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MINIREVIEWS

Emerging role of cell-free DNA in kidney transplantation

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Abstract

Monitoring kidney transplants for rejection conventionally includes serum creatinine, immunosuppressive drug levels, proteinuria, and donor-specific antibody (DSA). Serum creatinine is a late marker of allograft injury, and the predictive ability of DSA regarding risk of rejection is variable. Histological analysis of an allograft biopsy is the standard method for diagnosing rejection but is invasive, inconvenient, and carries risk of complications. There has been a long quest to find a perfect biomarker that noninvasively predicts tissue injury caused by rejection at an early stage, so that diagnosis and treatment could be pursued without delay in order to minimize irreversible damage to the allograft. In this review, we discuss relatively novel research on identifying biomarkers of tissue injury, specifically elaborating on donor-derived cell-free DNA, and its clinical utility.

Key Words: Biomarker; Donor-derived cell-free DNA; Kidney allograft outcomes; Kidney transplant; Allograft biopsy

Core Tip: Donor-derived cell-free DNA (dd-cfDNA) is now available as a noninvasive biomarker to evaluate the risk of rejection in kidney allografts and other organ transplants. The technology utilizes next generation sequencing and does not require donor genotyping. In this review we discuss the current literature on the utility of dd-cfDNA in kidney transplantation, the limitations, and future directions.

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INTRODUCTION

Kidney transplants offer the best survival to patients with end-stage kidney disease [1]. Conventional monitoring of kidney transplant recipients includes serum creatinine, proteinuria, and donor-specific antibodies (DSA), which are neither sensitive nor specific. Surveillance biopsies are performed for allograft monitoring in a few centers, but are invasive and have multiple disadvantages including bleeding risk, inconvenience, sampling error, and poor reproducibility in interpretation. They generally have low yield as the majority reveal normal histology and have not been validated to improve outcomes.

The risk of renal allograft dysfunction from acute rejection (AR) during the first year after transplant is around 10%-15%. AR can be either acute T cell-mediated rejection (TCMR) characterized by lymphocytic infiltration of tubules, interstitium, and in severe cases, vessels causing cytotoxic injury or acute antibody-mediated rejection (ABMR) caused by DSA resulting in complement activation and lysis of target cells. A rise of serum creatinine is a delayed marker of the assessment of AR. By the time the serum creatinine rises, significant histological damage has already occurred, thereby significantly lowering the chance of complete recovery from injury. There have been multiple efforts to develop a noninvasive biomarker that promptly, accurately, and inexpensively predicts immunological allograft injury at an early stage with high sensitivity, specificity, and predictive value[2]. An ideal biomarker should assess the risk of injury, diagnose and monitor the injury and the pharmacological response, have prognostic value, and assess the safety of treatment[3].

NONINVASIVE BIOMARKERS IN ORGAN TRANSPLANTATION

Over the last decade, the emphasis has been on finding the perfect biomarker, which will help to predict, diagnose, and treat rejection in order to improve short- and long-term transplant outcomes. Various biomarkers in different categories have been studied, including blood mRNA (Granzyme B, Perforin, FasL, HLA-DRA, and multigene signature); blood donor-derived cell-free DNA (dd-cfDNA); blood proteins (DSA, C1Q binding, Pleximune, Immuknow, kSORT, IFN-γ Elispot and, TCR repertoire); urinary mRNA (Perforin, Granzyme B, PI-9, CD103, FOXP3, CXCL10, NKG2D, TIM3, Granulysin, and multigene signature); and urinary proteins (CXCL9, CXCL10, and Fractalkine)[4]. The markers have varying degrees of sensitivity and specificity and are summarized in Table 1. dd-cfDNA has recently become as one of the most commonly used biomarkers. The purpose of this review is to outline the discovery and utility of dd-cfDNA and to evaluate the available data regarding its use in kidney transplantation.

DD-CFDNA

Plasma cell-free DNA has been used as a biomarker in prenatal testing, cancer diagnosis, and organ transplantation[5-8]. Multiple studies have shown that allograft-derived cell-free DNA can be detected and quantified as a fraction of total cell-free DNA in the plasma or serum of various solid organ transplant recipients[8-10] such as kidney[11], heart[12], lung[13], pancreas[14], and liver[15]. Similar studies were also done looking at cell-free DNA excretion in urine[16]. This noninvasive marker was extensively studied in heart transplant recipients by Snyder *et al*[9], where significantly increased levels of dd-cfDNA were noted with biopsy-proven AR. Severity of rejection worsened with increasing levels of dd-cfDNA. In addition to being a marker of rejection, dd-cfDNA can also be used as an individualized tool to assess the efficacy of immunosuppressive treatment. In a study of liver transplant recipients, higher tacrolimus levels were associated with lower amounts of dd-cfDNA, suggesting that the relationship could be used as a tool for optimizing immunosuppressant drug dosing[17]. The presence of dd-cfDNA in the plasma of solid organ transplant patients was first described in 1990's, and involved measurement of Y chromosome DNA

Table 1 Biomarkers studied in the field of transplantation

Ref.	Biomarker name	Biomarker assay method	Sample size	Rejection type	Sensitivity/ specificity	PPV/NPV	Comments
Patel *et al*[56]	CDC crossmatch	Micro-cytotoxicity assay	225	Hyper acute rejection/ early graft loss	0.75/0.97	0.80/0.97	FDA approved
Mahoney *et al* [57]	Flow crossmatch	Flow cytometry	90	Early graft loss	0.71/0.74	0.33/0.93	FDA approved
Pei *et al*[58]	Luminex	HLA beads; flow cytometry	10	Anti HLA Ab	-	-	FDA approved
Ashokkumar *et al* [59]	Pleximmune	T cytotoxicmemory cell	32	Acute rejection	0.88/0.94	0.93/0.88	FDA approved
He *et al*[60]	Cylex-Immuknow	Lymphocyte ATPgeneration	42	CD4 T cell function	-	-	FDA approved
Loupy *et al*[61]	C1q bindingassay	Flow cytometric C1q binding	1016	TCMR/ABMR/graft loss	-	-	FDA approved
Hricik *et al*[62]	IFN-γ ELISPOT	Donor-reactive memory T cell	21	De novo DSA/ rejection	1.0/0.67	0.67/1.0	Not FDA approved
Roedder *et al*[63]	KSORT	PBLRNA by qPCR	143	AR	0.83/0.91	0.81/0.91	Not FDA approved
Bloom *et al*[33]	dd-cfDNA	PBL single gene sequencing	102 (107 samples)	ABMR and I b or higher TCMR	0.59/0.85	0.61/0.84	FDA approved
Acquino–Dias *et al*[64]	FOXP3	PBL, urine (PCR)	65 (78 sample)	AR *vs* DGF	0.94/0.95	0.94/0.95	
Li *et al*[65]	Granzyme B, perforin	Urine mRNA (PCR)	85 (151 samples)	AR	0.79-0.83 /0.77-0.83	-	
Hricik *et al*[66]	Urine CXCL9	Urine ELISA	258	TCMR	0.85/0.81	0.68/0.92	Not FDA approved
Suthanthiran *et al* [67]	Urine 3 gene; CD3E, CXCL10, 18SrRna	Urine RNA by PCR	485 pts (4300 samples)	Diagnosis AR 20 d early	0.79/0.78	-	Not FDA approved
Renesto *et al*[68]	TIM-3	PBL, urine mRNA PCR	115 (160 samples)	Diagnose AR, values normal post treatment	0.87/0.95	0.87/0.93	
Valujskikh *et al* [69]	miRNA-210	Urine miR-210 PCR	81 (88 samples)	Diagnose AR, values normal post treatment	0.52/0.74	-	

ABMR: Antibody-mediated rejection; AR: Acute rejection; CDC: Complement dependent cell cytotoxicity; dd-cfDNA: Donor-derived cell-free DNA; ELISA: Enzyme-linked immunosorbent assay; FDA: Food and Drug Administration; HLA: Human leukocyte antigen; IFN: Interferon; PBL: Peripheral blood lymphocyte; PCR: Polymerase chain reaction; TCMR: T cell-mediated rejection.

particles in a female recipient from a male donor[18].

BIOLOGY AND KINETICS OF CELL-FREE DNA

Levels of cell-free DNA fluctuate randomly during the day[19,20] and vary with multiple factors including age[21], exercise, obesity[22], malignancy, transplant[20], acute coronary syndrome[23], stroke[24], and other pathological conditions. The concentration of cell-free DNA may vary from 3.5-100 ng/mL[20,25]. Cell-free DNA is rapidly cleared from the plasma. Its The mean clearance half-life of fetal Y chromosome DNA particles from maternal plasma was reported by Lo *et al*[26] to be 16 min. Cell-free DNA is primarily cleared by apoptosis, necrosis, and active secretion. Less than 20% is secreted in urine[27] and partly degraded by the liver[28] and endonucleases in the plasma and other tissues. Yu *et al*[29] found clearance of fetal cell-free DNA to occur in two phases, one with a half-life of about 1 h and a slower phase with a half-life of about 13 h. Nearly complete disappearance of fetal cell-free DNA occurs by 2 d postpartum.

In kidney transplant recipients, the kinetics of dd-cfDNA were described in detail by Shen *et al*[30], where the authors compared the dynamics of degradation of dd-cfDNA in the immediate post transplant period in kidney transplant recipients from

living donors (LD) compared with deceased donors (DD) where donors had cardiac death, and some experienced delayed graft function. Based on their analysis, the mean dd-cfDNA concentration was 20.69% at 3 h, 5.22% by about 16 h, and 0.85% by day 7. The concentrations were significantly higher in recipients of kidneys from DDs than LDs initially (45% *vs* 10%) and on day 7 d (1.11% *vs* 0.59%) probably because of higher levels of ischemia reperfusion injury in the former group. Other large solid organs, such as livers may have more cell turnover and larger proportions of dd-cfDNA released in the recipient. Beck *et al*[10] found dd-cfDNA fractions of 90% immediately after transplant with steady state levels below 15% by day 10.

MEASURING DD-CFDNA

The technology of measuring dd-cfDNA initially had some limitations as the assays required prior recipient and donor genotyping and were time consuming and expensive[8,9]. Newer technologies that have been validated as clinical-grade assays measure dd-cfDNA in transplant recipients by polymerase chain reaction (PCR) such as real-time quantitative PCR, droplet digital PCR, or next generation sequencing (NGS) as described by Grskovic *et al*[19]. Droplet digital PCR and NGS have been clinically investigated and validated over a wide range for detecting rejection in transplant recipients[31,32]. The basic principle of measuring dd-cfDNA is by measuring single nucleotide polymorphisms (SNPs) that are homozygous in the recipient and differ from those of the donor. That can be accomplished in the absence of donor genotyping[33]. There are no standardized assays to be used for transplantation, in terms of the number of SNPs. The commercially available assays using NGS technology so far are Allosure, (CareDx, Brisbane, CA, United States), which targets 266 SNPs[34]; Prospera, (Natera Inc, San Carlos, CA, United States), which targets 13392 SNPs[35], Viracor Transplant Rejection Allograft Check (TRAC) combined with TruGraf, and (Eurofins Viracor, (Lee's Summit, MO, United States), which targets 70000 SNPs[36]. There is one study that compared the results of two commonly available commercial assays in United States; Allosure and Natera, involving 76 kidney transplant recipients. It found no significant differences in the test results for predicting rejection or other test characteristics, but found some differences in the test result turnaround time[36,37]. The recipient genotype is determined at each SNP and the relative fraction of dd-cfDNA is computed using custom bioinformatics tools. The performance of the assay was validated in 1117 samples from related and unrelated transplant recipients with reliability and precision. The turnaround time of the test was 3 d, which was considered as a practical time frame for transplant recipients.

REPORTING DD-CFDNA AS A FRACTION *VS* ABSOLUTE VALUE

In clinical application, the dd-cfDNA value is expressed as a fraction of background circulating cell-free DNA fragments. This assumes that the recipient's DNA fragments remain constant. However the host's cell-free DNA fragment levels can vary in different scenarios such as exercise, inflammatory state, and body size[22,38,39]. In a recent report involving 121 stable kidney transplant recipients, there was a significant negative correlation of the average baseline dd-cfDNA fractions between 4-12 wk post-transplantation and increasing recipient BMI[22]. That indicates that dd-cfDNA fractions are influenced by recipient body size.

Previous studies have compared absolute dd-cfDNA values to fractional values[40-42]. The analysis by Whitlam *et al*[40] included 61 samples and reported similar areas under the curve (AUC) for diagnosing ABMR, with an absolute dd-cfDNA value of 0.91 [95% confidence interval (CI): (0.82-0.98)] and a dd-cfDNA fraction of 0.89 (95%CI: 0.79-0.98). Neither measure was very useful in diagnosing 1A and borderline TCMR rejection. In a prospective observational study, Oellerich *et al*[42] compared dd-cfDNA quantification of copies/mL plasma to dd-cfDNA fraction at prespecified visits in 189 patients over 1 yr post kidney transplant. Median dd-cfDNA (copies/mL) was 3.3-fold and the median dd-cfDNA fraction was 2.0 fold higher in patients with biopsy-proven rejection (n = 15 with 22 samples) compared with the median in stable patients (n = 83 with 408 samples). Measuring dd-cfDNA (copies/mL) showed superior performance (P = 0.02) with an AUC of 0.83 compared with the dd-cfDNA fraction, which had an AUC of 0.73. A subset analysis found a significant inverse correlation between tacrolimus levels and dd-cfDNA (copies/mL), implying that dd-cfDNA may be useful

in evaluating adequacy of immunosuppression. A subsequent study from the same group evaluated the longitudinal time-dependent changes in total cfDNA (copies/mL), dd-cfDNA (copies/mL) and dd-cfDNA fraction in 303 clinically stable kidney transplant recipients 12-60 mo post-transplantation[41]. Total cfDNA showed a significant decline over time, resulting in increasing dd-cfDNA fractions, with doubling of the 85th percentile value by 5 yr. In contrast, dd-cfDNA (copies/mL) values remained stable during the same period. The authors concluded that measurement of absolute dd-cfDNA concentrations minimize false positive results compared with dd-cfDNA fractions and were hence superior for long-term allograft monitoring. Further large scale studies are still needed to define the ideal method of dd-cfDNA monitoring.

DD-CFDNA IN DIAGNOSING AR IN KIDNEY TRANSPLANTATION

The Diagnosing AR in Kidney Transplant Recipients (DART) study by Bloom *et al*[33] focused more on dd-cfDNA (Allosure, CareDx, Brisbane, CA) as a novel biomarker in discriminating subclinical rejection from no rejection at an early stage, which could allow early intervention and hopefully better outcomes. It was a prospective multicenter study of renal allograft recipients (n = 102) that used targeted amplification of dd-cfDNA by sequencing of SNPs to quantify donor and recipient DNA contributions in the plasma without the need of donor genotyping. A dd-cfDNA level of < 1% had an AUC of 0.87 (95%CI: 0.75-0.97) for discriminating ABMR from no rejection. The positive predictive value (PPV) and negative predictive value (NPV) with a cutoff of < 1% were 44% and 96% respectively, which was quite significant, suggesting a dd-cfDNA value of > 1% may indicate active rejection (TCMR type ≥ 1 b or ABMR) where the sensitivity and specificity were 59% and 85% respectively. The hope is that this noninvasive biomarker could replace the need of surveillance biopsies done at some centers to monitor for rejection. A limitation of the study was that the test failed to pick up borderline TCMR type Ia rejection. Measurement of dd-cfDNA as a steady state fraction of recipient cfDNA in kidney transplants was first described by Bromberg *et al*[32], using the Allosure test. The study established that in steady state, a dd-cfDNA fraction above 1.2% could be abnormal and potentially predict AR. The results of the Prospera test were reported by Sigdel *et al*[35] in a single center retrospective study from a curated biobank. Along the same lines, a study by Jordan *et al*[34] combining the use of elevated DSA with dd-cfDNA > 1% increased the pro-bability of diagnosis of ABMR. That study involved 87 kidney transplant recipients, 16 had ABMR, and the PPV of a 1% threshold level of dd-cfDNA to detect active ABMR in DSA positive patients was 81%, whereas the NPV was 83%. The PPV for DSA positivity alone was 48%.

Based on pivotal validation studies, dd-cfDNA became Medicare reimbursable in October 2017 for noninvasive monitoring of rejection in transplant recipients. A subsequent external validation study by Huang *et al*[43] in 63 kidney transplant recipients with suspicion of rejection, revealed that the dd-cfDNA test did not discriminate TCMR from no rejection. The AUC for TCMR was 0.42 (CI: 0.17-0.66), although performance for diagnosing ABMR was much better, with an AUC of 0.82 (CI: 0.71-0.93). To better understand the long-term outcomes based on dd-cfDNA, a large prospective multicenter observational cohort study, the Kidney Allograft Outcomes AlloSure Registry (KOAR, ClinicalTrials.gov Identifier NCT03326076) is underway and plans to enroll 4000 kidney transplant recipients. KOAR is sponsored by CareDx, and will complete enrollment in December 2021. The ProActive study utilizing the Prosepra test and sponsored by Natera, Inc. (NCT04091984) is also underway and is targeting to enroll 3000 kidney transplant recipients prospectively from the time of transplant surgery. It will assess changes in the utilization of allograft biopsy and clinical outcomes based on physician-directed use of the Prospera test to rule in and rule out active rejection. The planned follow up for the study is 3 yr for most patients and 5 yr for a subset of patients at high immunologic risk.

The utility of dd-cfDNA in first time single kidney transplant recipients (SKTR) was clearly shown in the above mentioned studies, but the validity of the test in repeat kidney transplant recipients (RKTR) was unclear until Mehta *et al*[38] reported a median dd-cfDNA of RKTR (n = 12) in the surveillance group that was higher than in the SKTR group (0.29% *vs* 0.19%, P < 0.001). However, both were significantly lower than the established 1% dd-cfDNA rejection threshold[44]. Another study by Sureshkumar *et al*[45], showed that there were no significant differences in dd-cfDNA values for either deceased *vs* living donor (0.39% ± 0.42% *vs* 0.37% ± 0.20%, P = 0.35) or

repeat *vs* first time (0.34% ± 0.07% *vs* 0.39% ± 0.43%, P = 0.36) kidney transplant recipients. One possible reason for the latter observation could be that the limited number of viable cells in a failed allograft is insufficient to generate enough cell-free DNA fragments.

Using a slightly different platform from Natera to detect dd-cfDNA, Sigdel *et al*[35] have shown promising results. They measured plasma dd-cfDNA with a single SNP-based cell-free assay targeting 13392 SNPs using a massively multiplexed PCR method to detect allograft injury or rejection without knowing the donor genotype. Altuğ *et al* [31] further validated the performance of this method to detect the dd-cfDNA fraction with improved precision over other currently available tests, regardless of donor-recipient relationships. A major limitation was that the study was a retrospective analysis of archived samples from a single center comparing outcomes of patients who underwent for-cause biopsies, with an increased risk of rejection. The superiority in the technique of measuring dd-cfDNA and methodology of those studies was questioned in an editorial by Grskovic *et al*[46]. More studies are needed to prove the superiority of this technique over the other available techniques used to measure dd-cfDNA.

There have been multiple recent meta-analyses compiling the data from studies of the potential of dd-cfDNA as a biomarker to distinguish between different types of allograft rejection in kidney transplant recipients. A meta-analysis by Wijtvliet *et al*[47] included seven studies and one by Xiao *et al*[48] included nine studies. Both revealed significantly higher levels of dd-cfDNA in patients with ABMR compared with those with no rejection. The diagnostic accuracy was less for early TCMR, particularly Banff 1A and borderline. The meta-analysis by Xiao *et al*[48] revealed that the incidence of ABMR was 12%-37% in patients with elevated dd-cfDNA, with a pretest probability of 25%, positive likelihood of 58%, and negative likelihood of 6%, suggesting it may be a good test to rule out rejection. The presence of DSA can enhance the ability of dd-cfDNA to diagnose ABMR[34]. Zhang *et al*[49] showed that patients with positive DSA but without ABMR on biopsy had a higher baseline dd-cfDNA value compared with transplant recipients with neither DSA nor ABMR. The study suggests that the dd-cfDNA level may help in differentiating possibly "benign" DSA from the more damaging DSA that can cause ABMR. The majority of stable kidney transplant recipients have a median dd-cfDNA value of 0.21% with an NPV of 95%; suggesting that dd-cfDNA could be a reasonably accurate marker to rule out active rejection [33]A recent meta-analysis reported similar results[48].

DD-CFDNA IN SUBCLINICAL REJECTION

Subclinical rejections are usually diagnosed in protocol biopsies, and there has been some data to suggest that subclinical rejections portend worse long-term graft outcomes; yet there is no data to suggest that treatment of this improves outcomes [50]. A study by Gielis *et al*[51] using dd-cfDNA measured by NGS in 43 patients who had 107 protocol biopsy specimens did not differentiate subclinical rejection from pyelonephritis or acute tubular injury. Bloom *et al*[33] reported that in the DART study, dd-cfDNA did not predict early TCMR, the majority of which were subclinical rejections. Even though the efficacy of diagnosing subclinical rejection is low, use of dd-cfDNA in combination with other markers of graft dysfunction such a DSA, chemokines, gene transcripts, and other novel biomarkers, might be able to predict rejection in immunologically high risk recipients[50]. In a recently published multicenter study involving 79 patients with steroid-treated borderline/1A TCMR, those with dd-cfDNA ≥ 0.5% had a steeper decline in glomerular filtration rate (median 8.5% *vs* 0%), more frequent development of DSA (40.5% *vs* 2.7%) and recurrent rejection rates (21.4% *vs* 0%) at 3-6 mo post-initial diagnosis than patients with a value < 0.5%[52].

DD-CFDNA FOR SURVEILLANCE AND MONITORING

The ideal frequency of monitoring dd-cfDNA has not been established, but studies have shown that, depending on the type of donor organ (*i.e.* living or deceased with or without DGF), the dd-cfDNA value nadirs at 2 wk post transplant, from the ischemia reperfusion injury. Hence, the monitoring should begin at 2 wk post transplant[30]. Some studies, like as the DART study[33], measured dd-cfDNA monthly for 3 mo and quarterly thereafter for a year, which might be a good frequency to follow. Various

other studies to look at the outcomes of using this biomarker as a tool for surveillance to monitor rejection in all transplant recipients or a subset of those with high immunological risk are ongoing and are described in Table 2. Interestingly, dd-cfDNA was elevated in pathologies other than rejection, such as BK nephritis[53] and infection[54].

LIMITATIONS OF DD-CFDNA AS A BIOMARKER

The use of the dd-cfDNA assay has limitations that need to be kept in mind. The test may give inaccurate results if performed within first 2 wk of transplant, in pregnant women, within 24 h of kidney biopsy, in patients who received whole blood or WBC components within a month of testing, in those with history of allogenic bone marrow transplantation, kidney transplant from monozygotic twin and in multiorgan transplant recipients. In dual organ transplants from a single donor, a cutoff value above which one could anticipate an increased risk of rejection has not been defined, and an increased value will not distinguish which organ is experiencing the injury. A positive result in single organ recipients does increase the risk of rejection, but cannot distinguish the grade and type of rejection. Confirmatory diagnosis of type and intensity of rejection is still based on biopsy findings. Occasionally increased levels of dd-cfDNA were be seen in BK nephritis or other causes of allograft injury other than rejection.

FUTURE DIRECTIONS

The availability of dd-cfDNA for clinical use in recent years is a step in the right direction toward noninvasive monitoring of allograft health, especially following kidney transplantation. A number of recent publications have described the utility of dd-cfDNA in kidney transplant recipients. In general, studies have found that dd-cfDNA was more useful in diagnosing ABMR, with less clear impact toward diagnosing milder forms of TCMR. One possible reason for the early rise in dd-cfDNA levels in ABMR is the associated microvascular injury in the allograft, with earlier release of cell-free DNA fragments into the circulation. Emerging reports suggest that dd-cfDNA is predictive of short-term adverse graft outcomes in TCMR1A at a lower threshold dd-cfDNA level. Despite being clinically available as an attractive option for noninvasive allograft evaluation, there are still many unanswered questions on the optimal utilization of these biomarkers. More large studies and experience are needed. Some of these questions are: (1) Should we use absolute dd-cfDNA levels or dd-cfDNA fractions? (2) What is the role of surveillance using dd-cfDNA in stable kidney transplant recipients, and would there be a favorable impact on long-term transplant outcomes? And (3) Is it cost effective to perform serial dd-cfDNA measurements? Puttarajappa *et al*[55] used a Markov model to perform an economic analysis comparing noninvasive biomarker monitoring to protocol biopsy during the first 12 mo following kidney transplantation. Assuming an incidence of 12% subclinical TCMR and 3% subclinical ABMR, protocol biopsy yielded more quality-adjusted life years at a lower cost compared with biomarkers. Hopefully many of these questions will be answered once the results of large database studies such as KOAR and ProActive become available.

CONCLUSION

Noninvasive monitoring of early diagnosis of kidney allograft injury is a need of the hour. Among the various biomarkers that have been studied, dd-cfDNA captured the most attention and data is emerging. The available literature finds dd-cfDNA to be valuable for the early diagnosis of ABMR, but its role in milder forms of TCMR is less clear. Similarly, the favorable impact of dd-cfDNA in allograft surveillance on long-term outcomes is also not clear. Results from ongoing large outcome studies could shed further light onto this.

Table 2 Trials of donor-derived cell-free DNA in kidney transplantation

NCT02424227	Noninvasive blood test to diagnose acute rejection after kidney transplantation (DART)	Completed
NCT03765203	Utility of a novel dd-cfDNA test to detect injury in renal posttransplant patients (QIDNEY)	Completed
NCT03326076	Evaluation of patient outcomes from the kidney allograft outcomes allosure registry (KOAR)	Recruiting
NCT04091984	The Prospera kidney transplant active rejection assessment registry (ProActive)	Recruiting
NCT04057742	Allosure for the monitoring of antibody-mediated processes after kidney transplantation (All-MAP)	Recruiting
NCT03759535	Study in detection cfDNA for the early stage diagnosis of acute rejection post-renal transplantation	Not yet recruiting
NCT03984747	Study for the prediction of active rejection in organs using donor-derived cell-free DNA detection (SPARO)	Recruiting
NCT04130685	Donor-derived cell-free DNA for surveillance in simultaneous pancreas and kidney transplant recipients	Recruiting
NCT04166149	Eliminating the need for pancreas biopsy using peripheral blood cell-free DNA (PancDX)	Recruiting
NCT03859388	Longitudinal changes in donor-derived cell-free DNA with tocilizumab treatment for chronic antibody-mediated rejection	Enrolling
NCT04225988	Comparison of tacrolimus extended-release (envarsus xr) to tacrolimus immediate-release in HLA sensitized kidney transplant recipients	Recruiting
NCT04177095	Immune monitoring to facilitate belatacept monotherapy	Recruiting
NCT04239703	Intercomex donor-derived cell-free DNA study	Recruiting

dd-cfDNA: Donor-derived cell-free DNA; HLA: Human leukocyte antigen.

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MINIREVIEWS

Potential role of intermittent fasting on decreasing cardiovascular disease in human immunodeficiency virus patients receiving antiretroviral therapy

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Abstract

Cardiovascular disease (CVD) has become one of the commonest causes of comorbidity and mortality among People living with human immunodeficiency virus (HIV) (PLWH) on antiretroviral therapy (ART). Nearly 50% of PLWH are likely to

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have an increased risk of developing CVD, including coronary heart disease, cerebrovascular disease, peripheral artery disease and aortic atherosclerosis. Aside from the common risk factors, HIV infection itself and side effects of antiretroviral therapy contribute to the pathophysiology of this entity. Potential non-pharmacological therapies are currently being tested worldwide for this purpose, including eating patterns such as Intermittent fasting (IF). IF is a widespread practice gaining high level of interest in the scientific community due to its potential benefits such as improvement in serum lipids and lipoproteins, blood pressure (BP), platelet-derived growth factor AB, systemic inflammation, and carotid artery intima-media thickness among others cardiovascular benefits. This review will focus on exploring the potential role of intermittent fasting as a non-pharmacological and cost-effective strategy in decreasing the burden of cardiovascular diseases among HIV patients on ART due to its intrinsic properties improving the main cardiovascular risk factors and modulating inflammatory pathways related to endothelial dysfunction, lipid peroxidation and aging. Intermittent fasting regimens need to be tested in clinical trials as an important, cost-effective, and revolutionary coadjutant of ART in the fight against the increased prevalence of cardiovascular disease in PLWH.

Key Words: Human immunodeficiency virus; Intermittent fasting; Antiretroviral therapy; Metabolism; Cardiovascular disease; Mortality and morbidity

Core Tip: Intermittent Fasting of 14-18 h/d (Time Restrictive Feeding) or 2 d fast/5 d fed (Alternate d Fasting) is a widespread practice that has aroused great interest in the scientific community. Many reviews have postulated the potential benefits of intermittent fasting in different diseases. It has been shown to improve weight loss, cardiovascular effects, and glucose metabolism. It consists of periods of strict caloric restriction alternating with variable feeding schedules. Hence, we aimed to present the first literature review regarding the role of intermittent fasting as a potential nonpharmacological and cost-effective strategy in decreasing the burden of cardiovascular disease among human immunodeficiency virus patients on antiretroviral therapy.

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INTRODUCTION

The 2019 Heart Disease and Stroke Statistics update of the American Heart Association (AHA) reported that 48 percent of persons ≥ 20 years of age in the United States have some form of Cardiovascular Disease (CVD)[1]. In USA, roughly 16.3 million of people have Coronary Heart Disease (CHD)[2], secondly with approximately 7 million of Americans had at least one episode of stroke. Moreover, almost 82.6 million US citizens present at least one or more forms of CVD[2], which encompasses four major areas: CHD, cerebrovascular disease, peripheral artery disease and aortic atherosclerosis as well as thoracic or abdominal aortic aneurysm[1].

Current data suggest that every 36 s Americans die from CVD, accounting for 1 in 4 deaths in the country[3]. Furthermore, this illness it is characterized as a chronic low grade inflammatory condition that has atherosclerosis as its most common pathological substrate. In People living with HIV (PLWH), CVD risk has been shown to be 50% higher than in uninfected individuals[4]. Aside from the well-known risk factors for CVD such as smoking, changes in lipid profile and insulin resistance; HIV infection itself and some side effects of antiretroviral therapy (ART), especially protease

inhibitors, are further contributing factors among this population[5-7]. In that sense, Cardiovascular disease (CVD) has become one of the commonest causes of death in the PLWH under treatment with virological and immunological control[8].

Intermittent fasting (IF), consisting of periods of strict calorie restriction (CR) alternating with variable feeding schedules, is a widespread practice gaining high level of interest in the scientific community and the media followed by millions of people around the globe[9,10]. Different regimens of intermittent fasting have been reported in the literature with two of them being the most notorious: *Time Restrictive Feeding (TRF)*, where the fasting period is about 14-20 h/d, and *Alternate d Fasting*, traditionally 2 d fast/5 d fed[9,11,12]. It is important to remark that intermittent fasting does not necessarily involve limiting the total number of daily calories as in a typical caloric restriction regimen; therefore, it may be implemented in pathologies that do not require a reduction in the number of calories ingested[12]. Multiple potential benefits of IF have been described such as improvement in glucose metabolism and insulin sensitivity, weight loss, delayed aging, systemic inflammation, beneficial neurocognitive effects and cardiovascular benefits[12,13]. Additional metabolic benefits are still being investigated with promising paths for future research[12].

To the best of our knowledge, there is a large literature on the benefits of IF in cardiovascular disease, but none on the case of PLWH. Therefore, we aimed to explore the potential role of intermittent fasting as a non-pharmacological and cost-effective strategy in decreasing the burden of cardiovascular diseases among HIV patients on ART due to its intrinsic properties improving the main CVD risk factors and modulating the systemic inflammatory state.

EPIDEMIOLOGY

People living with HIV are almost 38 million distributed throughout all the continents [14]. PLWH on ART are disproportionately affected by an increase in the incidence of CVD compared with age-matched HIV-negative controls[4]. To date, it is known that people living with HIV present more than twice increased risk of cardiovascular disease in general[4,14]. For instance, from 1999 to 2013 the rate of deaths in the US caused by CVD in PLWH increased from 2% to almost 5%[15]. Furthermore, CVD is one of the main non-AIDS- related complications, since between 9% and 20% of PLWH in developed countries are at moderate to high risk of suffering a myocardial infarction (MI)[16].

Lately, there has been an increase prevalence of smoking in the HIV population which could be explained by a variety of factors including anxiety and other mental illnesses, alcohol and illicit drug use, sociodemographic stressors due to social discrimination, increased risk-taking behaviors and impulsiveness, or false perception of smoking risks[17,18]. It was seen in a Danish study that HIV smokers had a higher relative risk of suffering a Myocardial infarction (MI) compared to negative controls [19]. Furthermore, some of the ART regimens that include protease inhibitors (PIs) can also contribute to the increase in the incidence of CVD[7]. On a different note, the fact that the Framingham Score underestimates the MI risk in PLWH, which was clearly observed in a cohort study, complicates even more the early detection and treatment [20]. The intensity of CVD in HIV patients (measured objectively as Intimal Media Thickness = IMT) may also be directly related to the HIV duration, meaning that the arterial damage is most likely accumulative over the years[21]. The accelerated atherosclerosis formation is thought to be independent of viral replication (at least in plasma) and multifactorial[22-26] being the microbial translocation at the level of the Gut-mucosa one of the main culprits and generators of chronic inflammation[21,27-30].

PATHOPHYSIOLOGY

The increase in the CVD risk on PLWH can be explained due to the significant increase of systemic inflammation and immune-activation compared to HIV uninfected controls even in the presence of effective ART (Figure 1). Other identified contributing factors are increased clotting, altered lipid metabolism, macrophage/T-cell infiltration of arteries, residual viral replication, direct toxicity of ART, and immune-senescence [29,31]. Early immune senescence may contribute directly to accelerated CVD since senescent cells promote the secretion of pro-inflammatory cytokines (termed "senescent-associated secretory phenotype or SASP")[32]. In that sense, it was found that elimination of senescent cells from prematurely aged mice prevented aging of

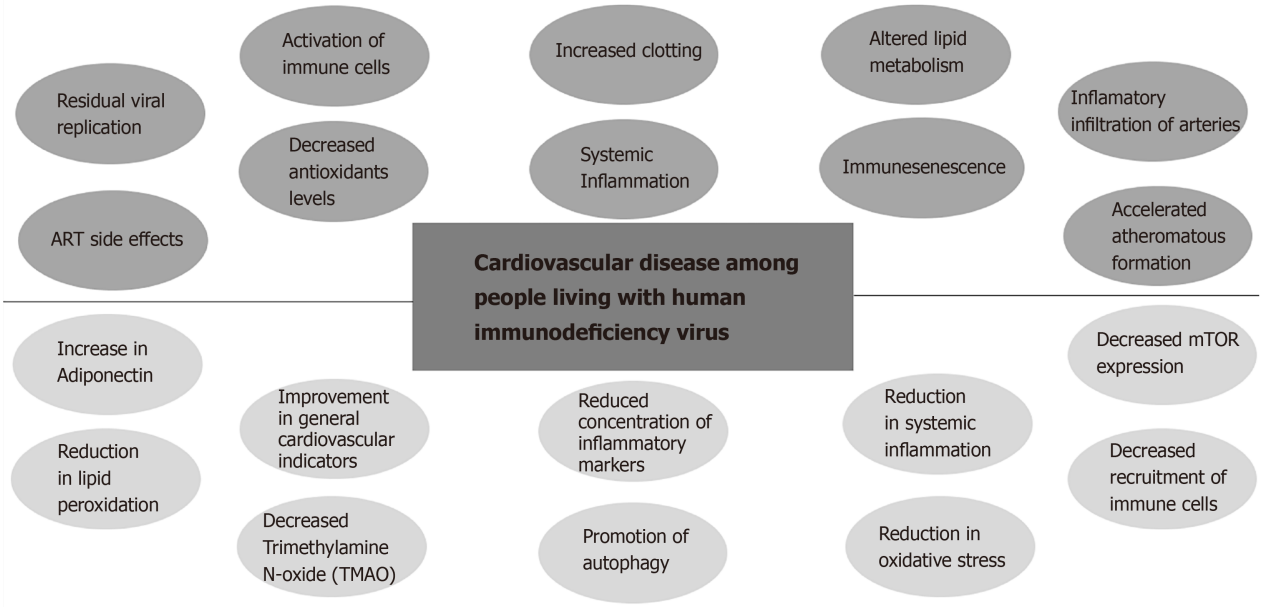

Figure 1 Summary of the interplay of the human immunodeficiency virus- antiretroviral therapy related contributing factors to cardiovascular disease and intermittent fasting potential benefits among People living with human immunodeficiency virus.

some organs[32]. Also, HIV is associated with decreased levels of antioxidants such as ascorbic acid, tocopherols, selenium, superoxide dismutase, and glutathione[33,34] along with an increase in the levels of hydroperoxides and malondialdehyde[35]. In addition, peroxides and aldehydes are not only passive markers of oxidative stress, but also really toxic compounds for cells being lipid peroxidation and LDL oxidation involved in the pathophysiology of CVD[36]. Endothelial dysfunction is associated with many of the traditional risk factors for atherosclerosis described above. The endothelial dysfunction is induced by oxidized low density lipoprotein (LDL) and should be considered as a common final pathway of multiple vascular insults[37]. On the other hand, metabolic side effects of ART are continuously being updated. Besides the well described metabolic side effects of some Protease inhibitors, new concerns regarding weight gain and subsequent metabolic disturbances are raising with the use of first line drugs such as Tenofovir Alafenamide (TAF) and Integrase strand transfer inhibitors (Raltegravir, Elvitegravir (EVG), Dolutegravir (DTG), and Bictegravir (BIC) [38]. The combination of the later generation ISTIs (Dolutegravir and Bictegravir) along with TAF presents the highest risk[38] (Figure 1).

The genesis of inflammation and immune-activation in PLWH most likely starts at the Gut-mucosal level early after the infection. It has been extensively studied that simian immunodeficiency virus SIV (in non-natural hosts) and HIV infection lead to breaches in the tight junctions between epithelial cells in the gut mucosa that allow microbial products, and chemokines to cross over[36,39-41]. These abnormalities are not only anatomical but functional as well. It is well known that bacterial products from the "gut-microbiome" like lipopolysaccharides (LPS) can stimulate the innate immune system through the pattern recognition receptors such as toll-like receptors (TLRs) mainly TLR-4 generating a local and systemic proinflammatory state[36,39]. Actually, it has been shown that an increase in the sCD14 (a soluble marker of monocyte activation after binding to LPS) predicts early mortality in HIV patients[42]. This finding is the first link between microbial translocation and mortality on HIV individuals particularly related to CVD.

The increased systemic inflammation and immune-activation in PLWH can be objectively measured through a specific cytokine profile. In HIV patients on effective ART with excellent immunological response (CD4 cell count > 500), fibrinogen and C-reactive protein (CRP) still remain strong and independent predictors of mortality[43]. In addition, interleukin 6 (IL-6), CRP, tumor necrosis factor (TNF), interferon gamma (IFN-gamma) and D-dimer all remain elevated even after effective ART[44]. It was shown that elevated CRP and HIV are independently associated with increased myocardial infarction (MI) risk, and that patients with HIV with increased CRP have a markedly increased relative risk of MI. Similarly, IL-6 and D-dimer were strongly related to all-cause mortality in this population[45]. Also, chemokines like interleukin 8 (IL-8), Regulated upon Activation Normal T Cell Expressed and Presumably

Secreted (RANTES), C-C motif ligand 2 (CCL2), and interferon gamma- induced protein 10 (IP10) remain elevated in PLWH[46], which is evidence of active recruitment of immune cells to the plaque. The above points toward a well-defined mechanism of accelerated atheromatous formation in PLWH related to systemic inflammation and local recruitment of inflammatory cells to the atheromatous plaque, a process that starts off at the level of HIV-associated gut mucosal dysfunction.

INTERMITTENT FASTING AND PREVENTION OF CARDIOVASCULAR DISEASE IN HUMAN STUDIES: TRANSLATION TO PLWH

Multiple strategies directed to decrease inflammation and immune-activation in PLWH on effective ART have shown partial and non-definitive results. In an attempt to look for nutritional and non-pharmacological approaches to face this problem, IF looks extremely attractive. IF has shown to decrease the CVD risk either directly (through improvement on the main CV risk factors) or indirectly (decreasing inflammation, immune-activation, immune cells migration, Trimethylamine N-oxide (TMAO) formation, and local oxidative stress)[12,47,48] (Figure 1). Multiple animal studies of IF have consistently proven to increase lifespan, decrease inflammation, treat diabetes and other metabolic diseases, improve cardiovascular health, and promote innumerable neurocognitive benefits (including neuro-protection against stroke) which has been described in detail in previous reviews by Mattson, M. and Longo[47,48]. Even though there is less robust evidence in human studies, multiple recent clinical trials have proven that IF decreases the overall CV risk through the improvement of each of its main modifiable risk factors. There is some discussion as to whether the decrease in the CVD risk with IF is due to its intrinsic characteristics or due to the weight loss secondary benefits. Of note, a very recent clinical trial showed the health benefits regardless of the daily calorie intake in a group of patients with metabolic syndrome[49]. As explained before, the health benefits are beyond weight loss since IF not necessarily implies a decrease in the daily caloric intake.

Direct Mechanism: improving modifiable traditional CVD risk factors

A recent study showed that a scheduled calorie restriction and IF (24 mo) in healthy, non-obese individuals was proven to be beneficial in improving risk factors for cardiovascular and metabolic disease such as visceral adipose tissue mass, ectopic lipid accumulation, blood pressure, and lipid profile, but improvements in insulin sensitivity were only transient[50]. Individuals that had been in a prolonged calorie restriction (CR) program had better outcomes in terms of serum lipids and lipoproteins, fasting plasma glucose and insulin, blood pressure (BP), high-sensitivity C-reactive protein (CRP), platelet-derived growth factor AB (PDGF-AB), body composition, and carotid artery intima-media thickness (IMT). Importantly, patients that were in the CR group had 40% less IMT, which is an important surrogate for coronary artery disease[51] (Figure 2). A very recent comprehensive review by Mattson M *et al*[52] showed that IF improves multiple indicators of cardiovascular health including blood pressure, resting heart rate, LDL and HDL levels, cholesterol, triglycerides, glucose and insulin resistance. The same review encouraged practitioners to start applying this strategy to patient care always under close professional supervision and progressively over weeks or months. Another recent study (single-arm, paired-sample trial) showed that 19 participants with metabolic syndrome who were exposed to a TRF (Time Restricted Feeding) protocol on which they ate for only 10 h, showed significant improvements in health indicators including: weight loss; reduced waist circumference, percent body fat, and visceral fat; reduced blood pressure, atherogenic lipids, and glycated hemoglobin[49]. Since PLWH are disproportionately affected by the traditional reversible CV risk factors IF could provide a significant improvement of health indicators, improvement in quality of life, and a marked reduction in the risk of CVD (Figure 2).

Indirect mechanisms

Indirectly, IF can decrease the CVD risk in PLWH through the decrease in systemic inflammation, reduction of lipid peroxidation, decrease in Trimethylamine N-oxide (TMAO), promotion of autophagy of cellular debris, and decrease in oxidative stress which in turn, shall decrease the accelerated atheroma plaque formation (Figure 3). It is important to clarify that even though IF showed much of its anti-inflammatory properties in animal studies, HIV patients present inflammatory levels way above the

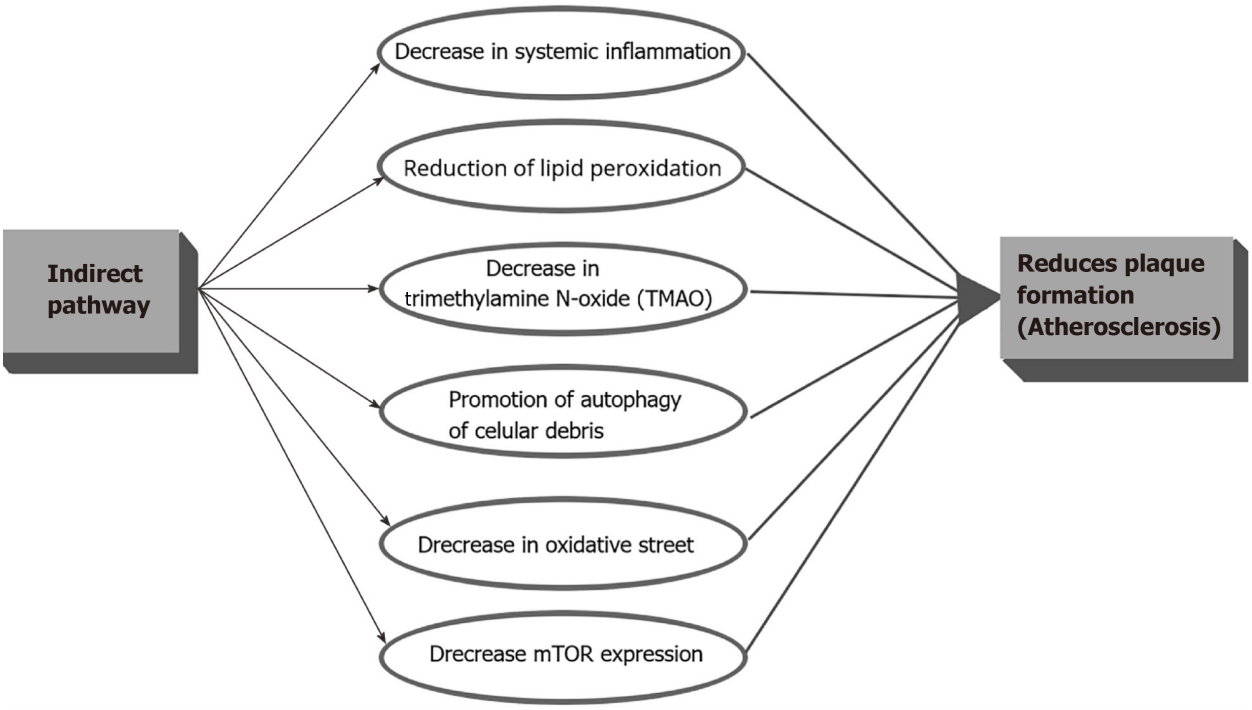

Figure 2 Summary of the potential benefits of the direct intermittent fasting pathway among People living with human immunodeficiency virus.

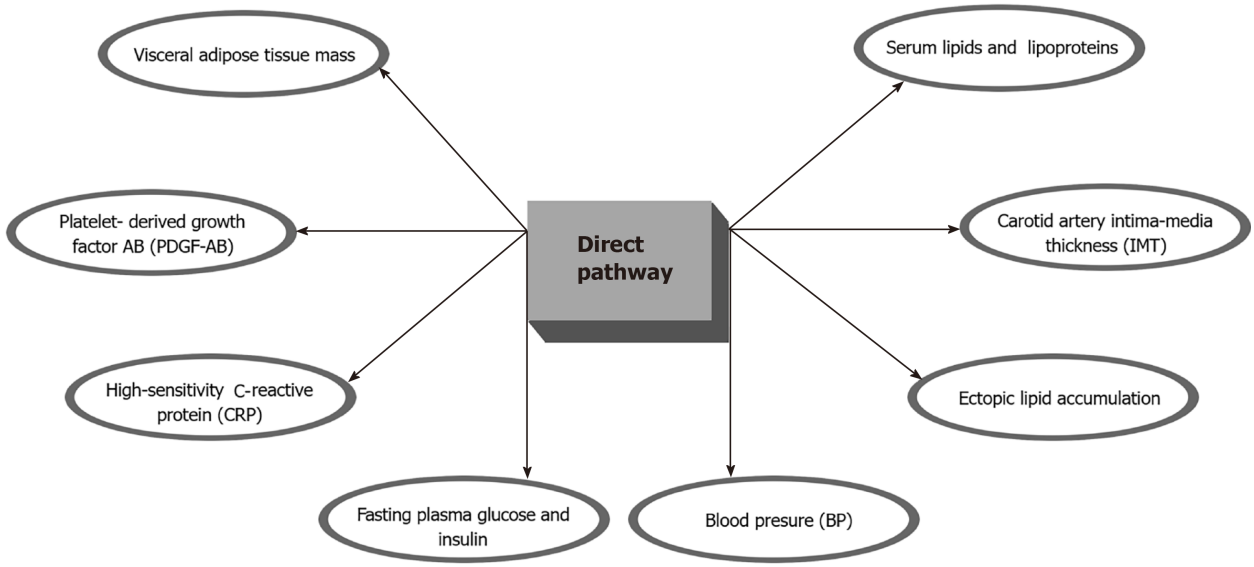

Figure 3 Summary of the potential benefits of the indirect intermittent fasting pathway among People living with human immunodeficiency virus.

mean levels compared with HIV negative controls which means that any change may correlate with a significant decrease in the CVD risk and clinical events. Trimethylamine N-oxide is an amine oxide produced in humans by intestinal microbiota from excess trimethylamine (TMA), and intermediate of choline metabolism. It has been linked to increase inflammation in adipose tissue and accelerate atherosclerosis [53]. A mean level of 14.3 ng of TMAO during fasting versus a baseline mean of 27.1 ng in control subjects (P = 0.019) was found in an IF study in humans[54], which means than IF can have implications on decreasing inflammation in the atheromatous plaque not only by decreasing the recruitment of activated monocytes but by decreasing the TMAO levels.

This ancient mechanism was probably not only created to use alternative sources of energy when food is lacking but also to clear cells from toxic molecules, reactive oxygen species (ROS), deoxyribonucleic acid (DNA) damage, and cellular debris probably through autophagy. As we explained above, oxidative stress and decreased antioxidants with lipid peroxidation is important for the plaque formation (Figure 3). The anti-atheroma formation mechanisms of IF may be mediated through: Possible endothelial improved cellular stress adaptation to ischemia and inflammation (mainly against ROS generation), decreased DNA damage, decreased inflammation, decrease recruitment of immune cells, decrease mammalian target of rapamycin (mTOR) expression[47], and promoting autophagy. In rats exposed to IF in stroke experimental models (which causes brain inflammation), decreases of Interleukin 1 beta (IL1-b), TNF-alpha, IL-6, and suppression of the "inflammasome" was observed[55]. IF also resulted in reduced levels of messenger ribonucleic acid (mRNAs) encoding the LPS receptor TLR4 and inducible nitric oxide synthase (iNOS) in the hippocampus of rats exposed to systemic LPS. Moreover, in another study IF prevented the LPS-induced elevation of IL-1α, IL-1b, IFN-γ, RANTES, TNF-α and IL-6[56]. Those two studies could have implications to decrease the LPS-driven activation of TLRs in innate immune cells, and, hence, gut inflammation in PLWH. The decrease in the gut inflammation shall decrease monocyte activation, migration, and generation of CD14's, which is directly implicated in the accelerated atheromatous plaque formation (Figure 3). IF could interrupt the "Gut-Heart axis" and significantly decrease the endothelial dysfunction. Following the same line of thoughts, IF may also inhibit the development of the atheroma plaque in HIV patients by reducing the local concentration of inflammatory markers, such as IL-6, homocysteine, and CRP, and, at the same time, decreasing the migration of immune cells to the subendothelial area through the increase of adiponectin[57]. Recently was shown that isocaloric TRF (Time Restricted Feeding) during 8 wk in males, reduced many markers of inflammation such as TNF alpha, IL-6, and IL-1b, and, increased adiponectin (an anti-inflammatory cytokine) [58]. Considering that this was a study in healthy human subjects and due to the fact that the HIV patients on ART have much higher levels of inflammation, the decrease in the CVD risk could be clinically significant. There are no theoretical biological barriers for which the above physiologic events would not happen in PLWH exposed to IF.

To understand the pathophysiology of chronic inflammation some big players need to be explained more in detail. The NLRP3 inflammasome is a multiprotein platform which is activated by infection (including HIV) or some sort of cellular stress (including ischemia). Its activation leads to caspase-1-dependent secretion of proinflammatory cytokines like interleukin-1β (IL-1β) and IL-18, and leads to an inflammatory form of cell death termed as "Pyroptosis"[59]. The inflammasome activation as a generator of inflammation will contribute to the increased CVD risk. The inflammasome can be activated directly by HIV through TLR8 activation after contact with viral RNA[46] but also by other TLRs-mediated pathways (like TLR4 with LPS in the gut mucosa as explained above). It was proved that the ketone bodies β-hydroxybutyrate (BHB) and acetoacetate, both elevated during starvation, inhibits the NLRP3 inflammasome. BHB and acetoacetate were shown to reduce the NLRP3 inflammasome-mediated interleukin (IL)-1β and IL-18 production in human monocytes[60] which will be extremely important for latently-HIV-infected monocytes to prevent activation and further recruitment with migration to the atheroma plaque. In another experimental model in rats with an experimentally induced stroke (which causes local inflammation), IF could attenuate the inflammatory response and tissue damage by suppressing NLRP1 and NLRP3 inflammasome activity[61]. A stressed Endoplasmic Reticulum (ER) is known to generate ROS which, in turn, activates the NLRP3 inflammasome and secretion of IL-1b. A recent study in rats also showed a potential therapeutic role of β-hydroxybutyrate in suppressing the ER (stressed)-induced inflammasome activation[62]. It was revealing the study that showed that patients with Rheumatoid Arthritis (RA) had significant clinical improvement (pain and inflammation) after a period of fasting if a vegetarian diet was followed thereafter [63]. Another study in overweight asthmatic female patients exposed to IF showed a significant decrease in the levels of TNF-alpha and markers of oxidative stress (8-isoprostane, nitrotyrosine, protein carbonyls, and 4-hydroxynonenal adducts) with improved clinical response. It showed that prolonged fasting blunted the NLRP3 inflammasome and T Helper 2 (Th2) cell activation in steroid-naive asthmatics as well as diminished the airway epithelial cell cytokine production[64]. These two studies highlight the possibility of using the "survival-mode" of IF to fight chronic inflammatory conditions, which, in turn, promote accelerated aging and CVD. In fact, HIV is a perfect example of a chronic inflammatory disease. We think that in all these

conditions (RA, Asthma, and HIV) the baseline level of inflammation is so high that any change will have significant clinical implications. There is no reason to think that the decreased levels of inflammation seen in these two studies will not be translated to PLWH, and, actually, it may be exacerbated. The decrease in the migration of inflammatory cells to the atheromatous plaque during IF is due to the decrease in the expression of the vascular cell adhesion molecule 1 (VCAM-1), endothelial-leukocyte adhesion molecule 1 (ELAM-1), and intercellular adhesion molecule 1 (ICAM-1) on vascular endothelial cells - all molecules highly implicated in the pathogenesis of atherosclerosis-[57]. Migration and trafficking of activated immune cells are highly involved in the pathogenesis of CVD in PLWH (Figure 4). Interestingly, Proteobacteria was identified as one of the main producers of TMAO which is increased in the dysbiosis caused by HIV[53]. IF may in fact cause a reversal of the HIV- associated - dysbiosis with decrease in the Proteobacterias (mainly inflammatory and Pro-glycolytic) with possible switch to a healthier microbiome (with less production of TMAO) like Lactobacillus and Firmicutes (Figures 3 and 4).

OTHER DIETARY REGIMENS AND HIV

Different dietary regimens have been evaluated with mixed results in PLWH on ART. A recent systematic review explored the potential benefits of micronutrients including but not limited to Vitamin A, D, Zinc, and Selenium[65]. The administration consisted in either each macronutrient or in combination. However, after a period of follow up to 6-18 mo, the study revealed minimal or no relevant benefits[65]. Another study compiled the interventions of some diets such as low-fat diet, hypocaloric diet, omega-3 fatty acids, carnitine, micronutrient supplements, formula, amino acids, uridine, among others, on HIV-infected patients receiving combination antiretroviral therapies. Where oral nutrition support (protein and energy intake) has been demonstrated to promote weight gain and fat mass overall[66]. Besides this, formula supplementation has not demonstrated further benefits. Whereas amino acids in combination showed to increase lean body mass in HIV-infected patients undergoing weight loss. The use of a low-fat diet was suggested to be implemented carefully and tailored accordingly in order to avoid a severe reduction in body mass[66,67]. Despite the paucity of controlled randomized trials with larger sample sizes, above results in small but significant findings. Further larger randomized blinded clinical trials are needed to ensure confirmatory results.

When it comes to assessing diet adherence among PLWH, it was previously seen in a study that overweighted HIV positive individuals tend to have a higher adherence to Mediterranean diet compared to the rest of the group[68]. It is hypothesized that due to the moderate risk of CVD and a diagnosis of metabolic syndrome, there is an increased awareness towards a healthier food pattern to avoid further complications [68]. In that sense, when introducing IF to PLWH we believe that adherence will not be a real problem indeed and PLWH with higher risk factors would be more prone to adhere to the new dietary regimen. Nevertheless, nutritional education strategies should be implemented early and routinely to optimize adherence among patients.

FUTURE TRIALS IN PLWH AND POTENTIAL CLINICAL IMPLICATIONS

To the best of our knowledge this is the first review addressing the possibility of applying IF in PLWH on effective ART. Due to the evidence presented above and due to the fact that PLWH are aging with increased prevalence of CVD, IF strategies need to be tested in clinical trials through proof-of-concept studies or large prospective randomized clinical trials. There are no obvious absolute contraindications that we can think of besides the obvious harm associated with extreme weight loss in patients with AIDS and wasting syndrome being off ART. Inclusion and exclusion criteria will need to be carefully defined in prospective clinical trials in order to be safe.

IF studies did not include pregnant women and were not tested in the extremes of age (pediatrics or frail elderly subjects) in which case its use is discouraged and the possible consequences are unknown. One caveat is that some HIV medications needs to be ingested with food and not on an empty stomach, but given the posology of current antiretrovirals (1 or two pills a d usually once daily in naïve patients) the recommendation would be to take the medication when the patient ingest the first meal of the d (when the patient "*breaks the fast*"). In the case of more complex regimens in PLWH with multidrug resistance and twice daily regimens personal accommod-

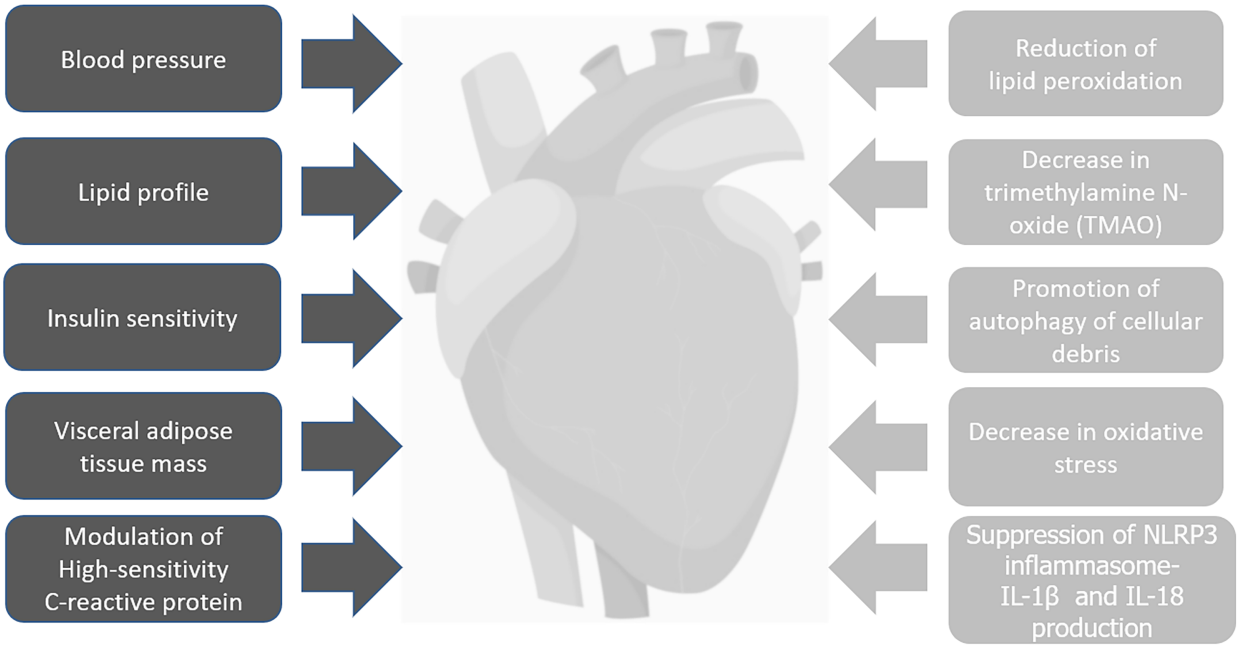

Figure 4 Summary of the direct (black) and indirect (gray) mechanisms of intermittent fasting in cardiovascular disease in People living with human immunodeficiency virus.

ations will need to be taken into account. Monthly injections of Cabotegravir and Rilpivirine were recently approved on which case IF protocols will be easier. However, first line initial regimens for most people with HIV -which generally consists of the combination of two nucleoside reverse transcriptase inhibitors (NRTIs) with an integrase strand transfer inhibitor (INSTI)-, suggest to use the combination of Bictegravir, Tenofovir alafenamide and Emtricitabine (BIC/TAF/FTC)[69]. Current indications from the Food and Drug Administration (FDA) suggest taking the drugs with or without food[70-72]. Rilpivirine (oral formulation only) regimens, in the contrary, will require a high caloric meal to increase its absorption when the feeding window starts.

Ruling out any impediment with the practice of IF within these patients. Of note, this strategy that we propose will need to be applied only to PLWH on stable ART with immunological and virological response (< 20 copies in two different occasions at least 6 mo apart in a stable regimen with good CD4 response which is not well defined but definitely more than 200 cells or more than 14%), without active opportunistic infections, active malignancy, malnourishment, or any other chronic debilitating disease. The inclusion and exclusion criteria will need to be clarified in detail by future investigations since this is a new concept so far unexplored. For sure, pregnancy and extremes of age with frailty and weight loss will be excluded during the initial trials.

CONCLUSION

The burden of Cardiovascular Diseases among HIV patients on ART is continuously growing. Intermittent fasting, through direct and indirect mechanisms, could play a role in the management and prevention of CVD among PLWH on ART. If these concepts are proven to be true in future clinical trials IF could be considered as an extremely important, cost-effective and revolutionary coadjutant of ART in the fight against the increased prevalence of CVD in PLWH which could, in turn, improve survival, decrease CV clinical events, and improve quality of life. Therefore, we recommend further longitudinal and experimental studies to ensure the safety, efficacy and effectiveness of IF on CVD among PLWH.

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