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Effect of Age on the Enteric Nervous System of the Human Colon

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Abstract

The effect of age on the anatomy and function of the human colon is incompletely understood. The prevalence of disorders in adults such as constipation increase with age but it is unclear if this is due to confounding factors or age-related structural defects. The aim of this study was to determine number and subtypes of enteric neurons and neuronal volumes in the human colon of different ages. Normal colon (descending and sigmoid) from 16 patients (9 male) was studied; ages 33–99. Antibodies to HuC/D, ChAT, nNOS, and PGP9.5 were used. Effect of age was determined by testing for linear trends using regression analysis. In the myenteric plexus, number of Hu-positive neurons declined with age (slope = -1.3 neurons/mm/10yrs, $p=0.03$). The number of ChAT-positive neurons also declined with age (slope = -1.1 neurons/mm/10yrs of age, $p=0.02$). The number of nNOS-positive neurons did not decline with age. As a result, the ratio of nNOS to Hu increased (slope = 0.03 per 10yrs of age, $p=0.01$). In the submucosal plexus, the number of neurons did not decline with age (slope = -0.3 neurons/mm/10 yrs, $p=0.09$). Volume of nerve fibers in the circular muscle and volume of neuronal structures in the myenteric plexus did not change with age. In conclusion, the number of neurons in the human colon declines with age with sparing of nNOS- positive neurons. This change was not accompanied by changes in total volume of neuronal structures suggesting compensatory changes in the remaining neurons.

Keywords

colonic transit; enteric neurons; myenteric plexus; PGP 9.5

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INTRODUCTION

Organ function changes with age. As a result many organs exhibit structural changes (1) and diseases such as hypertension increase in prevalence with age (2). Similar to other organs, diseases and disorders in the adult gastrointestinal tract such as reflux disease and constipation are also more prevalent with age. However, overall, the gastrointestinal tract ages well, with function of the gastrointestinal tract relatively well preserved with increasing age (3). Colonic transit has been reported to slow with age but most studies have been carried out in animals other than humans, and the data for humans are equivocal with both a slowing (4) and no change in transit (5) reported. Increased prevalence of constipation with age can be attributed to several causes including increased medication use, decreased mobility, changes in food and fluid intake and changes in enteric nerves.

Loss of enteric neurons occurs with age (6). Several studies have shown that there is a loss of neurons with age in mice (7), rats(8), guinea pigs (9) and humans (10). In the rat, the loss of enteric neurons with age does not appear to be generalized to all subtypes of neurons but appears to be limited to neurons that express choline acetyltransferase (ChAT). This selective loss of ChAT-positive neurons was accompanied with sparing of neuronal nitric oxide (nNOS) expressing neurons (11). Similar results were seen in different rat strains (12-14) suggesting it was a generalizable finding in the rat with the exception of two studies. The first study showed that neuronal nitric oxide synthase (nNOS)-positive neurons were significantly decreased in aged rats (15). The other study, in the esophagus, showed that loss of nitrergic and non-nitrergic neurons in the esophagus is dependent on the region of the esophagus and the strain of rat. Sparing of nitrergic neurons with age was observed in one rat strain (16). Selective age-related loss of ChAT-positive neurons with sparing of nitrergic neurons would result in a progressive increase in inhibitory neurons as the animal ages, which could alter colonic function. It is unclear if a similar loss occurs in the human colon with age. There has been one study that looked at the effect of aging on neuronal cell numbers in myenteric ganglia of the human colon. In this preliminary study carried out on necropsy specimens, the number of myenteric neuronal cell bodies was counted using a Giemsa stain in two age groups, younger than 35 and older than 65 yrs. When analyzed by region no change in neurons was seen, however, when all regions were combined an overall loss of myenteric neurons was seen (10). Another study found that myenteric ganglia abnormalities increase with age (17). Whether a loss of neuronal cell bodies occurs in the submucosal ganglia, and whether there is a concomitant loss of enteric nerve fibers innervating the muscle layers is not known. The aim of this study was therefore to quantify enteric neuronal cell body, ChAT and nNOS positive neurons in both plexuses and volume of neuronal structures in the human colon across a range of ages.

METHODS

Subject selection

Normal descending and sigmoid colonic tissue was obtained as surgical surplus tissue as a result of hemicolectomies carried out for non-obstructing colon cancer. To ensure that we had tissue from patients of different age, we prospectively collected colonic tissue until we had 16 patients ranging in age from 33–99 years (9 male, 7 female, Table 1).

Procedures

Collection and use of tissue for this study was approved by the Mayo Clinic College of Medicine Institutional Review Board. Tissues were obtained in the operating room, transferred in F12 medium over ice to the laboratory and fixed overnight in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (pH 7.2). The next day the tissue was washed 4×15 minutes with 1X 0.1 M phosphate buffered saline (PBS, pH 7.2) then incubated in 30% sucrose in 1X PBS

overnight before freezing and storage at -80°C until needed. The tissue obtained was at least 5 cm away from the tumor and there was no tumor present in the sections examined. 10 or 16 μm sections were cut from the blocks.

The total number of neuronal cell bodies and the number of ChAT and NOS positive neurons in both the submucosal and myenteric plexuses was determined using a monoclonal antibody to the neuron specific antigens HuC/HuD (Invitrogen, Carlsbad, CA), and polyclonal antibodies to ChAT (Millipore, Billerica, MA), and NOS1 (Santa Cruz Biotech., Inc., Santa Cruz, CA). Antibodies to HuC/HuD do not allow visualization of nerve fibers. To do so we used antisera against Protein Gene Product 9.5 (PGP9.5, AbD Serotec, Oxford, UK). These antisera recognize the entire neuron and therefore allow measurement of the volume of nerve fibers in the circular muscle. Preparations were either single labeled with HuC/HuD (1:200), PGP 9.5 (1:2000) or double labeled with HuC/HuD and ChAT (1:100) or NOS1 (1:1000). Sections of tissue were warmed to room temperature in a desiccator then rinsed twice in 1X PBS followed by a blocking step for non-specific antibody binding by incubating the tissue with a solution of 1X PBS, 10% normal donkey serum (NDS; Jackson ImmunoResearch Lab, Inc., West Grove, PA) and 0.3% Triton X-100 (Pierce, Rockford, IL) for one hour. The antibodies were diluted in 1X PBS, 5% NDS, 0.3% Triton X-100 (0.5ng/ μl) and incubated overnight at 4°C . On day 2, slides were rinsed in 1X PBS three times followed by a one hour incubation with secondary antibodies (Cy3 or Cy5 donkey anti-mouse 1:800, Cy3 donkey anti-goat 1:800, Cy3 donkey anti-rabbit, 1:800; Jackson ImmunoResearch Lab, Inc., West Grove, PA; Cy3 donkey anti-rabbit, 1:800; Chemicon, Billerica, MA) diluted in 1X PBS, 2.5% NDS, 0.3% Triton X-100 at room temperature. Slides were rinsed three times in 1X PBS and SlowFade Gold with DAPI (Invitrogen, Carlsbad, CA) was used as a mounting medium. Seven slides per patient were labeled for Hu, 4 for ChAT and 3 for PGP9.5 and for nNOS. There were two sections per slide and all tissue on each section was examined. Sections that were not properly oriented were discarded. Number of ganglia and number of neurons in both the submucosal and myenteric plexuses were counted and expressed as number of neurons/ganglion and number of neurons/mm length. A ganglion was defined as a structure containing more than one neuron. This method of quantification avoided problems where the ganglia in the sections were not uniformly distributed or where the section was not straight. This is particularly true for the submucosal plexus which is diffuse and has large spaces between ganglia. We applied a trace tool in the software program AnalyzeTM (Biomedical Imaging Resource, Mayo Clinic College of Medicine, Rochester, MN, USA) to draw a line that followed the contours of the plexuses (Figure 1A). The line delineated the length of the section. Within that length, all neurons and ganglia in the myenteric and submucosal areas were counted (Figure 1B).

For quantification of PGP9.5 immunoreactivity, images of immunolabeled tissues were collected with a laser scanning confocal microscope (Olympus, Melville, NY). Cy 3 fluorescence was visualized using a HeNe laser and the appropriate emission and excitation filters. Images were collected using a 40X, 1.2 NA water objective lens. Optical sections (512×512 pixels) were recorded at 0.65 μm (optical z-axis) increments through each scanned area. From each of the patient samples, 3 slides were labeled. From each slide, four random areas were selected from circular muscle, submucosal plexus and myenteric plexus (12 total stacks per area from each patient). Confocal digital image files from each area of the colon were examined using the AnalyzeTM software as described previously (18,19). Digital confocal image files were imported into AnalyzeTM for three-dimensional reconstruction and PGP 9.5 fluorescence quantification (Figure 1C). Images were thresholded to remove interfering background fluorescence. Images containing non-homogeneous background fluorescence were processed manually using multiple thresholds and a connect algorithm to separate the background noise from the image of interest (Figure 1D). Voxels containing structures positive

for PGP 9.5 were counted and their number expressed as a percentage of the number of voxels in the part of the image that represented the region of interest.

Data Analysis

The association of age with number of neurons and ganglia was assessed using linear regression models. The univariate relationships with age were summarized graphically by plotting the mean (per subject) values against age along with the simple linear regression line (the corresponding slopes from these regression lines are given in the text and figure legends). A P value of 0.05, or less, was considered significant for each specific model examined.

RESULTS

An average of 634 ± 65 and 290 ± 27 Hu positive neurons were counted per patient in the myenteric and submucosal plexuses respectively. In tissues doubly labeled for Hu and ChAT, 326 ± 61 and 121 ± 20 Hu positive and 230 ± 41 and 54 ± 8 ChAT-positive neurons per patient counted in the myenteric and submucosal plexuses respectively. In tissues doubly labeled for Hu and nNOS, 326 ± 61 and 121 ± 20 Hu positive and 138 ± 31 and 47 ± 9 nNOS-positive neurons per patient were counted in the myenteric and submucosal plexuses respectively.

Myenteric plexus

The number of Hu-positive neurons per mm length declined significantly with age (slope = -1.3 neurons/mm/10yrs, $p=0.03$, Figure 2A) as did the number of ChAT-positive neurons (slope = -1.1 neurons/mm/10 yrs, $p=0.02$, Figure 2B). The numbers of ganglia/mm length and the number of neurons/ganglion for the myenteric plexus appeared to also decrease with age, but the slopes were only borderline statistically significant (slope = -0.12 ganglia/mm/10yrs, $p=0.06$, Figure 2C and -0.5 neurons/ganglion per 10 yrs, $p=0.06$, Figure 2D, respectively). The number of nNOS-positive neurons/mm length did not change significantly with age (slope = -0.3 neurons/mm/10 yrs, $p=0.18$, Figure 2E). The ratio of nNOS/Hu neurons increased with age (slope = 0.03 per 10yrs, $p=0.01$, Figure 2F) suggesting a sparing of nNOS positive neurons. When the total volume of neuronal structures (nerve cell bodies and intrinsic and extrinsic nerve fibers) in the myenteric plexus was determined using antibodies to PGP9.5 no change in the total volume of PGP9.5 immunoreactivity was seen ($-0.9\%/10$ yrs, $p=0.65$, Figure 3A).

During quantification of the number of ChAT-positive neurons in the human colon, it became apparent that there were ChAT-positive neurons that were not HuC/D positive (Figure 4). This suggested that not all human enteric neurons are HuC/D positive. 13.6% of ChAT positive neurons were Hu C/D negative. This figure did not change with age (data not shown).

Submucosal region

The number of neurons/mm length or neurons/ganglion (slope = -0.3 neurons/mm/10yrs, $p=0.09$ Figure 5B and -0.02 neurons/ganglion/10 yrs, $p=0.8$, Figure 5C, respectively) did not change significantly with age. There was a decrease in number of ganglion/mm length with age in the submucosal plexus (slope = -0.1 ganglion/mm/10 yrs, $p=0.04$, Figure 5A). The volume of PGP 9.5 immunoreactivity did not change with age ($+16.2\%/10$ yrs, $p=0.22$, Figure 3C). There was no significant change in the number of ChAT or nNOS-positive neurons with age (slope = -0.14 neurons/mm/10yrs, $p=0.2$, Figure 5D and -0.05 neurons/mm/10 yrs, $p=0.68$, Figure 5E, respectively). There also was no change in the number of ChAT and nNOS neurons per ganglion (slope = -0.02 neurons/ganglion/10 yrs, $p=0.9$ and -0.04 neurons/ganglion/10 yrs, $p=0.12$ respectively) nor in the ratio of ChAT/Hu or nNOS/Hu (slope = -0.004 per 10 yrs, $p=0.18$ and -0.03 per 10 yrs, $p=0.14$ respectively).

Nerve fiber volume in the circular muscle

The nerve fiber volume in the circular muscle did not change significantly with age ($-0.04\%/10$ yrs, $p=0.59$, Figure 3B).

DISCUSSION

The main findings of this report are the loss with age of Hu-positive and ChAT-positive enteric neurons in the human colon with sparing of nitrergic neurons. How does this loss of enteric neurons with age observed in the normal human colon compare with that found in other animals? In one study on rats, the number of colonic myenteric neurons, measured by NADH-staining, decreased by 64% from age 6 months to 24 months with no change in neuronal cell size (8). In another study on Sprague Dawley rats, the number of myenteric neurons was reduced by 15% in aged rats, also measured by NADH staining. In this study, there was no significant difference in cell numbers between the age groups as measured by PGP 9.5 staining (20). In another study, no change in PGP 9.5-immunoreactivity, as measured by Western blots, was seen (15). Gabella (9) has convincingly shown that the number of myenteric neurons, measured by NADH staining, falls with age in the small intestine (50% decrease from 3–50 months). This fall was due to both a decrease in the actual number neurons and due to an increase in the length of the small intestine. This decrease was confirmed in a later study (21) that also showed a more pronounced loss of calretinin immunoreactive neurons. Our results in the normal human colon suggest that the human colonic myenteric plexus is similar to smaller animals with a decrease of approximately 38% in the number of myenteric neurons per mm by the age of 60 yrs as compared to the number of myenteric neurons at age 30. There are not enough data available in the literature to make a meaningful comparison for submucosal neurons. The decrease in the total number of myenteric neurons with age is consistent with a previous study that reported changes in morphology of human colonic myenteric ganglia with age. Hanani et al (17) reported a significant increase with age of ganglia with “cavities” (empty areas defined as an area with a diameter of at least 40 μ m where nerves were missing) and a decrease with age of normal appearing ganglia. The increase of ganglia with cavities may represent loss of neurons in the ganglia. Hanani also noted an increase in ganglionic area with age (17).

In rats, it has been shown that the loss of enteric neurons with age is not non-specific, rather there is a relatively greater loss of ChAT-positive neurons, with sparing of nitrergic neurons (11). However, in other studies, nitrergic neurons were not decreased but there was evidence for neuronal damage, including axonal swelling (13,22) and loss of expression of nitric oxide synthase as measured by Western and Northern blot analysis (15). The presence of ChAT in neurons was used as a marker for cholinergic neurons and hence for excitatory neurons and of nitric oxide synthase as a marker for inhibitory neurons, thereby suggesting a loss of excitatory neurons. A hypothesis put forward to explain the sensitivity of ChAT-positive rat enteric neurons to age is that as these neurons are excitatory they have a higher susceptibility to calcium dysregulation and increased reactive oxygen species (23). However, while the data on selective loss of ChAT positive neurons has been interpreted as selective loss of excitatory neurons, ChAT is not an exclusive marker of excitatory neurons as it is found in a large population of enteric neurons. We found that 70% of HuC/D-positive neurons were ChAT-positive, a number similar to that found in other studies (24,25). Also, ChAT is expressed in a subset of neurons that express nitric oxide synthase. In humans it appears that 4% of neurons that are nitric oxide synthase positive also express ChAT (24,25) potentially suggesting other functions for ChAT. Our findings in the human colon also suggest that there was sparing of nitrergic neurons with dropout of Hu and ChAT-positive neurons. The rate of drop out of ChAT positive neurons (1.1 neurons/mm/10yrs) was similar to that for Hu (1.3 neurons/mm/10yrs) suggesting that while there was nitrergic neuronal sparing there did not appear to be selective targeting of ChAT-

containing neurons. The relative sparing of nitrergic neurons coupled with overall decline in number of neurons resulted in an apparent increase in nitrergic neurons with age although this is a change in proportions and not actual numbers. This finding is similar to the findings of another study, in human small intestine that also found a larger percentage of nitrergic neurons with age (26).

HuC/D is a useful marker of enteric neurons (24) because it can be easily used in conjunction with other fluorescently labeled antibodies and because, unlike PGP9.5 it only labels neuronal cell bodies and not processes. The density of neuronal processes in the human colon is such that it is not possible to count individual cell bodies in the myenteric plexus using PGP9.5 as a marker. However HuC/D also has its disadvantages. A finding of particular interest in this report is that, apparently unlike in smaller animal species, HuC/D appears to not label a significant number of cell bodies. 13.6% of ChAT positive myenteric neurons were HuC/D negative. Even if one uses the number of HuC/D positive neurons as the denominator (an underestimate) and uses the figure that 75 of neurons are ChAT positive then HuC/D is not picking up at least 20% of human myenteric neurons. It has been suggested that HuC/D, RNA binding proteins, expression decreases with age therefore resulting in a lower identification of human enteric neurons than would otherwise occur in younger patients (6). However our data do not bear this out as there was no change in the percent of ChAT positive HuC/D negative neurons with age (data not shown) a finding that confirms a previous report (27) also showing no effect of age on Hu staining.

Another finding in the current study was that while there was a dropout of neurons in the human colonic myenteric plexus with age, this dropout was not accompanied by a loss of total myenteric neuronal volume. The number of neurons in the myenteric plexus was determined using antibodies to HuC/D. The total neuronal myenteric volume was determined using antibodies to PGP9.5. Anti HuC/D antibodies stain enteric neuronal cell bodies allowing assessment of cell numbers while antibodies to PGP 9.5 stain enteric nerves cell bodies and their processes as well as any process from the extrinsic nervous system that innervates the enteric nervous system. This apparent discrepancy may be due to several possible factors. It is possible that anti HuC/D antibodies and anti PGP9.5 antibodies stain different populations of neurons and that the neuronal loss is different in the two populations. The density of nerve fibers in the human colon confounds attempts to accurately count cell bodies with PGP 9.5. However, the potential explanation that anti HuC/D antibodies and anti PGP9.5 antibodies stain different populations of neurons and that the neuronal loss is different in the two populations is unlikely to be correct. HuC/D are 35–40 kilo Dalton proteins that are cytoplasmic RNA binding proteins. PGP 9.5 is a soluble 27 kDa protein corresponding to an ubiquitin COOH-terminal hydrolase that plays a modulating role in intracellular proteolysis (28). There is no evidence that expression of either HuC/D or PGP 9.5 is linked to expression of a particular neurotransmitter, or a particular neuronal subtype making it quite unlikely that the differences observed between the two methods was due to differential staining of neuronal subtypes. A more likely explanation is a compensatory increase in individual neuronal cell body size with age and/or an increase in the number or volume of processes per remaining neuron. There is good evidence in the literature that individual neuronal size increases with age (9). Phillips et al (13) reported an increase in average individual neuronal size of 19% in the aged rat colon. It is unknown whether this increase in the size of the neuronal soma is related to aging or is a compensatory mechanism to the loss of neurons with age. Our data from the circular muscle layer where no neuronal cell bodies are present suggests that the volume of nerve fibers in the circular smooth muscle layer does not change with age, arguing in favor for the compensatory hypothesis.

What is the effect of a drop out of myenteric neuronal cell bodies on colonic function? Current understanding of the enteric nervous system suggests that there is considerable reserve of

neurons in the enteric nervous system and that therefore a loss of neurons may not translate into a change in function until a critical mass is lost. What that critical mass is, is not known. A study on colonic transit in aged (65 yrs upwards) (4) compared to younger healthy controls (<40 yrs) showed an increased colonic transit time with age. Also nerve stimulation results in a smaller smooth muscle response with age (29) suggesting that the drop out of neurons may indeed have physiological consequences. However another study on colonic transit failed to show any difference in colonic transit with age (5).

In summary, the data presented in this report show that there is a significant loss of enteric neurons in the human colonic myenteric plexus. ChAT-positive neurons were lost at the same rate as Hu-positive neurons while there was sparing of nitrergic neurons. This loss was not accompanied by a concomitant loss of total neuronal volume nor a loss of circular muscle nerve fibers, suggesting a compensatory increase in the size of the remaining neuronal structures.

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Figure 1.

Methodology used to count neurons and determine volumes. Panel A shows a tissue section marked with a trace tool applied from Analyze™ on myenteric and submucosal plexuses to determine length. Scale bar 1 mm. Panel B shows a ganglion immunolabeled with an antibody to HuC/D to identify neurons. Scale bar, 20µm. Panel C shows a confocal image immunolabeled with an antibody to PGP9.5 to identify neuronal structures. The image was imported into Analyze™ for thresholding and reconstruction of the myenteric plexus neuronal volume (panel D). Scale bar, 100µm. SMP=submucosal plexus, MP = myenteric plexus.

Figure 2.

Changes in neuronal numbers in the myenteric plexus of the normal human colon with age. The number of Hu-positive (A) and ChAT-positive (B) neurons/mm decreased with age (slope = -1.3 neurons/mm/10yrs, $p=0.03$, -1.1 neurons/mm/10 yrs, $p=0.02$ respectively). The number of ganglia per mm and neurons/ganglion trended to but did not reach a statistically significant decrease with age (slope = -0.12 ganglia/mm/10yrs, $p=0.06$, Figure 2C; -0.5 neurons/ganglion per 10 yrs, $p=0.06$, Figure 2D, respectively). The number of nNOS-positive neurons/mm length (E) did not change with age (slope = -0.3 neurons/mm/10 yrs, $p=0.18$). The ratio of nNOS/Hu neurons (F) increased with age (slope = 0.03 per 10yrs, $p=0.01$). Panels G-L show representative examples of the changes seen with age. Panels G, H and I are from

a 40 yr old colon and panels J, K and L from a 76 yr old colon. Panels G and H show neurons identified with HuC/D, panels H and K neurons identified with nNOS (arrows) and panels I and L the merged images. Two neurons out of 8 were immunoreactive for both HuC/D and nNOS (arrows) in the 40 yr old patient and two neurons out of 3 in the 76 yr old. Scale bar, 20 μ m.

Figure 3.

PGP 9.5 immunoreactivity. Panel A shows data from the myenteric plexus, panel B from circular muscle and panel C from the submucosal plexus (C). Confocal images of PGP 9.5 stained colonic tissue were reconstructed and volumes measured. The data represents the percentage of PGP 9.5 fluorescence containing voxels as compared to the number of voxels in the image space. Each data point is the mean of 12 observations. No change in PGP 9.5 immunoreactivity was seen with age for the myenteric (A) plexus ($-0.9\%/10$ yrs, $p=0.65$), the circular muscle (B) ($-0.04\%/10$ yrs, $p=0.59$) or the submucosal plexus ($+16.2\%/10$ yrs, $p=0.22$).

Figure 4.

HuC/D does not label all human colonic neurons. Panel A shows a ganglion immunolabeled with HuC/D and panel B the same ganglion immunolabeled with ChAT.(arrows and arrowheads). Panel C shows the merged image showing a ChAT-positive neuron that was not HuC/D positive (arrowhead). Scale bar, 20µm.

Figure 5.

Changes in neuronal numbers in the submucosal plexus of the normal human colon with age. The number of Hu-positive neurons/mm (A) and neurons/ganglion (B) did not change with age (slope = -0.3 neurons/mm/10yrs, $p=0.09$, -0.02 neurons/ganglion/10 yrs, $p=0.8$, respectively). The number of ganglia per mm declined with age (C, slope = -0.1 ganglion/mm/10 yrs, $p=0.04$). There was no significant change in the number of ChAT (D) or nNOS (E)-positive neurons with age (slope = -0.14 neurons/mm/10yrs, $p=0.2$ and -0.05 neurons/mm/10 yrs, $p=0.68$, respectively).

Table 1
Details of Tissue samples studied

AGE	SEX	DIAGNOSIS
33	M	Colon cancer
40	M	Colon cancer
40	F	Colon cancer
40	F	Colon cancer
45	F	Colon cancer
46	M	Colon cancer
50	M	Colon cancer
53	M	Colon cancer
57	F	Colon cancer
58	M	Colon cancer
68	M	Colon cancer
69	M	Colon cancer
71	M	Colon cancer
76	F	Colon cancer
82	F	Colon cancer
99	F	N/A