributed variables showed not statistically significant differences in terms of transcript loads in the two groups. P: P-value.

	10^2	10^3	10^4	10^5
TRUENESS				
Mean concentration	151	1192	11158	130550
Standard deviation	84,46	268,57	1108,18	25651,56
t-calc	1,35	1,60	2,34	2,66
t-tab (n=5)	2,776	2,776	2,776	2,776
PRECISION				
Intratest variability-Repeatability	55,99	22,53	9,93	19,65
Intertest variabilty- Intermediate reproducibility	75,84	46,87	34,34	43,01
Repeatability limit	331,59	1054,37	4350,55	100704,35
Accuracy (%)	50,86	19,23	11,58	30,55

Table 1. Trueness and precision of UL99 Real Time PCR. Five repetitions of standard concentrations ranging from 10^2 to 10^5 were amplified and trueness and precision evaluated.

TREATED

ID	HCMV	UL99
448	312928	39417
1034	<4141	NEG
2961	41360	NEG
3270	<4141	NEG
3478	<4141	NEG
3895	18480	NEG
5773	804320	78833
7757	5808	NEG
8574	<4141	NEG
9504	73744	NEG
9959	4224	33000
1694	16896	NEG

NOT TREATED

ID	HCMV	UL99
2237	4576	30250
4527	14080	41250
6213	466928	23833
8175	10208	16500
8778	17776	13750
1464	<4141	NEG

Table 2. HCMV DNA, UL99 RNA results in antiviral “treated” or “not treated” patients. Results are expressed as copies/ml. <4141: value assigned to a positive sample below the limit of quantification; NEG: negative result.