**TITLE:**

**Method to Obtain Pattern of Breathing in Senescent Mice through Unrestrained Barometric Plethysmography**

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Apnea, frequency, minute ventilation, tidal volume, VCO2, augmented breath

**SUMMARY:**

Unrestrained barometric plethysmography is used to quantify the pattern of breathing in awake mice. We show that 15 s segments under a standardized protocol display similar values to an extended duration of quiet breathing. This methodology also allows for the quantification of apnea and augmented breaths during the first hour in the chamber.

**ABSTRACT:**

Unrestrained barometric plethysmography (UBP) is a method for quantifying the pattern of breathing in mice, where breathing frequency, tidal volume, and minute ventilation are routinely reported. Moreover, information can be collected regarding the neural output of breathing, including the existence of central apneas and augmented breaths. An important consideration for UBP is obtaining a breathing segment with a minimal impact of anxious or active behaviors, to elucidate the response to breathing challenges. Here, we present a protocol that allows for short, quiet baselines to be obtained in aged mice, comparable to waiting for longer bouts of quiet breathing. The use of shorter time segments is valuable, as some strains of mice may be increasingly excitable or anxious, and longer periods of quiet breathing may not be achieved within a reasonable timeframe. We place 22-month-old mice in a UBP chamber and compare four 15 s quiet breathing segments between minutes 60–120 to a longer 10 min quiet breathing period that takes 2–3 h to acquire. We also obtain counts of central apneas and augmented breaths prior to the quiet breathing segments, following a 30 min familiarization period. We show that 10 min of quiet breathing is comparable to using a much shorter 15 s duration. Additionally, the time leading up to these 15 s quiet breathing segments can be used to gather data regarding apneas of central origin. This protocol allows investigators to collect pattern-of-breathing data in a set amount of time and makes quiet baseline measures feasible for mice that may exhibit increased amounts of excitable behavior. The UBP methodology itself provides a useful and noninvasive way to collect pattern-of-breathing data and allows for mice to be tested over several time points.

**INTRODUCTION:**

UBP is a common technique for the assessment of breathing patterns1–4. In this method, mice are placed in a closed chamber where pressure differences between the main chamber (where the animal is housed) and a reference chamber are filtered through a pneumotachograph to obtain values. The resulting UBP setup is noninvasive and unrestrained and allows for respiratory measures to be assessed without the requirement of anesthesia or surgery. Additionally, this technique is suitable for studies requiring multiple measurements in the same mouse over time. Variables such as breathing frequency, tidal volume, and minute ventilation can be quantified with this method, during a single trial or over several trials. Whole-body UBP also provides measures of peak flows and respiratory cycle duration. Together, these parameters quantify the pattern of breathing. The recorded breathing traces also make it possible to review the data and count the number of central apneas displayed within a given time period. This count can be used alongside an analysis of tidal volume and inspiratory times to gauge other alterations in the pattern of breathing.

While several noninvasive plethysmography techniques exist for the direct assessment of pulmonary physiological parameters, whole-body UBP allows for a way to screen for respiratory function with minimal undue stress to the mouse. Head-out plethysmography, which utilizes tidal midexpiratory flow measures and is also noninvasive, relies on restraint, like many other types of plethysmography (e.g., double-chamber plethysmography). While these methods have been used in rodent models to measure airway responsiveness5, the use of neck collars or small restraint tubes may take mice (vs. other species) longer to acclimate to and return their breathing to resting levels.

Obtaining an optimal air-breathing segment is an important consideration for baseline comparisons. The increased use of commercially available plethysmography systems makes collecting pattern-of-breathing data possible in many laboratories. Importantly, pattern of breathing is variable throughout the collection period, particularly for mice. With that said, it is necessary to standardize baseline analysis as a means of ensuring that the training level of experimenters does not confound results. There are numerous ways to collect an air-breathing segment, serving as one area of variation between experimental designs. One example includes averaging the final 10–30 min of data following a previously defined set of time within the chamber1, while another method involves waiting until the mouse is visibly calm for 5–10 min6. The latter can take 2–3 h to achieve and in some cases, a trial may need to be abandoned if the mouse is not calm for long enough. This concern is an especially important consideration for strains of mice where observed behaviors are more anxious and excitable7. These mice may take longer to adjust to the chamber environment and only remain calm for short bursts of time. Limiting the time devoted to baseline collection standardizes the chamber time for each mouse.

It is crucial that experimenters obtain a suitable baseline that encompasses resting behavior values in the mouse but also occurs in a timely manner. Hence, the goal of this report is to provide a description of methods used to obtain short quiet baseline values for breathing parameters in mice. Moreover, we report that apneas and augmented breaths can be quantified during the first hour in the chamber.

**PROTOCOL:**

All procedures were approved by the Le Moyne College Institutional Animal Care and Use Committee. All use of animals was in agreement with the policies described in the Guide for the Care and Use of Laboratory Animals8.

NOTE: **(Critical)** Prior to experimentation, obtain all necessary approvals and training required for animal use. It is important the experimenters are familiarized with the mouse behaviors and activity levels, including signs of sleep, distress, and/or movement artifact vs. normal sniffing and breathing.

**1. Whole-body barometric plethysmography chamber**

1.1. Read the appropriate user manuals for the barometric plethysmography chamber, including connectors, O-rings, etc., and create a standard protocol file to define analyzers (i.e., metabolic) and parameters specific to the software.

1.2. Make sure all hoses and tubes are connected to the chamber. Connect a gas flow tube (flow-in) and a vacuum tube (flow-out) directly to the barometric plethysmography chamber.

NOTE:The inflow must be attached to the opening marked **bias flow**.

1.3. Attach CO2, O2, and N2 gas tanks to the gas mixer. Make sure all gas tanks are in the open position prior to experimentation.

**2. Calibration of the barometric plethysmograph chamber**

2.1. Calibrate a high and a low flow of gas by selecting the **7700-Amplifier Setup** under the **Hardware** tab of the barometric plethysmography software.

2.2. Set a vacuum (flow out of the chamber) appropriate for the experimental design, and set gas analyzers (~0.1 L/min).

NOTE:The outflow rate must remain the same throughout the calibrations and experiment for accurate metabolic recordings.

2.3. Set a low flow of air by removing the flow tube from the chamber and turning off the vacuum.

2.4. Record the zero flow by entering a **0** into the **Low Unit** cell. Double-click the **Low Cal** cell, change the time to 3 s, and hit **Measure**.

2.5. Reattach the flow tube and allow gas (20.93% O2, balanced N2) to flow through the barometric plethysmography chamber from the gas mixer.

2.6. Convert the inflow from liters/minutes into milliliters/second. Click the **High Unit** cell and enter the value in milliliters/second. Double-click **High Cal**, change the time to 3 s, and click **Measure**.

2.7. Leave the **7700-Amplifier Setup** tab open to calibrate the metabolic analyzers to the barometric plethysmography software.

**3. Metabolic analyzer calibration**

3.1. In the gas mixer program, set the gas mixer to release a flow of gas containing 20.93% O2 and 79.2% N2.

3.2. On the metabolic analyzers, set the O2 calibration level to 20.93% and the CO2 to read 0%. Turn the dial back to **Sample** once the appropriate values are entered.

3.3. Set the high O2 percentage. Click on the **ABCD-4** tab of the barometric plethysmography software and then enter **20.93** under **High Unit** of the C2 line. Under **High Cal**, change the time to 3 s and hit **Measure**.

3.4. Set the low CO2 percentage. Enter **0** under **Low Cal** of the C3 line,and then change the time to 3 s and click **Measure** under **Low Cal**.

3.5. In the gas mixer program, change the O2 value to 10% and the CO2 value to 5%. Wait several minutes for the gas flow to adjust to these values. On the metabolic analyzers, turn the adjustment knobs to calibrate CO2 equal to 5% and O2 equal to 10%. Be sure to turn the dial back to **Sample** once the values are calibrated.

3.6. Set the high CO2 percentage. Ensure the analyzer readings are stable before inserting appropriate values into the O2 and CO2 on the barometric plethysmography software. Click **High Unit** under C3 and enter **5**. Change **High Cal** to 3 s and hit **Measure**.

3.7. Set the low O2 percentage. Click **Low Unit** under the C2 option and enter **10**. Click **Low Cal**, input 3 s, and click **Measure**.

3.8. Change the gas values on the gas mixer back to 20.93% O2 and 79.2% N2. Wait several minutes for the chamber to adjust to these values. Repeat the steps 3.1‒3.7 if the metabolic analyzers do not automatically read 20.93% O2 and 0% CO2, to ensure proper calibration.

3.9. Recheck the flow meters connected to the barometric plethysmography chamber. Adjust the air flow into and out of the chamber to rates appropriate for the experiment (typically, 0.1–0.3 L/min).

3.10. Once all settings have been applied to the barometric plethysmography software, click **OK** to begin recording.

**4. Unrestrained barometric plethysmography**

4.1. Record the mouse’s weight and initial body temperature. Wait 10 min before placing the mouse in the chamber, to collect O2 and CO2 data from an empty chamber. Work in a quiet area familiar to the mice so noise and smells do not interfere with the data collection. Avoid any possible disruptions, including the opening and closing of doors or personnel moving in/out of the data collection room.

NOTE: This specific protocol employed 22-month-old male C57BL/6J mouse.

4.2. During the first hour, document the behaviors of the mouse and take detailed notes, including specific values of the flow in/out of the chamber.

4.3. After 60 min of chamber habituation, watch for segments of quiet breathing for the following 60 min. List any segments lasting at least 15 s in length without sniffing and grooming**.** Take body temperature measures every 10 min when using an implantable device.

4.4. At the end of the experiment, remove the mouse from the chamber and place it back in its cage. All equipment should be cleaned and wiped down thoroughly.If droplets of water remain, use pressurized air to remove them.

**5. Analysis of pattern of breathing and metabolism**

5.1. Open the barometric plethysmography review file and consult the recorded notes for the animal of interest.

5.2. Open the **Metabolic** panel in the software and take the average of the first 10 min of O2 and CO2, when the chamber was empty. Record these values as the FiO2 and FiCO2.

5.3. View the **Flow** panel of the barometric plethysmography software. Right-click **Analyze Attributes** and set appropriate parameters. Under the **Meta 1** tab, enter the FiO2 and FiCO2 from step 5.2, as well as the flow into the chamber under **Meta 2**, to calculate VO2 and VCO2.

5.4. For a pattern-of-breathing analysis, enter the times for the 15 s intervals of quiet breathing under **Open Data Parser Dialogue** from the **Data Parser** tab. Click **Parser View Mode** to only show the specific 15 s segments of interest.

5.5. Click **Save Parsed Derived Data**. Open the data file in a spreadsheet to obtain the binned data.

**6. Analysis of apneas and augmented breaths**

6.1. In the open review file, exit **Parser View Mode**. Go into the **Graph Setup** option under **Setup** > **P3 Setup** and select **Page View** under **Type**. Select **5** for the number of panes. Enter **-2** into the box labeled **Low** and **2** into the box labeled **High** for flow measures in milliliters/second. Apply the changes.

6.2. Scroll to the 30 min mark on the flow tracings panel.

6.3. Count apneas and augmented breaths for the 30–60 min after the mouse was placed in the chamber. Quantify periods of suspended breathing lasting longer than or equal to 0.5 s, indicative of an apnea. Augmented breaths are indicated by a sharp rise in the breathing trace above 1.25 mL/s followed by a sharp decrease below -0.75 mL/s.

**REPRESENTATIVE RESULTS:**

The results of UBP as an evaluation of pattern of breathing in 16 aged (22-month-old) mice performed under normal air gas (20.93% O2 with balanced N2) are reported. The analysis first included a comparison of a longer 10 min quiet breathing segment (which took over 2 h to obtain) versus the average of four short 15 s segments (quantified within minutes 60–120). A representative flow tracing of quiet breathing, where breathing is consistent with no active breathing behaviors, is provided in **Figure 1A**. When similar tracings are collected from animals, 100% of the breaths should be accepted by the software. However, **Figure 1B** represents breathing from a more active segment, where the mice are exploring the chamber, sniffing, and/or grooming. Tracings similar to that shown in **Figure 1B** are less likely to be accepted by the software and are not ideal for the type of breathing collection used and explained by this methodology. The parameters selected for the assessment of possible pattern-of-breathing differences between the two time points were breathing frequency (**Figure 2A**), tidal volume (VT, **Figure 2B**), minute ventilation (VE, **Figure 2C**), tidal volume/inspiratory time ratio (VT/Ti, **Figure 2D**), and minute ventilation/expelled carbon dioxide ratio (VE/VCO2/g, **Figure 2E**), which were all calculated using the barometric plethysmography software and Drorbaugh and Fenn equation. Values reported for measures are within the range of what we have previously reported for the mouse model6,9. No significant differences (*p* < 0.025 was considered significant) were elucidated between groups; post hoc corrections for multiple comparisons of breathing frequency and VT data were accounted for with Bonferroni. These results show that the use of a simplified protocol using 15 s baselines provides similar results to that of a longer baseline protocol.

Further analysis was performed with each of the four 15 s baseline segments for frequency, VT, VE, VT/Ti, and VE/VCO2/g (**Figure 3**). No significant differences (*p* > 0.05) between any of the time points were found. There were also no differences in the variability between any of the four time segments for any pattern-of-breathing measure. Additionally, we tested the variability of the segment of the 15 s group vs. the 10 min group and found no significant differences using Levene’s test when comparing the averaged group data.

The number of apneas and augmented breaths observed for each animal during minutes 30–60 of the UBP protocol are presented in **Figure 4**. These results show that aged animals showcase a high number of apneas and augmented breaths within a 30 min span (tracing shown in **Figure 1C**). The data are indicative of changes during the aging process, as these findings were observed in 22-month-old mice. To confirm interrater reliability for apnea and augmented breath analyses, Pearson correlation was calculated for two different investigators. A high degree of agreement between raters was found, as indicated by a value of *r* = .99 for apneas and *r* = .86 for augmented breaths. In future studies, an increased number of apneas compared to a control group would be telling for a breathing dysfunction stemming from a neural component.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Representative flow tracings.** (**A**) Flow tracing from a quiet baseline, where the mouse does not showcase any active behaviors such as sniffing or grooming. (**B**)Flow tracing from an active breathing period not included in our analyses, where mice are moving about the chamber and many breaths are not routinely accepted. (**C**)Flow tracing displaying an augmented breath followed by a period of apnea. A 5 s window is shown for all traces.

**Figure 2:** **Breathing parameters are similar for calm breathing segments of 10 min and 15 s in 22-month-old mice.** Barometric plethysmography was used to collect breathing data in aged mice (*n* = 16, 22 months old). Breathing data were calculated for mice during two different time points, namely the average of four 15 s calm intervals within the 60–120 min mark of the mouse being in the chamber and for 10 min of consistent calm breathing. (**A**) Breathing frequency (breaths/minute). (**B**) Tidal volume (VT; milliliters/breath). (**C**) Minute ventilation (VE; milliliters/minute). (**D**) Ratio of tidal volume to inspiration time (VT/Ti; milliliters/second). (**E**) Ratio of minute ventilation to carbon dioxide expelled, normalized to weight (VE/VCO2/g). There are no statistically significant differences between the groups after post hoc corrections (*p* > 0.025). Values of >3 SD above the mean were considered outliers and removed from the data set. Data are presented as mean ± SD.

**Figure 3:** **Comparison of four 15 s intervals.** Breathing data were calculated in calm breathing mice (*n* = 16, 22 months old) for four separate 15 s intervals within the 60–120 min of chamber placement. (**A**) Breathing frequency (breaths/minute). (**B**) Tidal volume (VT; milliliters/breath). (**C**) Minute ventilation (VE; milliliters/minute). (**D**) Ratio of tidal volume to inspiration time (VT/Ti; milliliters/second). (**E**) Ratio of minute ventilation to carbon dioxide expelled, normalized to weight (VE/VCO2/g). There are no statistically significant differences between the time segments (*p* > 0.05). Outliers are defined as >3 SD above the mean and removed. Data are presented as mean ± SD.

**Figure 4: Apnea and augmented breath counts in mice.** Apneas (≥0.5 s without breathing) and augmented breaths (ABs; a sharp increase in inhalation over 1.25 mL/s followed by a sharp exhalation below -0.77 mL/s) were counted in aged mice (*n* = 16, 22 months old) between 30–60 min. The counts were analyzed over 30 min and the total for that time period are reported. Data are presented as mean ± SD.

**Figure 5: Schematic of the unrestrained barometric plethysmography (UBP) setup.** The overall UBP setup should be similar to that described in the figure. Flow measurements must be measured for the gases entering and leaving the chamber, and the gas composition must be known for data interpretation.

**DISCUSSION:**

The protocol provides information regarding a quiet breathing baseline in mice, as well as collecting data about central apneas and augmented breaths. The representative results show that a 10 min quiet baseline has a similar pattern of breathing when compared to an average of four 15 s bouts for a cohort of old mice. Importantly, the 15 s bouts are not statistically different, nor do these groups have differences in variation from one another using Levene’s test. These data demonstrate that even one short bout is sufficient for monitoring quiet breathing. However, it is entirely possible that analyzing individual variation within a mouse at 15 s vs. 10 min may result in different findings, as the 10 min bout could encompass minimal sniffing and grooming activities. However, using Levene’s test for a comparison of individual mouse baseline segments provides a different analysis than the one described in this protocol. Overall, the design of this methodology uses 15 s breathing segments that can be acquired during minutes 60–120 in the chamber, versus having to wait for each mouse to achieve longer durations of quiet baseline.

The shorter duration required for baseline allows for more anxious/agitated strains of mice to be tested for quiet breathing. The use of a longer breathing segment (i.e., 10 or 2 min) lengthens the protocol duration, to a point where a trial may need to be abandoned if the mice do not display a quiet breathing trace within 3 h. Since many experimental designs also incorporate respiratory challenges (i.e., hypoxia), the extended time allotted for other gases highlights the need for baseline collection time to be standardized. The use of a single 15 s bout of quiet breathing helps to relieve the concern of working with mice (and strains of mice) that may be particularly excitable in the chamber. While working with barometric plethysmography, we found that ~10% of mice per study had to be excluded because of their inability to perform as little as 2 min of continuous quiet breathing within the chamber. The implementation of previous familiarization trials was unsuccessful in getting mice to calm down faster when placed in the chamber on the day of experimentation. However, because different strains, sexes, and ages of mice may all react differently to the chamber environment10,11, it is possible that habituation techniques may be helpful12,13 for some cohorts. Our familiarization trials consisted of placing the mice in the UBP chamber in the testing room for 1–2 h for several days prior to experimentation. While we observed no changes in animal behavior following this procedure, a previous study has shown that 24 h of habituation was needed to eliminate novelty effects resulting in spontaneous physical activity in mice12. Additionally, Kabir et al. found that placing plastic cylinders similar in size to the barometric plethysmography chamber in the home cage was advantageous in getting rats to familiarize themselves with the setups prior to experimentation13.

This protocol also uncovers possible respiratory dysfunction in mice via the quantification of central apneas, indicative of neural control issues. Thirty minutes of observation prior to the baseline pattern-of-breathing collection showed that all 16 aged mice displayed a high number of apneic episodes and augmented breaths (represented in **Figure 1C**). The numerous apneas in this aged mouse cohort highlight the ability of this protocol to quantify another important breathing measure without adding additional time to experimentation. It should be noted that age and disease progression (if applicable) can affect the presence and number of apneic episodes.

In order to characterize quiet breathing, it is important to continuously observe the barometric plethysmography chamber and mouse throughout the duration of the protocol. For the quantification of quiet breathing, mice should be awake but not partake in any active behaviors such as sniffing, grooming, or exploring (represented in **Figure 1A**). Since patterns of breathing during sleep can differ from those in an awake animal14,15, the collection of calm breathing during the awake state is critical. It is possible that longer segments of quiet breathing could include periods of sleep, which may not be desired depending on the experimental design. In this case, shorter segments of quiet breathing would be ideal to document, as the likelihood of data collection during sleep is reduced when active segments flank the short (15 s) quiet breathing segments. We have observed that longer segments of quiet breathing can be challenging to acquire in the mouse model, as mouse behavior in the chamber seems to be very different compared to that of rats. It is important both to critically observe mouse respiratory flow for appropriate breathing segments and to document animal behavior. In cases of reduced ventilation or unstable breathing, this method can still be utilized. In these instances, it is essential the experimenter is blinded to the cohort when selecting the 15 s segments. The software program should distinguish the breaths, with an acceptance rate of 100% during the 15 s period. We advise taking note of the breathing tracings in addition to ensuring that the animals meet the behavioral criteria for baseline since it is possible that stationary mice may still be anxious. A previous study reported that although rats exhibited calm behavior, they still showed altered breathing patterns (i.e., increased frequency) in response to controlled stimuli within the testing room13.

Measures of frequency, VT, VE, inspiratory and expiratory time, and VE/VCO2 are all quantified using analyzers and the UBP software and are frequently reported in the literature. Particularly, VT and VE calculations use the Drorbaugh and Fenn equation16, which requires body temperature, ambient chamber temperature, humidity, and barometric pressure. It is recommended to collect these measures throughout the experiment for the most accurate VT and VE values. Other variables that are calculated by the system should be used with caution. UBP is not a direct measure of pulmonary mechanics; thus, variables related to airway resistance (e.g., enhanced pause [Penh]) should be interpreted with this caveat in mind5. Additional components of the UBP setup that can impact variables calculated by the software include flow rates and the general calibration of the system. Confirm seals and gaskets are working properly (no leaks) and ensure proper connection of all equipment to the barometric plethysmography chamber (**Figure 5**). Flow rates in and out of the chamber should be kept consistent. Required flow rates can differ between UBP setups, so it is important to check these values prior to experimentation. The flow rate into the chamber should be enough to provide fresh air or gas challenges in a timely manner. The flow rate should also be sufficient for allowing the metabolic analyzers to measure O2 and CO2 without having CO2 build up within the chamber environment, which poses the risk of a changing pattern of breathing. Similarly, gas mixer/analyzer calibrations need to be regularly implemented to ensure that the metabolic parameters are accurately measured.

Other considerations for conscious UBP include reducing distractions within the experimental room while animals are being tested. Loud noises, different smells, and the presence of nonessential personnel in the room can all add to anxious behaviors exhibited by mice. Using smaller rooms as testing areas may help, but if this is not possible, cardboard walls (with a small viewing window) can be set up surrounding the chamber to lessen distractions for the mice. Electrical activity within the room should be kept at a minimum to prevent additional noise within the barometric plethysmography tracings. Therefore, it is important to take note of the flow tracings during the 10 min period when the software is collecting data from an empty chamber. These tracings should remain flat, and any interruptions or slight waves are signs of noise and should be addressed. Pressure changes from opening and closing doors or from HVAC functioning can also add to erroneous fluctuations, and ensuring that these actions occur minimally (and noting them when they do occur) is critical. Humidity can also affect the calculated tidal volume and minute ventilation, making it very important to confirm that the chamber and connecting tubes are dried before use. If necessary, the use of Drierite beads in sequence with the flow-in tubes can help remove all condensation in the air prior to chamber entrance. This step would be instituted in cases when the humidity has routinely been higher than levels listed in The Guide for the Care and Use of Animals8 (30%–70%, ideally within 10% of setpoint). Humidity can also build up in the chamber due to the presence of the animal. Although some humidity is normal, it may continue to build if the animal is excessively active or placed in the chamber for longer durations. If humidity levels reach maximum levels (99.99%), the chamber may need to be opened and wiped down during experimentation to maintain comparable breathing measures. The software accounts for changes in barometric pressure, ambient temperature, animal temperature, and humidity. Best practice is to maintain the values within a reasonable range so that “apples to apples” are being compared across ages, strains, and sexes. Moreover, the circadian cycle of mice and the time range of testing, as well as specific lighting conditions of the room, are important details to consider13,17. For instance, we typically test mice in lighting similar to their housing room (either light or dark cycle) and within a 3 h range18. Experimenters should also remain blinded to the animal groups during data collection and analyses to prevent differences in the selection of a baseline. When possible, the same experimenter should collect all data and/or analyze all tracings in a given experiment. Steps to keep experimenters blinded to the animal groups, as well as randomization and testing during similar times of the day, are crucial to the rigor of investigation. Ultimately, there are extraneous factors that may alter the flow tracings, and these concerns should be considered when performing UBP.

The UBP method is a technique used to characterize the pattern of breathing in mice. Baseline measures can be collected within 2 h when using a 15 s breathing segment. Here we report a method which can be performed with aged mice, who are often more agitated in the chamber than younger mice, suggesting that other anxious or active mouse strains could also be tested with this protocol. The data collected from UBP is noninvasive and allows for testing over multiple time points, which is useful for studies about aging, drug therapy, and disease progression.

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**DISCLOSURES:**

The authors have nothing to disclose.

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