Metagenomic analysis of intestinal mucosa revealed a specific eukaryotic gut virome signature in early-diagnosed inflammatory bowel disease

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ABSTRACT
Intestinal dysbiosis is one of the causes underlying the pathogenesis of inflammatory bowel disease (IBD), encompassing ulcerative colitis (UC) and Crohn’s disease (CD). Besides bacteria, microbiota comprises both prokaryotic and eukaryotic viruses, that together compose the gut virome. Few works have defined the viral composition of stools, while the virome populating intestinal mucosae from early-diagnosed IBD patients has never been studied. Here we show that, by in-depth metagenomic analysis of RNA-Seq data obtained from gut mucosae of young treatment-naïve patients, early-diagnosed for CD and UC, and from healthy subjects (Ctrl), UC patients display significantly higher levels of eukaryotic Hepadnaviridae transcripts by comparison with both Ctrl and CD patients, whereas CD patients show increased abundance of Hepeviridae versus Ctrl. Moreover, we found that UC gut mucosa is characterized by lower levels of Polydnaviridae and Tymoviridae, whereas the mucosa of patients with CD showed a reduced abundance of Virgaviridae. Our findings support the idea that certain eukaryotic viruses might trigger intestinal inflammation and contribute to IBD pathogenesis and pave the way not only for the discovery of novel diagnostic biomarkers but also for the development of anti-viral drugs for the treatment of IBD.

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Introduction
The human intestine is progressively colonized after birth by several microbial strains that change during lifespan according to anatomical, dietary and nutritional statuses.1 Alterations in the gut microbiota composition (dysbiosis) are well-recognized contributors to the pathogenesis of gastrointestinal disorders, such as inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn’s disease (CD). In fact, bacteria, fungi, archaea, and viruses, all populating the human intestine, were found to control gut homeostasis, with a continuous pathogen-host interplay that results in both local (mucosal and luminal) and systemic (metabolic and nutritional) effects.1,2

Although bacteria have always gained the greatest attention in gastrointestinal disorders, the viral component of the human gut microbiome, termed the “gut virome”, is understudied.1 The viral community of the microbiota is mainly composed of prokaryotic-infecting viruses (bacteriophages),3,4 thus rendering the gut a dynamic community structure, characterized by continuous “predator-prey interactions” that cause either horizontal gene transfer (viruses to bacteria), or modification in the bacterial composition of the microbiota, impacting on both health and disease.5

Besides bacteriophages, the gut virome hosts also DNA- and RNA-based eukaryotic-targeting viruses able to directly transfer their genetic information to
host cells, and demonstrated to be associated with the pathogenesis of both UC and CD. Furthermore, eukaryotic viruses have been shown to contribute to intestinal dysbiosis in mice carrying mutations in the IBD-associated Il10 or Atg16L1 genes, suggesting that the gut virome might cooperate with genetic factors, ultimately leading to chronic intestinal inflammation.

Although a consistent number of studies described the viral composition of the intestinal microbiota in human stools, mainly focusing on bacteriophages, the whole eukaryotic virome that colonizes the intestinal mucosa of IBD patients still needs to be defined. A metagenomic analysis performed on a small cohort of UC and CD patients revealed that the inflamed colonic mucosa of these subjects hosts a high level of transcripts belonging to the *Herpesviridae* family, in comparison with healthy controls. However, since they selected late-stage IBD patients undergoing pharmacological treatments, they were not able to assess whether specific eukaryotic viral entities infecting the gut mucosa might have influenced disease onset.

In this work, by exploiting state-of-the-art metagenomics pipelines, we profiled the gut eukaryotic virome in young treatment-naïve patients with early-diagnosed IBD and identified the eukaryotic viral communities that might be involved in IBD onset.

**Results**

To identify any putative eukaryotic viral entity populating the gut mucosa during early phases of intestinal inflammation, we exploited the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database (www.ncbi.nlm.nih.gov/geo/) to search for high-throughput sequencing data performed on endoscopic mucosal biopsies from young, treatment-naïve IBD patients at their first diagnosis. Our age-range selection criteria encompassed subjects not older than 20 years. Among all the publicly available datasets, only one RNA-Seq experiment (GSE57945) performed on total RNA from biopsies derived from a large cohort of age-matched healthy subjects and patients with IBD (RISK study) fulfilled all patient inclusion criteria (patients’ characteristics are listed in Figure 1A).

In details, the RNA-Seq experiment included i) not inflamed ilea from colitic CD patients (cCD), ii) non-involved ilea from patients with UC, iii) ilea from CD patients with macroscopic inflammation (ileal CD [iCD]), and iv) ilea from healthy subjects undergoing colonoscopy surveillance for IBD-unrelated disease (Ctrl). Of note, the comparison between not inflamed mucosae from CD, UC and non-IBD subjects gave us the opportunity to identify those eukaryotic viruses activated independently of the mucosal inflammatory milieu, that might confer to an individual a virotype susceptible to IBD development. On the other hand, the comparison between not involved and inflamed CD allowed us to define whether a specific eukaryotic viral composition of the gut mucosa is inflammation-site specific.

We first questioned whether mucosae from IBD patients were characterized by a viral response-related transcriptional signature in comparison with the Ctrl. By Gene Set Enrichment Analysis (GSEA) we observed a statistically significant over-representation of viral infection-related gene sets in IBD samples (Figure 1B). Specifically, datasets related to “cellular response to virus”, “regulation of defense response to virus” and “virus receptor activity” were found enriched in cCD, iCD, and UC versus Ctrl, confirming that a physiological response against viral infections at transcriptional level occurred at early stages of CD and UC.

**Metagenomic analysis revealed a specific eukaryotic virome signature in early-diagnosed IBD**

To overcome the limit given by the extensive genetic variation existing in the human gut viral genomes, we took advantage of Kraken, an ultrafast metagenomic sequence classification tool based on exact alignments of sequencing reads to several reference genomes simultaneously. Such tool is able to achieve genus-level sensitivity and precision that are very similar to that obtained by the BLAST program (Figure 1C).

By this analysis, the 0.02–0.03% of the total RNA-Seq FASTQ reads resulted to be viral transcripts (Supplementary Figure 1A). To verify the correct assignment of Kraken-classified reads to the viral genomes, we subsequently performed the
same metagenomics analysis against the currently available microbial genome collection, finding a very small residual contamination (8.28 ± 0.4% bacterial over the total viral sequences). Additionally, classified viral reads were submitted to BLAST\textsuperscript{19} in order to further exclude possible artifacts and to ensure their viral identity. Finally, we manually inspected the average base quality of classified reads; this was higher than 30 in a scale from 0 to 41 (Illumina 1.8+ Phred+ 33, Supplementary Figure 1B), thus confirming the good quality of the data prior to downstream analysis.

We next assessed the presence of the classified viral transcripts in endoscopic mucosal biopsies derived from patients with IBD who were recruited independently of disease stage, pharmacological treatments or age (Supplementary Table 1). In these samples viral amplicons were detected by RT-PCR, using multiple sets of primers (Supplementary Figure 1C, 1D, 1E and Supplementary Table 2). Not all mucosal samples presented viral amplicons (Supplementary Figure 1E), confirming the heterogeneity of biopsies in terms of viral composition. To further strengthen the reliability of our metagenomic analysis, RT-PCR-detected viral amplicons were Sanger-sequenced and successfully aligned to the respective viral genomes, in the expected region (Supplementary Figure 2A), conclusively indicating that the computational methodology identified real viral sequences.\textsuperscript{17,20–22}

Being the relative composition of viral communities more important than the absolute number of viral reads\textsuperscript{18,23}, we then analyzed the fraction of specific viral reads on the total viral reads (hereafter referred as relative sequence abundances), rather than the absolute Reads Per Kilobase of transcript, per Million mapped reads (RPKM). By excluding prokaryotic-infecting viruses and viral
entities with relative abundances lower than 1%, we found the Herpesvirales order to be the main component of both IBD- and Ctrl-gut viromes (~40% of total orders detected), followed by Picornavirales and Tymovirales (~40% of total orders detected). However, no statistically significant variation was observed (Figure 1D).

Moreover, Principal Component Analysis (PCA) performed on the relative sequence abundances of viral orders failed to distinguish Ctrl- from IBD-derived samples (Figure 1E). Similar results were obtained when PCA was performed on viral families’ relative abundances (Figure 1F), thus highlighting the extreme heterogeneity among the gut viromes of these patients. Nevertheless, at family level, we observed Partitiviridae to be the most abundant in all the experimental groups, followed by Herpesviridae, accounting for ~25% and ~12% of total families detected, respectively (Figure 2A and Supplementary Table 3). Of note, the Hepadnaviridae family resulted to be highly enriched in UC patients by comparison not only with Ctrl, in which this viral family was not detected at all, but also with cCD and iCD (Figure 2B-E), pointing out these transcripts as a peculiar feature of the gut virome populating the mucosa in the early stages of UC pathogenesis.

The most investigated and characterized member of the Hepadnaviridae family is Hepatitis B virus (HBV). HBV genome encodes for a small protein named HBx, whose function has yet to be fully elucidated. Interestingly, the not-inflamed mucosa of UC patients showed a higher level of HBx transcripts by comparison with the other groups (Figure 2F and Supplementary Figure 2B), thus suggesting this protein to be a conceivable factor that may influence the mucosal immune response in UC.24

A different signature was found in CD-specific eukaryotic viromes. In fact, both iCD and cCD samples showed higher levels of Hepeviridae by comparison with Ctrl (Figure 2G-I), indicating this family to characterize the intestinal mucosa of early-diagnosed CD patients. We also observed that the eukaryotic virome signature in CD patients was inflammation-specific; in fact, Baculoviridae were

Figure 2. Early-diagnosed IBD patients showed increased abundance of specific eukaryotic viruses. A. Viral family relative abundance in Ctrl, UC, cCD, and iCD. B-E. Dot-plot (B) and MA-plots (C-E) showing Hepadnaviridae differential abundance for the indicated comparisons. F. Dot-plot showing HBx transcript differential enrichment in UC vs Ctrl, iCD, and cCD. G-K. Dot-plots for Hepeviridae (G) and Baculoviridae (J) and MA-plots (H, I, K) showing the differential abundance of the indicated comparisons. Red dots in MA-plot represent statistically enriched viral families (P ≤ 0.05). Data are represented as mean ± s.e.m. *P < 0.05; ***P < 0.005.
significantly enriched in actively inflamed iCD in comparison with unaffected ilea from cCD (Figures 2J and 2K).

Since the intestinal microbiota is characterized by a dynamic equilibrium among its residents, we asked whether the over-representation of defined families in the human gut virome might be counterbalanced by the reduction of others.

Pearson correlation analysis revealed that higher levels of Hepadnaviridae in UC patients correlated with low levels of Polydnaviridae and Tymoviridae (Figure 3A and 3B); consistently, both Polydnaviridae and Tymoviridae were significantly decreased in UC patients by comparison with Ctrl (Figure 3C and 3D). Similarly, higher levels of Hepeviridae in cCD and iCD was compensated by the reduced abundance of the Virgaviridae family, versus Ctrl (Figure 3E), with a significant negative correlation in cCD (Figure 3F). Finally, the enrichment of Baculoviridae in iCD was associated with lower levels of both Partitiviridae and Bromoviridae by comparison with cCD (Figure 3G and 3H). Also in this case we found a negative correlation between Baculoviridae and Partitiviridae and Bromoviridae (Figure 3I-J).

Overall, our metagenomic analysis revealed that this cohort of young, treatment-naive early-diagnosed patients displayed a peculiar eukaryotic gut virome signature that characterizes mucosae at early stages of IBD pathogenesis.

**Discussion**

The human virome includes human, plant and insect viruses, ancient virus-derived elements inserted in our chromosomes, and bacteriophages infecting bacteria that reside within our organism. Since systemic viruses are frequently inherited from parents very early in life, either by vertical or horizontal transmission soon after birth, the virome may be seen as a significant part of the individual genetic identity.

Viruses may have both beneficial and detrimental effects on human health, depending on their

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**Figure 3.** Defined viral entities prevail over others in the IBD-specific eukaryotic gut virome. Dot plots displaying statistical significant Pearson $R^2$ correlation coefficients for the following comparisons: Hepadnaviridae vs Polydnaviridae (A), Hepadnaviridae vs Tymoviridae (B), Hepeviridae vs Virgaviridae (F), Baculoviridae vs Partitiviridae (I), Baculoviridae vs Bromoviridae (J), and histogram showing relative sequence abundance for the Polydnaviridae (C), Tymoviridae (D), Virgaviridae (E), Partitiviridae (H), and Bromoviridae (I) viral families. Data are represented as mean ± s.e.m. $*P < 0.05; **P < 0.01; ***P < 0.005.$
interactions with other viruses and bacteria within the host. Of note, in the gut of patients with CD, the specific expansion of bacteriophages Caudovirales has been associated with decreased bacterial diversity, supporting the concept that an unbalanced virome composition may contribute to bacterial dysbiosis and intestinal inflammation. On the other hand, the concept that viruses might be protective has been already introduced by pioneering studies demonstrating that lymphocytic choriomeningitis virus (LCMV) prevents diabetes in mice; this finding was reinforced by several other epidemiological observations in animal models. In this regard, some works in mice reported that viral entities, such as Herpesvirus, might enhance resistance to Listeria monocytogenes and Yersinia pestis, or activate NK cells and gain resistance to tumor grafts, as well as increased susceptibility to autoantigen-driven experimental allergic encephalomyelitis.

Eukaryotic viruses residing in the gut may infect host cells without symptoms; however, even if asymptomatic, a virus-carrying host may hold a persistent immune response with a “continuum” of inflammatory mediators that might increase host susceptibility to disease. The scientific community now agrees with the concept that viruses are sensed by host’s specific molecules thus eliciting a specific transcriptional response not only in infected cells but also in bystander cells that may be induced to release inflammatory cytokines, eventually influencing systemic immunity.

So far, the occurrence of gut viral infections has been evaluated only in cohorts of IBD patients with long-lasting chronic intestinal inflammation. However, in these studies, either immunosuppres- sant or disease-induced stressing conditions may have led to the activation of latent viral infections such as those from Hepatitis B and C (HBV and HCV), CMV and Epstein-Bar virus (EBV). For example, a recent metagenomic analysis on colonic mucosae revealed Herpesviridae as the most enriched viral community in IBD patients. Nonetheless, since in these works most of the enrolled patients were under pharmacological treatments and not early diagnosed, the identification of any putative viral entity responsible for the onset of IBD might not have been accurate.

Here we report for the first time a comprehensive metagenomic analysis on gut mucosae from a large cohort of treatment-naïve young patients with IBD at their first diagnosis. We included data from not inflamed mucosae to understand whether a host-specific virotype might occur and lie beneath IBD pathogenesis, independently of the inflammatory milieu. In agreement with the study performed by Wang and colleagues on long-lasting IBD patients, also in our cohort Herpesviridae family was highly abundant in all the analyzed eukaryotic gut viromes, although not differentially enriched among the groups.

By contrast, we found Hepadnaviridae to be highly abundant in UC patients, along with the small protein HBx. Despite the huge efforts in characterizing the life-cycle of Hepadnaviridae, and in particular of HBV, the function of HBx has yet to be elucidated. This has been proposed to indirectly impact on host’s transcriptional activity by interacting with nuclear transcription factors or by activating specific signal transduction pathways. Moreover, many cell-signaling cascades, including JAK/STAT and Ras-Raf-MAPK pathways, seemed to be affected by HBx. Thus, we may argue that HBx, or similar proteins, might influence host’s immune response, ultimately leading to gut chronic inflammation in some subjects predisposed to UC, although this concept definitely needs further investigations.

Analogous events may occur in those CD patients that displayed a high abundance of Hepeviridae, the viral family encompassing Hepatitis E virus (HEV). HEV anti-viral response is very complex and intricate and leads to the release of multiple cytokines and antiviral molecules. Thus, similarly to HBx, also Hepeviridae-derived proteins may have an impact on host immunity, eventually triggering intestinal inflammation.

Conversely, other viral families, such as Polydnaviridae and Tymoviridae in UC, and Virgaviridae in CD, that we observed to be less enriched in IBD patients and to negatively correlate with the presence of other viruses, might be somehow considered protective in the human host. This is interesting, because Polydnaviridae, Tymoviridae, and Virgaviridae are viruses that typically infects plants and insects and may have reached the gut through the diet. The trans-kingdom interaction between viruses and hosts, such as plant and insect
viruses that colonize human tissues, has already been reported in the past for Tobacco Mosaic Virus (TMV), against which antibodies were found in human sera. Unlike animal viruses, plant viruses cannot replicate in humans or other animals because of the lack of specific receptors. Nevertheless, they still can induce the host immune response, as shown for the cowpea mosaic virus in mice.

Altogether, our metagenomic analysis introduces a quite new concept that sheds new light on IBD aetiogenesis as likely being related to a peculiar mucosal eukaryotic virome composition where some viral entities clearly prevail over others (Figure 3K). These virome signatures could be acquired early in life possibly because of environmental factors (i.e., diet), and ultimately be responsible for higher host susceptibility to IBD.

Notably, the metagenomic analysis we performed for accomplishing this study exploited pre-existing RNA-Seq data, generated with the final goal to characterize the entire human transcriptomic profile of mucosal biopsies derived from pediatric IBD patients. This means that the sequencing coverage used in the original work did not properly cover the non-human extra genomes present in the preparation, as the authors’ intentions were not indeed to look for exogenous viral sequences. We believe that future works aimed to finely dissect this aspect will need to consider this important technical limitation and increase sequencing power accordingly.

Conclusively, further studies adequately planned to elucidate the molecular mechanisms through which these viruses may lead to gut chronic inflammation are urgently needed, as this may help to develop novel biomarkers for the diagnosis and drugs for the treatment of IBD.

Material and methods

RNA-Seq transcriptome and metagenomic analyses

Patient cohort

The National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database (www.ncbi.nlm.nih.gov/geo/) was interrogated to search for high-throughput sequencing data performed on endoscopic biopsies collected from IBD patients and healthy controls satisfying the following criteria: (1) all subjects had to be not older than 20 years; (2) IBD patients enrolled had to be at their first diagnosis (with clinical symptoms for less than 5 years) and treatment-naïve. One RNA-Seq experiment (GEO accession number GSE57945) displaying these patients’ characteristics was performed on total RNA isolated from ileal biopsies obtained during diagnostic colonoscopy of early-diagnosed CD, UC, and non-IBD (Ctrl) patients, with an age comprised between 2 up to 17 years and belonging to the RISK study, an ongoing, prospective observational inflammatory bowel disease (IBD) inception cohort sponsored by the Crohn’s and Colitis Foundation of America (CCFA) (www.crohnscolitisfoundation.org). Confirmation of active colitis/ileitis was done by histology prior to diagnosis and treatment, in a standardized fashion. Information about diagnosis and biopsy collection is available in Haberman et al., 2014 and at http://www.crohnscolitisfoundation.org/science-and-professionals/research/current-research-studies/pediatric-risk-successes.html?referrer=https://www.google.it/#!.

Transcriptomics and metagenomics

FASTQ reads were retrieved from the NCBI GEO repository, quality filtered, and adaptor trimmed, with NCBI fastq-dump. For transcriptome analysis, reads were aligned to the hg38 reference human genome with TopHat for splice junction specific mapping. Gene expression read counts were measured with featureCounts. Functional enrichment analysis was performed with GSEA. For metagenomics analysis, the reads that failed to be aligned to the human genome with TopHat were subsequently mapped to the complete collection of all available virus genomes (https://www.ncbi.nlm.nih.gov/genome/) with Kraken for exact alignment of k-mers and accurate viral read classification. Relative abundances were calculated with Pavian. Viral read calls were confirmed by manually aligning Kraken classified reads to the respective viral genomes with TopHat and visualizing the resulting BAM alignments with the Integrative Genomics Viewer (IGV).

Prior to statistical analysis, classified reads were double checked with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to confirm quality filtering and adaptor trimming, and
then submitted to BLAST\textsuperscript{19} in order to exclude possible artifacts resulting from the \textit{in silico} analysis. Analyzed data and FASTQ read quality check can be found at https://doi.org/10.17605/OSF.IO/EKD3C.

**Statistical analysis**

All statistical analysis and plotting were performed with either GraphPad Prism 7 or the IBM SPSS statistical packages. Differential relative viral read abundances were calculated by one-way ANOVA, with Bonferroni post-hoc test and FDR correction for multiple comparisons (see Supplementary Table 3). Pearson correlation coefficients were used for multivariate analyses with linear regression assumptions.

**Collection of human biopsies for viral amplicon detection after \textit{in silico} analysis**

Colonic biopsies were collected from patients with UC and CD undergoing endoscopy. IBD patients were diagnosed by physicians based on clinical, endoscopic and histological criteria. Endoscopic activities were assessed using the endoscopic Mayo score\textsuperscript{49}, and the Riley histological scoring system\textsuperscript{50} as described in Ungaro et al., 2017\textsuperscript{51} Healthy colonic biopsies were obtained from patients undergoing routine check-up for non-IBD related diseases. For patients’ characteristics see the Supplementary Table 1. Samples were not randomized, and allocation and evaluation of clinical disease parameters were not blinded. The study was approved by the Humanitas Research Hospital ethics committee. All subjects provided written informed consent.

**Reverse transcription polymerase chain reaction (RT-PCR) and sanger sequencing**

Total RNA was extracted from human biopsies using the PureZOL\textsuperscript{®} RNA isolation reagent (catalog #7326880; Bio-Rad) according to manufacturer’s instructions. RNA retro-transcription was performed with High Capacity cDNA Reverse Transcription Kits (catalog #4368814; Applied Biosystems). RT-PCR reaction was performed with GoTaq\textsuperscript{®} DNA Polymerase (catalog #M300; Promega Corporation) according to the manufacturer’s instructions. For melting curve analysis, RT-PCRs was performed with SYBR\textsuperscript{®} Green Real-Time PCR Master Mix (catalog #4309155; Thermo Fisher Scientific) according to the manufacturer’s instructions and analyzed on ViiA7 Real-Time PCR System (Applied Biosystems). PCR products were loaded on 2% agarose gel and then visualized at ChemiDoc™ MP Imaging System (Bio-Rad Laboratories), using Quantity One software. GAPDH expression was used as internal control. Primer pairs were specifically designed for viral nucleotide sequences obtained by metagenomic analysis, tested for the viral identity by BLAST and are listed in the Supplementary Table 2.

RT-PCR products obtained with Expand High Fidelity PCR System (Roche®) were loaded on 1% agarose gel and run at 130 V in TAE1X buffer. For each viral amplicon, the band of the expected molecular weight (Supplementary Table 4) was purified with Wizard\textsuperscript{®} SV Gel and PCR Clean-Up System (Promega Corporation) and underwent Sanger Sequencing at Hunimed Genomic Unit. Primers used for Sanger sequencing are listed in the Supplementary Table 4. Sanger sequencing results are available at https://doi.org/10.17605/OSF.IO/EKD3C.

**Author Contributions**

Conceptualization, F.U., L.M., and S.DA.; Methodology, F.U., L.M. and V.R.; Formal Analysis, F.U., and L.M.; Investigation, F.U., L.M.; Resources F.F. and S.D.; Writing – Original Draft, F.U., L.M., and S.DA.; Writing – Review & Editing, F.U., L.M., F.F., S.DA., L.P., V.R. and S.D.; Visualization, F.U., and L.M.; Supervision, S.DA. and S.D.; Project Administration, F.U., and S.D.; Funding Acquisition, F.U.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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