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Approval Page

Impact of an Ultrasensitive *Cytomegalovirus* (CMV) Quantitative Nucleic Acid Test (qNAT) on CMV Detection and Therapy in Renal Transplant Recipients

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Abstract Cover Page

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M.D. Baylor College of Medicine 2018

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An abstract of

A thesis submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Master of Science

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Abstract

Impact of an Ultrasensitive *Cytomegalovirus* (CMV) Quantitative Nucleic Acid Test (qNAT) on CMV Detection and Therapy in Renal Transplant Recipients

By Vivek Beechar M.D.

Background: Cytomegalovirus (CMV) infection has broad implications for morbidity and mortality in renal transplant recipients (RTR). Routine surveillance for CMV replication with PCR-based quantitative nucleic acid testing (qNAT) assays is standard practice in most transplant centers, but the impact of assay sensitivity on antiviral decision-making and virologic outcomes has not been studied. We investigated the effects of an ultrasensitive CMV qNAT assay on clinical outcomes, including time to detection and duration of CMV DNAemia.

Methods: We conducted a single-center cohort study comparing RTRs monitored with a qNAT with a higher lower limit of quantification (LLOQ> 300 IU/mL) with those monitored with a more sensitive qNAT (LLOQ> 35 IU/mL). Patients were stratified by donor (D)/recipient (R) CMV serostatus (D+/R-, high-risk; any R+, moderate-risk). CMV viral load monitoring was performed monthly post-transplantation according to Emory Transplant Center protocols, with the primary outcomes being time to CMV DNAemia and its duration.

Results: 1382 patients were analyzed from 2014-2016 and 2019-2021. Moderate-risk RTRs monitored with the more sensitive assay experienced a greater hazard for the development of a first episode of CMV DNAemia (aHR- 1.95 95% CI- 1.55 to 2.46) and an average of 24 (95% CI- 16.40 to 31.98) additional days of DNAemia after reaching the 1,000 IU/mL threshold compared to those tested with the less sensitive assay. There was no difference in CMV end-organ disease or one-year all-cause mortality between moderate-risk RTRs.

Conclusions: The more sensitive assay was associated with earlier detection and extended durations of CMV DNAemia in moderate-risk RTRs, without altering clinical outcomes. These findings inform optimal use of these assays and antiviral stewardship in RTRs.

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Acknowledgements

I would like to thank Dr. Michael Woodworth for his mentorship throughout the MSCR course. His advice and guidance have been invaluable to me as I hope to establish myself as a physician scientist in the field of transplant infectious diseases. Dr. Woodworth has taught me the value of patience and persistence in the face of adversity.

I would like to thank my transplant infectious disease mentors, Dr. Varun Phadke and Dr. Stephanie Pouch. Their assistance in guiding me with the thesis content and analysis has been invaluable and they both serve as role models for me in my clinical career.

I would like to thank Dr. Christian Larsen and Ms. Geeta Karadkhele for their assistance with navigating the renal transplant database.

I would like to thank Dr. Monica Farley and Dr. Wendy Armstrong for giving me the opportunity to pursue the Masters of Science in Clinical Research while being an infectious diseases fellow at Emory.

I would like to thank the course instructors and thesis chair, Dr. Amita Manatunga, for the Masters in Clinical Research whose instruction laid the foundation for my thesis. I would also like to thank Dr. Ahmed Babiker for being a thesis reader and for the guidance he provided me in the project.

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Introduction

CMV is a leading driver of solid organ transplant recipient (SOTR) morbidity[1]. CMV establishes latency in myeloid cells and a large proportion (estimated to be 83%) of the population demonstrate prior serologic evidence of infection[2,3]. When immunosuppressive agents are administered in the post-transplant period to reduce risk of rejection, latent CMV can reactivate and cause a wide spectrum of disease ranging from asymptomatic infection to life threatening organ failure and increased risk for developing other opportunistic infections[4].

The three CMV related clinical illnesses seen in SOTRs are asymptomatic CMV DNAemia, CMV syndrome, and CMV disease. Asymptomatic CMV DNAemia is defined as the presence of detectable CMV nucleic acid in the plasma or whole blood, by quantitative nucleic acid testing (qNAT). CMV syndrome is defined by the detection of CMV via qNAT along with at least two of the following features: 1) Fever greater than 38°C for a minimum of two days 2) Increased fatigue 3) Neutropenia/leukopenia detected on two separate samples 4) Thrombocytopenia 5) The detection of at least 5% atypical lymphocytes 6) Transaminitis to at least two times the upper limit of normal (excluding patients with liver transplants). CMV disease manifests as end-organ dysfunction of a variety of organs ranging from the retina to the colon[4,5].

CMV Preventative Strategies

Given the substantial morbidity associated with CMV reactivation and disease in SOTRs, CMV prevention and control are cornerstones of management. The two main strategies employed for the prevention of CMV-related illnesses include prophylactic and preemptive antiviral therapy. Each of these strategies has associated advantages and disadvantages. Two categories of factors influence the adoption of one of these strategies: 1) logistical - capacity to obtain, monitor, and respond to laboratory test results, either for virologic or therapeutic drug monitoring, as well as drug/test costs; 2) biological - risks of drug toxicity, delayed CMV disease, and impact of prophylaxis on CMV immunity. Antiviral prophylaxis carries the risks of drug-induced neutropenia, delayed onset CMV disease, and antiviral resistance but requires less frequent viral load monitoring and therefore is easier to coordinate. Preemptive antiviral therapy requires significant viral load monitoring and its associated costs but carries less of the biological risk factors[4,6].

CMV Diagnostic Tools

The diagnostic tools for detecting CMV related illnesses have evolved over time and include CMV viral culture, pp65 antigen detection, serology, histopathology, cell mediated immune assays, and qNAT testing[4,7]. The development of new diagnostic tests over time for CMV has been motivated by the need to have rapid turnaround time, ease of testing, and the ability to quantify the severity of the illness. A test that possesses these qualities allows transplant clinicians to rapidly diagnose and treat patients with asymptomatic CMV DNAemia before the progression to end-organ disease[4,8].

Viral Culture

CMV viral culture is no longer routinely performed as the assay takes between 2-4 weeks to complete, which severely limits its clinical utility, and more sensitive assays are available for the detection of CMV[9]. Historically, CMV viral culture provided a method of testing for the phenotypic expression of viral resistance. However, with the advent of genotypic mutational testing the need to test for phenotypic expression of viral resistance is largely obsolete[4].

pp65 antigen testing

The pp65 antigen is a CMV protein that is expressed within leukocytes and the assay works by detecting antibodies to pp65[10,11].The pp65 antigen assay is no longer routinely performed and has been replaced by the CMV qNAT assay[4,9]. The assay's benefits include its quick turnaround time, high sensitivity, quantitative nature, and utility in tracking response to therapy. However, the limitations that prevent its routine clinical use include technical expertise in performing and interpreting the assay, the need for the patient's sample to be processed within 6 hours of collection, its limited use in patients who are leukopenic, and the lack of standardization across laboratories[9].

Serology

Serology plays a pivotal role in the pre-transplant risk-stratification of patients. The serostatus (IgG) of the donor and recipient is assessed using an enzyme-linked immunosorbent

assay (ELISA)[4,9]. Table 1 shows the risk stratification of patients based on serostatus. CMV serostatus informs several clinical decisions including duration of anti-viral prophylaxis post-transplantation and threshold at which anti-viral therapy is initiated in asymptomatic CMV DNAemia[4]. Outside of risk stratification, CMV serology plays a very limited to no role in the management of patients with SOTRs. When patients develop CMV DNAemia, syndrome, or disease there is no utility in checking either the CMV IgM or IgG for diagnostic purposes given that the immunosuppression regimens that these patients are on to prevent rejection also blunts the production of these antibodies[10].

Histopathology

When CMV end-organ disease is suspected, biopsy and histological analysis of the affected organ may be needed for definitive diagnosis[10-13]. CMV causes cellular and nuclear enlargement along with the aggregation of amphophilic and basophilic inclusions[10-12,14]. After compatible histological features are noted, the diagnosis of CMV is confirmed with *in situ* hybridization (ISH) and/or immunohistochemical staining (IHC) of the sample[14]. The most prominent limitation of histopathology is the need for an invasive procedure for tissue sampling. This also limits its utility as a method of tracking clinical improvement after anti-viral therapy is initiated[9,12].

Cell-Mediated Immune Assays

Cell-mediated immunity is a SOTR's central defense against CMV viral replication, and several assays have been developed to measure T-cell responses to CMV. Notable among these

are the CMV QuantiFERON (Qiagen, Hilden, Germany) and the CMV enzyme-linked immunosorbent spot (ELISpot) assay, which are interferon gamma release assays (IGRA). The assays work by using CMV antigens to stimulate T-cell production of interferon gamma, which is measured in platform-specific approaches. The CMV QuantiFERON (Qiagen, Hilden, Germany) measures the CD8+ T-cell IFN-gamma response using ELISA, while the CMV ELISpot (TSPOT® CMV, Oxford Immunotec) quantifies the frequency of IFN-gamma-producing CD4+ and CD8+ T-cells utilizing ELISpot[11]. A third assay, the CMV inSIGHT T-Cell Immunity test (Eurofins Viracor, Lenexa, Kansas USA), combines intracellular interferon gamma cytokine staining in tandem with flow cytometry. This platform provides a quantification of the activated, CMV-specific CD4+ and CD8+ T-cells. Several studies have highlighted the predictive capacity of these assays with varying results for CMV DNAemia and disease[15-20]. However, they have not been widely integrated into current transplant care and warrant larger validation and implementation studies.

CMV Quantitative Nucleic Acid Testing (qNAT)

qNAT for CMV serves as the bedrock of CMV diagnostics as it provides information that can guide a plethora of clinical decisions. The assay works by using PCR to amplify CMV-specific viral DNA targets allowing clinicians to rapidly quantify the CMV viral load in a patient's sample relative to standard curves of known CMV DNA concentration. This information can then be used for diagnostic, therapeutic, prognostic, and preventative purposes[9,10]. The degree of DNAemia informs the severity of the illness with low level CMV DNAemia more likely to represent latent CMV replication and high level CMV DNAemia more

suggestive of end-organ disease[21-27]. Viral kinetics describes the change of the viral load over time. Patients who have a rapidly rising CMV viral load over one week, defined as an increase in viral load greater than one-log10[28,29], are more likely to have CMV disease[30]. If a patient is diagnosed with either CMV DNAemia, syndrome, or disease, the CMV viral load can be monitored on a weekly basis to assess for clinical response after anti-viral therapy is initiated[9]. It should be noted that it can take up to two weeks after the initiation of anti-viral therapy before the CMV viral load starts to decline[31]. Refractory CMV is diagnosed if the CMV viral load increases by one-log10, two weeks after anti-viral therapy is initiated[32]. Drug resistant CMV[33,34], intense immunosuppression[35,36], and subtherapeutic anti-viral treatment[37] are all possible etiologies of refractory CMV.

While CMV qNAT has revolutionized the management of patients with SOTs who develop CMV related illnesses, there are several limitations in its clinical applications. The most prominent of these is the lack of inter-platform precision when reporting the degree of CMV DNAemia[4]. In 2010, the WHO published an international calibration standard to improve the precision of CMV qNAT between laboratories[38]. However subsequent studies utilizing this international standard have found that there still exists a significant degree of variation between different CMV qNAT assays particularly when clinical samples are tested compared to tissue culture derived CMV. One such study found a median variance of 1.5 log IU/mL with maximal variances as high as 2.82 log IU/mL[39]. As a result, current CMV guidelines strongly recommend that each transplant center establish internal guidelines with one validated laboratory assay with institution specific thresholds for the initiation of treatment[4]. Another limitation to the CMV qNAT assay is that patients can have end-organ CMV disease without having CMV DNAemia.

This has been described as compartmentalized disease and has been known to occur with CMV retinitis and CMV colitis[12,40]. In the latter case, patients require direct sampling of the affected organ through biopsy[4].

The sensitivity of CMV qNAT assays have improved over time creating a challenging gap in knowledge related to the clinical impact of low level CMV DNAemia. The lack of universal CMV viral load thresholds for antiviral management is further complicated by the differing sensitivity of CMV testing platforms. While the advent of more sensitive qNAT assays has enabled the detection of previously undetectable CMV DNAemia, the clinical implications of treating or not treating these cases are unexplored.

To address this knowledge gap, we analyzed the impact of a change in our institution's qNAT testing platform on viral kinetics of previously undetectable levels of CMV DNAemia, and investigated the relationship between type of qNAT used and a variety of clinical outcomes in RTR, including: the time to the first episode of CMV DNAemia, the total duration of detectable CMV DNAemia, the duration of low-level CMV DNAemia, the duration of CMV DNAemia after the 1,000 IU/mL threshold is reached in moderate-risk patients, peak CMV DNAemia, the magnitude of the initially detected CMV viral load, the odds of developing end-organ CMV disease, and one-year all-cause mortality.

Methods

Study Design and Participants

We conducted a cohort study of renal transplant recipients (RTR) at a single large tertiary-care hospital and transplant center. In April 2018, the CMV qNAT assay platform at this institution changed from one whose lower limit of quantitation (LLOQ) was 300 IU/mL ("high LLOQ qNAT"- Thermo Fisher 7500 using the Qiagen ARTUS assay) to one whose LLOQ was 35 IU/mL ("lower LLOQ qNAT" – Roche COBAS). With the higher LLOQ qNAT assay (run on the patient's plasma), viral loads <100 IU/mL were reported as undetectable, those between 100-300 were reported as "detectable but not quantifiable", and those ≥300 IU/mL were quantitated. Assay PCR primers were designed for specific amplification of a 105 bp region of the CMV Major Immediate Early Gene (MIE). In contrast, the lower LLOQ qNAT assay (run on patient plasma), viral loads that were 1-35 IU/mL were reported as "detectable but not quantifiable", and those ≥35 IU/mL were quantitated. The target for this assay is the highly conserved region of the CMV DNA polymerase (UL54) gene.

RTRs undergo routine post-transplant surveillance for CMV DNAemia with at least monthly CMV qNAT assays for the first 12 months post-transplantation. For this analysis, we included RTRs who had been transplanted over a three-year period before (January 1, 2014-December 31, 2016) and after (January 1, 2019-December 31, 2021) the assay change; we excluded those transplanted in 2017 and 2018 since these patients could have undergone monitoring using either assay. Patients needed to have received all transplant-related care (e.g.,

CMV monitoring) at the study institution and completed one year of post-transplantation follow-up with CMV viral load monitoring after the conclusion of antiviral prophylaxis.

Patients were categorized based on the type of PCR platform used for CMV testing (higher LLOQ vs. lower LLOQ) and further stratified by CMV donor/recipient serostatus (high-risk, donor CMV positive/recipient CMV negative, or D+/R-; moderate-risk, any recipient CMV positive, or R+); patients who were D-/R- were excluded from the analysis.

Data Collection

We collected all CMV viral load measurements in the first 12 months post-transplant for RTRs included in the cohort. CMV viral loads collected during the period of routine post-transplant antiviral (valganciclovir) prophylaxis (given for 6 months and 3 months after transplant in D+/R- and R+ patients respectively) were excluded from the analysis due to the low occurrence of breakthrough CMV DNAemia, and to minimize confounding related to immunosuppression management and antiviral underdosing. Patients were considered lost to follow-up if they lacked any viral load measurements between the 10th and 12th months post-transplantation (See Figure 1).

Data were stored in the Emory renal transplant database, which directly retrieved the information from the electronic medical health record. IRB approval was obtained prior to data collection and analysis.

Variables and Definitions

Exposure

The primary exposure was the qNAT platform used to monitor patients for CMV DNAemia. We specifically compared outcomes among those patients monitored with the higher LLOQ qNAT assay with those monitored with the lower LLOQ qNAT assay and stratified our analysis by CMV risk status.

Outcomes

The primary outcomes of our study were the duration and the time to onset of CMV DNAemia. The time to CMV DNAemia was calculated as the time to the first episode of CMV DNAemia for each subgroup after the protocol-defined window of antiviral prophylaxis had ended. The duration of CMV DNAemia was measured by the number of days that a patient had detectable DNAemia. Each unique episode of CMV DNAemia was included. The duration of low-level CMV DNAemia was measured by the number of days a patient experienced CMV DNAemia where the first recorded viral load fell within the low-level DNAemia range. Low level CMV DNAemia was defined as the period of detectable but not quantifiable DNAemia, which differed between the two qNAT assays (100-300 IU/mL for the higher LLOQ assay and 1-35 IU/mL for the lower LLOQ assay). At our center, moderate-risk patients that are asymptomatic are typically only initiated on antiviral therapy after reaching a threshold of 1000 IU/mL, and so we also calculated the duration of CMV DNAemia in days after this threshold was reached. An episode of DNAemia was calculated from the first detected viral load, based on

the definitions above, to the first of two consecutive undetectable viral load results, which were collected at least 5 days apart.

Secondary outcomes included peak CMV DNAemia, the magnitude of the first detected CMV viral load, the odds of developing CMV disease, and one-year all-cause mortality. Peak CMV DNAemia was quantified as the highest viral load detected during an episode of CMV DNAemia. Magnitude of the first detected CMV viral load was defined as the viral load at the first detection of DNAemia. Using International Classification of Diseases (ICD)-9 and ICD 10 codes, patients who developed end organ CMV disease between the conclusion of antiviral prophylaxis and the first year after transplantation were identified. One-year all-cause mortality was defined as any death occurring during the first year after transplantation.

Covariables

Before the data analysis, we constructed a directed acyclic graph (DAG) to describe the expected relationship between the exposure and outcome. This graph helped identify potential confounding variables that might impact the results (see Figures 2-3). Induction and maintenance immunosuppression were identified as potential confounders because local policy changes in immunosuppression protocols changed during the same period as the change in qNAT assay. These potential confounders were included as covariables in the Cox proportional hazards model, linear regression model, and logistic regression model. Notably, these variables consisted of the type of induction immunosuppression used at the time of transplantation, the maintenance immunosuppressive regimen used after transplantation (specifically whether the patient was

252 treated with belatacept, tacrolimus, or other agent, in addition to an antimetabolite and
253 corticosteroid).
254
255 *Statistical Analysis*
256
257 The baseline characteristics of each subgroup (higher LLOQ moderate/high CMV risk
258 and lower LLOQ moderate/high CMV risk) were summarized by proportion and measures of
259 central tendency. Baseline categorical data were presented as percentages. The incidence rate of
260 CMV DNAemia was calculated for patients who were CMV moderate-risk (R+), with 9 months
261 of follow-up time, and CMV high-risk (D+/R-), with 6 months of follow-up time. Follow-up
262 time was defined as the period of monthly collection of CMV viral loads up to one year after
263 transplantation.
264
265 Kaplan Meier curves were generated to visualize the time to the first episode of CMV
266 DNAemia, contrasting the moderate-risk groups with each other and the high-risk groups with
267 each other. We used Cox proportional hazards models for time to CMV DNAemia, linear
268 regression for duration of CMV DNAemia, and logistic regression for the odds of developing
269 CMV end-organ disease. The assumption of proportional hazards was checked both graphically
270 and statistically using Schoenfeld residuals and graphical assessment of scaled Schoenfeld
271 residuals versus time. Mean peak CMV DNAemia and the mean first detected CMV viral load
272 were compared by T-test. After examination of the pattern of missing data suggested data were
273 missing at random, missing values were imputed by multiple imputation using the Multiple
274 Imputation by Chained Equations (MICE) method[41]. To assess the robustness of our findings and

the potential influence of unmeasured confounders, we performed a sensitivity analysis to compute the E-value for each model[42,43]. Data analyses were performed using R and R studio version 4.2.2.

Results

Baseline Demographics

We identified 611 RTRs (509 CMV moderate-risk, 102 CMV high-risk) who were monitored with the higher LLOQ platform and 771 (641 CMV moderate-risk, 130 CMV high-risk) monitored with the lower LLOQ platform (See Figure 1).

Baseline demographic characteristics were similar between the two groups (Table 2). A higher proportion of those tested with the higher LLOQ qNAT platform (8.3%) received thymoglobulin induction agent compared to those tested with the lower LLOQ qNAT platform (0.8%). A lower proportion of CMV high-risk patients received belatacept as part of their maintenance immunosuppression during the period when the lower LLOQ qNAT was used compared to the period when the higher LLOQ qNAT was used (36.9% vs 76.5%). A total of 12,993 CMV viral loads were measured corresponding to 428 discrete episodes of CMV DNAemia. The incidence rate of CMV DNAemia varied based on patient serostatus and qNAT testing platform.

CMV Viral Kinetics

Tables 3, 4 and Figure 4 present the findings for the primary outcomes of time to CMV DNAemia, duration of CMV DNAemia, duration of low-level CMV DNAemia, and duration of CMV DNAemia after the 1000 IU/mL threshold is reached in moderate-risk patients.

Among patients tested with the lower LLOQ platform, the hazard rate for the first episode of CMV DNAemia was 1.95 times higher (95% CI: 1.55 to 2.46) compared to those tested with the higher LLOQ platform, adjusting for the types of induction and maintenance immunosuppression regimens administered. For the high-risk group, a numerical trend suggested a greater hazard rate in patients tested with the lower LLOQ platform than those tested with the higher LLOQ platform (aHR- 1.35 CI- 0.84 to 2.15); however, this trend did not achieve statistical significance when accounting for the induction and maintenance immunosuppression regimens used. Non-significant p-values in the Schoenfeld residuals test and graphical assessment of scaled Schoenfeld residuals versus time indicated that the proportional hazards assumption was valid.

Figure 4 displays the Kaplan-Meier curves illustrating the time to the first episode of CMV DNAemia for both moderate and high-risk CMV patients. In the moderate-risk group, patients tested with the lower LLOQ platform had earlier onset of CMV DNAemia compared to those tested with the higher LLOQ platform. However, for the high-risk group, there was no difference in the time to the initial episode of CMV DNAemia between the lower and higher LLOQ testing platforms.

Linear regression modeling allowed precise estimates of CMV DNAemia duration produced by platforms with differing LLOQ. Regarding the duration of CMV DNAemia, on average, moderate-risk patients tested with the lower LLOQ platform experienced CMV DNAemia 24.79 days (CI: 17.73 to 31.85) longer than those tested with the higher LLOQ

platform, adjusting for induction and maintenance immunosuppression regimens. In the high-risk group, a numerical trend was noted toward longer durations (aLR- 20.75 CI: -6.43 to 47.93) of CMV DNAemia in patients tested with the lower LLOQ platform compared to those tested with the higher LLOQ platform, although this difference was not statistically significant. For moderate-risk patients who developed low level CMV DNAemia initially, on average, those tested with the lower LLOQ platform experienced CMV DNAemia 23.71 days (CI- 15.59 to 31.84) longer than those tested with the higher LLOQ platform, adjusting for induction and maintenance immunosuppression regimens. There was no significant difference noted for high-risk patients who developed low level CMV DNAemia when comparing those tested with the lower LLOQ platform to those tested with the higher LLOQ platform (aLR- -0.69 CI: -28.26 to 26.89). Finally, for CMV DNAemia duration after exceeding the 1000 IU/mL threshold, moderate-risk patients tested with the lower LLOQ platform had on average 24.19 days (CI: 16.40 to 31.98) of additional CMV DNAemia compared to those tested with the higher LLOQ platform, adjusting for induction and maintenance regimens used (See Table 4).

Regardless of CMV risk status, patients tested with the lower LLOQ platform had a lower peak CMV viral load and a lower first detected CMV viral load compared to those tested with the higher LLOQ platform (See Table 5). Moderate risk CMV RTRs tested with the lower LLOQ platform had a longer time to peak CMV and 1000 IU/ml viral loads compared to those tested with the higher LLOQ platform.

Clinical Outcomes

There was no difference in the odds of developing CMV disease based on qNAT platform used for the moderate-risk CMV patients (See Table 6). When considering one-year mortality, moderate-risk CMV patients tested with the lower LLOQ platform exhibited a risk that was 0.91 times (95% CI: 0.45-1.84) that of patients tested with the higher LLOQ platform, after adjusting for induction and maintenance immunosuppression regimens. Among high-risk CMV patients, no deaths occurred in the group tested with the lower LLOQ platform, whereas 3 patients tested with the higher LLOQ platform died within one year (See Table 7 for more information regarding cause of death).

Sensitivity Analysis

We conducted an E-value sensitivity analysis for the moderate-risk comparison for the primary outcomes. For the adjusted hazard ratio, the E-value was determined to be 2.57, with a lower confidence limit E-value of 2.09. For the linear regression coefficient for CMV DNAemia, the E-value was 3.32, with a lower confidence limit E-value of 2.62. For the linear regression coefficient for low level CMV DNAemia, the E-value was 3.46, with a lower confidence limit E-value of 2.53. For the linear regression coefficient for CMV DNAemia duration after the 1000 IU/mL threshold was reached, the E-value was 3.19, with a lower confidence limit E-value of 2.45.

Discussion

Impact of Ultrasensitive CMV PCR Platform

Our study, drawing on a robust renal transplant database at Emory of over 3,800 patients, examined CMV viral kinetics and clinical outcomes in this patient population. We found that use of a more sensitive qNAT platform was associated with earlier CMV DNAemia detection and longer durations of detectable DNAemia, regardless of initially detected viral load, in CMV moderate-risk patients. Among all patients, use of the more sensitive assay was also associated with lower peak and initial CMV viral loads.

Low-level CMV DNAemia

Prior research offers insight into the kinetics of low-level CMV DNAemia that can guide antiviral stewardship. A study by Natori et al. identified clinical predictors of clearance for low-level CMV DNAemia (viral loads between 137 and 999 IU/mL) in solid organ transplant recipients. Remarkably, spontaneous clearance of DNAemia was common even among high-risk CMV serostatus patients, especially if they had experienced a prior CMV DNAemia episode[44]. This suggests that careful monitoring without immediate antiviral treatment could be feasible for patients with low-level CMV DNAemia. Furthermore, antiviral stewardship programs have advocated for preemptive therapy in CMV prevention and the use of CMV cell-mediated immune assays to guide duration of surveillance monitoring[45,46]. Preemptive therapy in liver transplant recipients was shown to enable immune system priming, leading to the development of neutralizing antibodies and reducing the risk of delayed-onset post-prophylaxis CMV disease,

compared to those on antiviral prophylaxis[6]. These findings, coupled with our own, suggest that controlled exposure to low-level CMV DNAemia could potentially enhance CMV-specific immunity and antiviral stewardship, without compromising patient outcomes.

Mechanistic Insights

Our study demonstrates that the more sensitive assay detects a greater number of viral loads that fall within the low-level CMV DNAemia range, regardless of CMV risk status. For moderate-risk CMV RTRs this amounts to earlier detection before reaching the 1000 IU/mL threshold at which antiviral treatment is initiated. At the study institution, patients are treated with antivirals until they achieve two consecutive negative viral loads. The more sensitive assay detects these tail viral loads for a longer period for the moderate-risk CMV patients leading on average to an additional 24 days of CMV DNAemia. This bears significant implications for antiviral stewardship in renal transplant recipients. Given that we treat patients with a renally adjusted dose of valganciclovir, an extra 24 days of CMV DNAemia could lead to additional costs ranging from $103 to $821 per CMV DNAemia episode (based on current valganciclovir prices), underlining the economic impact detecting prolonged CMV DNAemia. For time to CMV DNAemia, the frequency of viral load monitoring could mask differences in the high-risk group, and a biweekly screening interval might provide more detailed insight into viral kinetics. The lack of a difference in CMV DNAemia duration in the high-risk patients may have been due to a smaller sample size and a shorter follow up period. We performed a power analysis and calculated an effect size of 0.372, which corresponds to an 80% chance of detecting a difference of 13.5 days or more.

Impact of Induction and Maintenance Immunosuppression Regimens

Prior studies have demonstrated that the induction agent, thymoglobulin, and maintenance regimen agent, belatacept, increases a patient's risk for developing CMV related illnesses[47,48]. In our study, patients who were tested with the higher LLOQ assay were more likely to receive thymoglobulin, regardless of CMV risk status, and the CMV high-risk patients were more likely to receive belatacept compared those tested with the lower LLOQ assay. After adjusting for both the induction and maintenance agent or adjusting for either the induction or maintenance agent received, we found that the hazard ratio for time to CMV DNAemia and the linear regression estimates for duration of CMV DNAemia increased for patients tested with the lower LLOQ assay compared to those tested with the higher LLOQ assay, which highlights the significant impact that these immunosuppression agents have on future CMV risk.

Limitations and Future Directions

We acknowledge some limitations, such as a smaller sample size and shorter follow up period for high-risk CMV patients, the potential lack of generalizability beyond the renal transplant population, and lack of direct antiviral use and immunosuppression reduction data analysis. Future investigations should extend to liver, lung, and heart transplant recipients to determine the implications of low-level CMV DNAemia detection in these patients. Direct analysis of antiviral use patterns was not possible with our database due to missing parameters such as creatinine clearance, which is essential to differentiate between an antiviral treatment

dose vs secondary prophylaxis dose. However, the differences in CMV DNAemia duration serve as a surrogate for differences in antiviral use duration given that the duration of antiviral use is dependent directly on the duration of CMV DNAemia. Future studies examining the impact that ultrasensitive assays have on the time to reduction of immunosuppression maintenance regimens are needed.

Conclusion

In summary, our study underscores the clinical implications of employing an ultra-sensitive PCR platform in monitoring CMV DNAemia in RTRs. We observed earlier detection of CMV DNAemia, longer durations of DNAemia, and lower peak and initial viral loads, particularly among moderate-risk patients. Despite these variations in viral kinetics, we did not observe significant differences in the odds of developing end-organ CMV disease or the risk for one-year mortality. Furthermore, our findings highlight the potential economic impact of prolonged DNAemia duration and point to the importance of optimized antiviral stewardship. Given these complex implications, our findings encourage a careful consideration of using ultrasensitive CMV qNAT assays when designing institutional protocols for the treatment of CMV DNAemia. Future research in diverse organ transplant populations will be crucial in further refining the role of these platforms in CMV surveillance.

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Tables

Table 1- CMV Risk Stratification by Serostatus: This table highlights the various combinations of CMV serology (IgG) tests results and indicates the level of CMV risk in SOTRs. Donor positive/Recipient negative patients are at the highest risk because the donor organ contains CMV and is then transplanted into a recipient who has no prior cell-mediated or antibody mediated immunity against CMV. Recipient positive patients are considered standard/moderate risk given the assumption that these patients have pre-transplant cell-mediated and antibody mediated immunity against CMV. Donor negative/Recipient negative patients are the lowest risk given that neither the donor organ nor the recipient have latent CMV[4].

Serostatus	CMV Risk
Donor positive / Recipient negative (D+/R-)	High
Donor positive / Recipient positive (D+/R+) Donor negative / Recipient positive (D-/R+)	Standard/Moderate
Donor negative / Recipient negative (D-/R-)	Low
CMV- cytomegalovirus	

635 **Table 2:** Baseline patient characteristics of renal transplant recipients stratified by CMV risk
636 status and quantitative nucleic acid amplification test performed (higher LLOQ vs. lower
637 LLOQ). Demographic and clinical variables are summarized as means with standard deviations
638 for continuous variables and as frequencies with percentages for categorical variables.

	Lower LLOQ			Higher LLOQ		
	High risk (n=130)	Moderate risk (n=641)	Combined (n=771)	High risk (n=102)	Moderate risk (n=509)	Combined (n=611)
Age						
Mean (SD)	50.0 (13.9)	52.4 (13.1)	52.0 (13.2)	49.5 (12.4)	50.9 (12.8)	50.7 (12.8)
Sex						
Female	43 (33.1%)	302 (47.1%)	344 (44.7%)	38 (37.3%)	229 (45.0%)	267 (43.7%)
Male	87 (66.9%)	339 (52.9%)	426 (55.3%)	64 (62.7%)	280 (55.0%)	344 (56.3%)
Donor Type						
Deceased	87 (66.9%)	490 (76.4%)	576 (74.8%)	58 (56.9%)	331 (65.0%)	389 (63.7%)
Living	43 (33.1%)	151 (23.6%)	194 (25.2%)	44 (43.1%)	177 (34.8%)	221 (36.2%)
Unreported	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.2%)	1 (0.2%)
Induction Agent						
Basiliximab	128 (98.5%)	624 (97.3%)	751 (97.5%)	88 (86.3%)	457 (89.8%)	545 (89.2%)
Other	0 (0%)	1 (0.2%)	1 (0.1%)	0 (0%)	3 (0.6%)	3 (0.5%)
Thymoglobulin	0 (0%)	6 (0.9%)	6 (0.8%)	12 (11.8%)	39 (7.7%)	51 (8.3%)
Missing	2 (1.5%)	10 (1.6%)	12 (1.6%)	2 (2.0%)	10 (2.0%)	12 (2.0%)
Maintenance Agent						
Belatacept	48 (36.9%)	551 (86.0%)	598 (77.7%)	78 (76.5%)	319 (61.0%)	462 (75.6%)
Tacrolimus	81 (62.3%)	72 (11.2%)	153 (19.9%)	7 (6.9%)	164 (31.4%)	74 (12.1%)
Unknown	0 (0%)	1 (0.2%)	1 (0.1%)	0 (0%)	0 (0%)	0 (0%)
Other	0 (0%)	9 (1.4%)	9 (1.2%)	15 (14.7%)	29 (5.5%)	64 (10.5%)
Missing	1 (0.8%)	8 (1.2%)	9 (1.2%)	2 (2.0%)	11 (2.1%)	11 (1.8%)
Number of patients with missing CMV viral loads month 10 – month 12	13	73	86	9	100	109
Number of discrete episodes of CMV DNAemia	24	241	265	35	128	163
Number of patients with more than one episode of CMV DNAemia	4	31	35	7	27	34
Number of recurrent CMV DNAemia episodes	4	33	37	7	29	36
Number of discrete episodes of low level CMV DNAemia	4	156	160	8	38	46

Number of discrete episodes of CMV DNAemia exceeding 1000 IU/mL threshold	--	64	--	--	62	--
Total number of CMV viral loads	1106	6412	7518	1055	4420	5475
Total number of low level CMV viral loads	103	671	774	86	178	264
Follow up time in person years	65.00	482.25	547.25	51.00	381.75	432.75
CMV DNAemia incidence rate per year	0.97	0.76	0.78	1.24	0.41	0.51
Number of patients with CMV end-organ disease	0	2	2	4	2	6
Abbreviations: CMV- cytomegalovirus. LLOQ- lower limit of quantitation. SD- standard deviation						

641 **Table 3- Hazard Ratios for time to CMV DNAemia:** Moderate-risk CMV RTRs tested with
642 the lower LLOQ assay have a greater hazard for developing a first episode of CMV DNAemia.
643 Univariable and multivariable Cox proportional hazards models for time to first episode of CMV
644 DNAemia among renal transplant recipients stratified by CMV risk status and quantitative
645 nucleic acid amplification test platform. Hazard ratios (HRs) and 95% confidence intervals are
646 reported for the comparisons. Multivariable models are adjusted for induction and maintenance
647 immunosuppression.
648

	Moderate Risk Comparison		High Risk Comparison	
Variable	Unadjusted hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)	Unadjusted hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)
Lower LLOQ	1.87 (1.51 to 2.31)	1.95 (1.55 to 2.46) $^{\Psi}$ 1.91 (1.53 to 2.38) $^{\Delta}$ 1.96 (1.56 to 2.46) $^{\Pi}$	1.21 (0.82 to 1.77)	1.35 (0.84 to 2.15) $^{\Psi}$ 1.24 (0.83 to 1.85) $^{\Delta}$ 1.35 (0.84 to 2.15) $^{\Pi}$
Higher LLOQ	REF	REF	REF	REF
$^{\Psi}$- Adjusted for induction and maintenance regimen used $^{\Delta}$- Adjusted for induction regimen used $^{\Pi}$- Adjusted for maintenance regimen used				
Abbreviations – CMV- cytomegalovirus. RTR- renal transplant recipient. LLOQ- lower limit of quantitation. CI- confidence interval				

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Table 4- Linear regression estimates for duration of CMV DNAemia: Moderate risk CMV RTRs tested with the lower LLOQ assay have on average longer durations of CMV DNAemia, low-level CMV DNAemia, and CMV DNAemia after reaching a threshold of 1000 IU/mL. Univariable and multivariable linear regression models for durations of CMV DNAemia, low-level CMV DNAemia, and CMV DNAemia duration after exceeding the 1000 IU/mL threshold among renal transplant recipients stratified by CMV risk status and quantitative nucleic acid amplification test platform. Point estimates and 95% confidence intervals are reported for the comparisons. Multivariable models are adjusted for induction and maintenance immunosuppression.

	Moderate Risk Comparison		High Risk Comparison	
Variable	Unadjusted Linear Regression Estimate (95% CI)	Adjusted Linear Regression Estimate (95% CI)	Unadjusted Linear Regression Estimate (95% CI)	Adjusted Linear Regression Estimate (95% CI)
CMV DNAemia duration				
Lower LLOQ	24.14 (17.24 to 31.04)	24.79 (17.73 to 31.85) [Ψ] 24.88 (17.85 to 31.91) [Δ] 25.02 (18.03 to 32.00) [Π]	10.31 (-8.93 to 29.55)	20.75 (-6.43 to 47.93) [Ψ] 13.83 (-5.88 to 33.55) [Δ] 20.75 (-6.17 to 47.67) [Π]
Higher LLOQ	REF	REF	REF	REF
Low-level CMV DNAemia duration				
Lower LLOQ	22.94 (14.85 to 31.03)	23.71 (15.59 to 31.84) [Ψ] 23.52 (15.45 to 31.59) [Δ] 23.88 (15.74 to 32.01) [Π]	-10.92 (-32.28 to 10.43)	-0.69 (-28.26 to 26.89) [Ψ] -8.63 (-30.95 to 13.70) [Δ] -0.69 (-28.26 to 26.89) [Π]
Higher LLOQ	REF	REF	REF	REF
CMV DNAemia duration after exceeding 1000 IU/mL threshold				
Lower LLOQ	22.87 (15.38 to 30.36)	24.19 (16.40 to 31.98) [Ψ] 24.16 (16.44 to 31.87) [Δ] 24.93 (17.19 to 32.67) [Π]		
Higher LLOQ	REF	REF		

[Ψ]- Adjusted for induction and maintenance regimen used
[Δ]- Adjusted for induction regimen used
[Π]- Adjusted for maintenance regimen used

Abbreviations- CMV- cytomegalovirus. RTR- renal transplant recipient. LLOQ- lower limit of quantitation. CI- confidence interval

663 **Table 5- Secondary outcomes:** Regardless of CMV serostatus, RTRs tested with the lower
664 LLOQ assay had lower mean peak CMV viral loads and lower first detected CMV viral loads.
665 Moderate risk CMV RTRs had a longer time to peak CMV viral load and time to the 1000
666 IU/mL threshold when tested with the lower LLOQ platform. Comparisons between higher
667 LLOQ and lower LLOQ moderate and high-risk groups including peak CMV DNAemia and first
668 CMV viral load detected. Means, standard deviations, and t-tests are reported where applicable.
669

	Moderate Risk (CMV R+) Comparison		T-test p-value (95% CI)	High Risk (CMV D+/R-) Comparison		T-test p-value (95% CI)
	Higher LLOQ (n=509)	Lower LLOQ (n= 641)		Higher LLOQ (n= 102)	Lower LLOQ (n= 130)	
Peak CMV DNAemia log10 mean (std dev) IU/mL	3.06 (0.77)	1.94(0.90)	<0.001 (0.94 to 1.29)	4.94 (1.01)	3.84 (1.29)	<0.001 (0.66 to 1.54)
Time to Peak CMV viral load (std dev) IU/mL	42.43 (60.80)	66.45(57.46)	0.03 (-46.76 to -1.28)	47.00 (47.43)	42.66 (38.38)	0.68 (-16.28 to 24.97)
Time to 1000 IU/mL threshold	9.45 (26.20)	42.17 (52.73)	<0.001 (-47.38 to -18.06)	--	--	--
First CMV viral load detected log10 mean (std dev) IU/mL	2.81 (0.74)	1.54 (0.58)	<0.001 (1.12 to 1.42)	3.90 (1.25)	2.87 (1.25)	<0.001 (0.54 to 1.51)
Abbreviations: CMV- cytomegalovirus. RTR- renal transplant recipient. LLOQ- lower limit of quantitation. CI- confidence interval						

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672 **Table 6 Odds ratios for end-organ disease**: Moderate risk CMV RTRs tested with either qNAT
673 assay show no difference in the development of CMV end-organ disease. Univariable and
674 multivariable logistic regression models for the odds of developing CMV disease among renal
675 transplant recipients stratified by CMV risk status and quantitative nucleic acid amplification test
676 platform. Odds ratios and 95% confidence intervals are reported for the comparisons.
677 Multivariable models are adjusted for induction and maintenance immunosuppression.
678

	Moderate Risk Comparison	
Variable	Unadjusted Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)[Ψ]
Lower LLOQ	0.79 (0.09 to 6.61)	1.12 (0.16 to 9.44)
Higher LLOQ	REF	REF
[Ψ]- Adjusted for induction and maintenance regimen used		
Abbreviations: CMV- cytomegalovirus. RTR- renal transplant recipient. qNAT- quantitative nucleic acid testing. CI- confidence interval		

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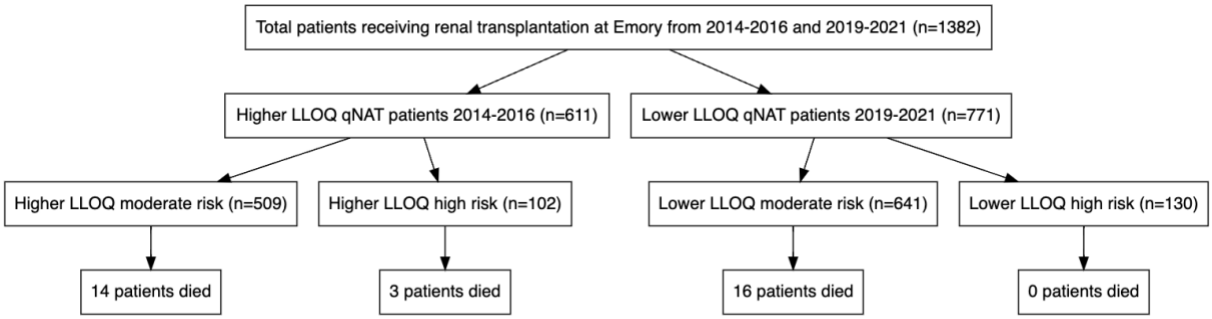
Table 7- Cause of death arranged by CMV risk status.

	Moderate Risk Lower LLOQ	High Risk Lower LLOQ	Moderate Risk Higher LLOQ	High Risk Higher LLOQ
Cardiac Arrest	2	-	5	1
Septic Shock	4	-	2	2
Abdominal Compartment Syndrome	-	-	1	-
Respiratory Failure	6	-	1	-
Status Epilepticus	-	-	1	-
Unknown	3	-	4	-
Multi-organ Failure	1	-	-	-

685 Figures
686



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689 **Figure 1:** Flow diagram illustrating the step-by-step patient selection process for the study,
690 detailing the number of patients in each subgroup and those who died. The higher LLOQ assay
691 quantifies viral loads greater than 300 IU/mL, reports viral loads between 100 – 300 IU/mL as
692 detected but not quantified, and is unable to detect viral loads less than 100 IU/mL. The lower
693 LLOQ assay quantifies viral loads greater than 35 IU/mL and reports viral loads between 1-35
694 IU/mL as detected but not quantified. Abbreviations: LLOQ- lower limit of quantitation. qNAT-
695 quantitative nucleic acid testing.

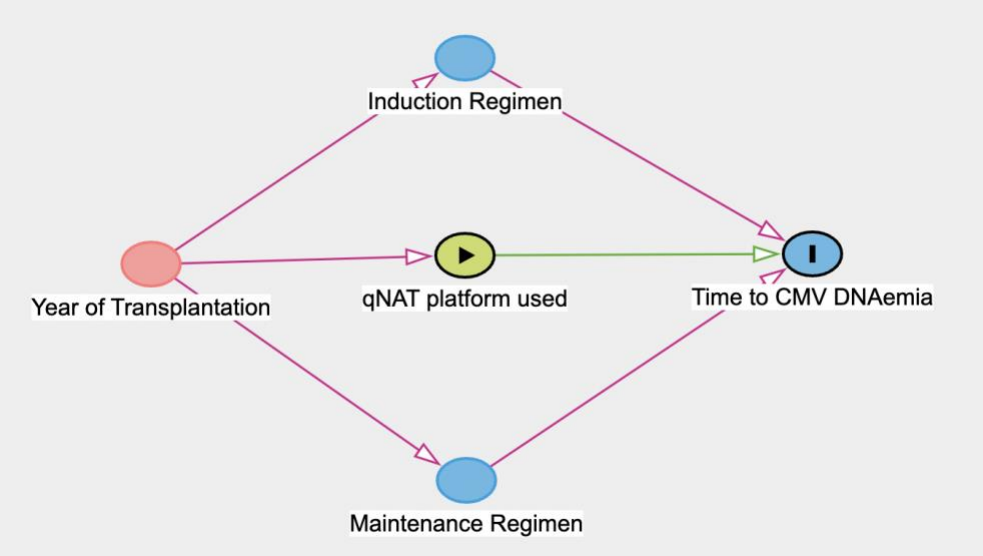

Figure 2: Directed Acyclic Graph for the primary exposure, PCR platform used, and outcome-time to CMV DNAemia. The confounding variables identified includes induction and maintenance immunosuppression regimen used. Abbreviations: CMV- cytomegalovirus.

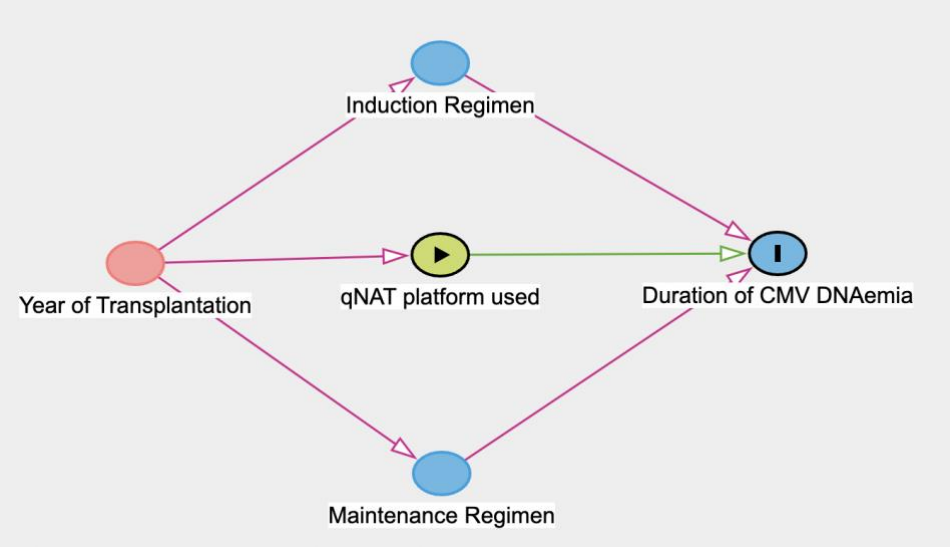

Figure 3: Directed Acyclic Graph for the primary exposure, PCR platform used, and outcome- duration of CMV DNAemia. The confounding variables identified includes induction and maintenance immunosuppression regimen used. Abbreviations: CMV- cytomegalovirus

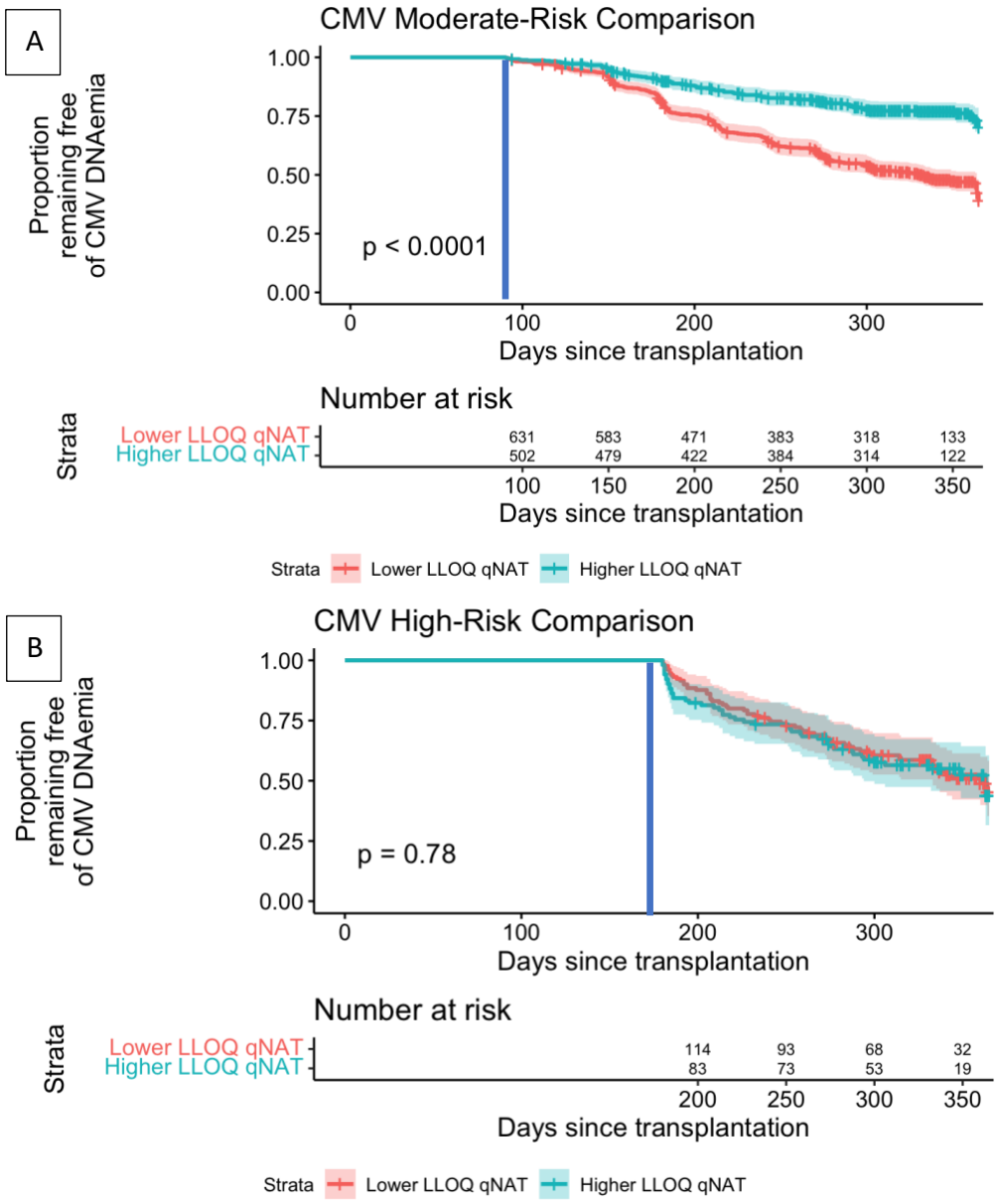

Figure 4- Kaplan Meier curve analysis: A- Kaplan Meier curve analysis comparing time to first episode of CMV DNAemia for higher LLOQ CMV moderate risk patients (n=509) with lower LLOQ CMV moderate risk patients (n=641) over a one year follow up period after transplantation. Time to CMV DNAemia was significantly shorter for patients monitored with the LLOQ platform (log-rank test p-value <0.0001). The median time to CMV DNAemia for the lower LLOQ group was 333 days and could not be calculated for the higher LLOQ group given that the survival probability was greater than 50% at the end of the study period. The vertical blue line represents the time at which antiviral prophylaxis was discontinued. B- Kaplan Meier curve analysis comparing time to first episode of CMV DNAemia for higher LLOQ CMV high risk patients (n=102) with lower LLOQ CMV high risk patients (n=130) over a one year follow up period after transplantation. Time to CMV DNAemia was not significantly different by (log-

rank test p-value = 0.78). The median time to CMV DNAemia for the higher LLOQ group and lower LLOQ group was 363 days and 360 days, respectively. The vertical blue line represents the time at which antiviral prophylaxis was discontinued. Abbreviations- CMV-cytomegalovirus. LLOQ- lower limit of quantitation. qNAT- quantitative nucleic acid testing.